Interactions in the rhizosphere of *Arabidopsis thaliana*: Effects of protozoa on soil bacterial communities

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ZUSAMMENFASSUNG

Die vorliegende Arbeit untersucht den Einfluss einer weit verbreiteten Bodenamöbe, *Acanthamoeba castellanii*, auf die Zusammensetzung bakterieller Gemeinschaften in der Rhizosphäre von *Arabidopsis thaliana.*

In einem ersten Experiment wurde eine molekularökologische Methode etabliert, die es erlaubt, den Einfluss von Prädatoren auf die Struktur und Funktion von bakteriellen Gemeinschaften in einem experimentellen Sand/Streu System zu untersuchen. Zur Etablierung der Methode wurden verschiedene Protokolle zur DNA und RNA Extraktion verglichen. Die Methode, die einen Aufreinigungsschritt mit Phenol/Chloroform enthielt, zeigte dabei das beste Resultat in Bezug auf die Verfolgung von Fraß-induzierten Veränderungen in der bakteriellen Gemeinschaft mittels Denaturierender Gradienten Gel Elektrophorese (DGGE).

Die Beweidung von Bakterien durch Protozoen verändert die Zusammensetzung bakterieller Gemeinschaften in der Rhizosphäre von Pflanzen. Diese Veränderungen gehen mit einem verbesserten Pflanzenwachstum einher. In einem zweiten Experiment wurde der Einfluss von Protozoen auf bakterielle Gemeinschaften mit Hilfe von DGGE und Fluoreszenz *In Situ* Hybridisierung (FISH) untersucht. DGGE Fingerabdrücke zeigten eine schnelle Veränderung der Bakteriengemeinschaft, da einzelne Banden schon zwei Tage nach Beimpfung mit Amöben nicht mehr sichtbar waren. Mittels FISH konnte eine Verringerung von metabolisch aktiven Bakterien in den phylogenetischen Hauptgruppen gezeigt werden, gleichzeitig veränderte sich der relative Anteil dieser Gruppen an der Gesamtheit der Bakterien. Hauptsächlich reduziert wurden *Betaproteobakterien* und *Firmicuten*. Die Anzahl an *Gammaproteobakterien* wurde nicht durch Protozoen beeinflusst aber durch den

Einsatz von spezifischen Primern für Pseudomonaden konnte eine funktionelle Veränderung im *gacA*-Gen beobachtet werden.

Protozoen sind dafür bekannt, dass sie das Pflanzenwachstum durch Rückführung von in bakterieller Biomasse gebundenen Nährstoffen verbessern. Außerdem kann die Beweidung bakterieller Gemeinschaften zu einer Dominanz wachstumsfördernder Rhizobakterien führen. In einem dritten Experiment wurde untersucht, ob Protozoen das Pflanzenwachstum indirekt über Veränderungen der bakteriellen Gemeinschaft oder direkt durch die Freisetzung von Nährstoffen beeinflussen. *Arabidopsis thaliana*-Pflanzen wuchsen in einem Sand/Streu System und wurden mit bakteriellen Einzelstämmen, einem diversen Inokulum und *Acanthamoeba castellanii* beimpft. Der Rosettendurchmesser, als Kriterium für pflanzliche Biomasse und Fitness, war generell in Behandlungen mit Amöben erhöht, gleichzeitig war die Wurzelbiomasse in den Behandlungen mit Einzelstämmen erniedrigt. Außerdem erhöhte sich der Stickstoffgehalt im Pflanzengewebe und resultierte in einer Verringerung des C/N-Verhältnisses. Das Wachstum und die Nährstoffgehalte der Pflanzen unterschieden sich nicht zwischen Ansätzen mit Einzelstämmen und diversen Bakteriengemeinschaften, was darauf hinweist, dass der wachstumsfördernde Einfluss von Protozoen unabhängig von der bakteriellen Zusammensetzung ist.

Die Ergebnisse der vorliegenden Arbeit lassen darauf schließen, dass nährstoffbezogene Effekte durch bakterielle Beweidung von Protozoen eine größere Rolle spielen als Veränderungen in der Zusammensetzung der bakteriellen Gemeinschaft.

ABSTRACT

The present study investigated the effect of a common soil amoeba, *Acanthamoeba castellanii*, on bacterial community composition in the rhizosphere of *Arabidopsis thaliana*.

In the first experiment a fingerprinting method was established to detect grazing induced shifts on bacterial communities in a sand/litter system. The effect of different extraction protocols on fingerprinting was assessed. The use of a method including a phenol/chloroform purification step proved to be most efficient for monitoring grazing-induced shifts with denaturing gradient gel electrophoreses (DGGE).

Bacterivorous protozoa alter the structure of bacterial communities in the rhizosphere of plants, and these changes likely contribute to plant growth-promoting effects of protozoa. In the second experiment effects of protozoan grazing on bacterial community composition was investigated with DGGE and fluorescence *in situ* hybridisation (FISH). DGGE fingerprinting pattern demonstrated rapidly induced changes in the composition of the bacterial community, some bands already disappeared two days past inoculation of amoebae. Using FISH with probes for major bacterial phyla a decrease in metabolically active bacteria in presence of amoeba could be demonstrated. However, simultaneously their proportion strongly increased. The decrease in numbers was most pronounced in *Betaproteobacteria* and *Firmicutes*. The quantity of *Gammaproteobacteria* was not affected by protozoan grazing but DGGE with specific primers for pseudomonads revealed functional shifts in the *gacA* gene.

Protozoa are known to increase plant growth by mobilization of nutrients due to grazing on bacteria and by grazing induced shifts in bacterial community composition favouring plant growth promoting rhizobacteria. In a third experiment it was

investigated if protozoan-mediated changes in plant performance are based on changes in the composition of the rhizosphere bacterial community or on nutrient based effects. *Arabidopsis thaliana* was grown in a sand/litter substrate inoculated with single strains of bacteria or a diverse bacterial soil filtrate and *Acanthamoeba castellanii*. Plant rosette diameter, as indicator for plant biomass and fitness, was generally increased in presence of amoebae, whereas root biomass was reduced in both single strain bacteria treatments. Further amoebae increased plant tissue nitrogen concentration resulting in lower C-to-N ratio. Amoeba-mediated changes in rosette diameter and plant tissue C-to-N ratio were not significantly different between the single strain and diverse bacterial communities suggesting that the effects were independent of bacterial community composition.

The results of the present study suggest that nutrient based effects caused by grazing of the amoeba on bacterial biomass and thereby mobilizing nutrients locked in bacterial tissue are more important than grazing induced shifts in bacterial community composition.

1 General introduction

1.1 Soil microorganism in the rhizosphere

Recycling systems of organic matter such as soils and sediments are loaded with species rich groups of indigenous bacteria (Horner-Devine et al., 2003). In the terrestrial subsurface the number of prokaryotic cells ranges between 25 and 250 x 10^{28} (Whitman et al., 1998), about half of the total carbon, up to 90% of nitrogen and phosphorous of the global biomass is bound in prokaryotes (Schleifer, 2004). In principle, soil organic matter is degraded by microbial primary consumers equipped with enzymes for digestion of complex plant and animal compounds thereby recycling carbon and nutrients. Further, the microbial primary consumers form the base of higher trophic levels and therefore for the microbial food web.

Microorganisms in soil are assumed to be predominantly limited by carbon and hence bottom-up controlled (Pace and Cole, 1994; Boenigk et al., 2002; Moore et al., 2003). Growth conditions for soil microorganism depend on several factors (e.g. carbon and energy sources, mineral nutrients, growth factors, ionic composition, available water, temperature, pH; Nannipieri et al., 2003). Since all microorganisms are aquatic, the role of water is most important (Nannipieri et al., 2003; Killham and Prosser, 2007).

Soil microbial communities are exceptionally divers. Based on cultivation techniques Janssen et al. (2006) highlighted nine genera of bacteria in soil: *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Micromonaspora, Nocardia* and *Streptomycetes*. However, many microorganisms were found in hot spots, i.e. sites with increased biological activity. In the terrestrial ecosystem the rhizosphere form the most prominent habitat where microbial activity is markedly increased. Rhizosphere as concept has been introduced by Hiltner in 1904 as root influenced zone, where microorganism and processes of central importance for the nutrition and

health of plants are located. The diverse array of rhizosphere microorganism is supported by resources released from plant roots (Phillips et al., 2003), e.g. carbon. The rhizosphere can be divided into endorhizosphere, i.e. the root itself with associated microorganisms, the rhizoplane, i.e. the root surface and the ectorhizosphere, i.e. the soil in close vicinity to roots. The abundance of microorganisms in the rhizosphere depends on the amount and composition of rhizodeposits with generally higher numbers of microbes near the root tip, branching points and root base (Semenov et al., 1998).

1.2 Plant-microbe interactions

Community composition and quantity of microbes in the soil influence plant nutrient acquisition and can be controlled by plant rhizodeposits which either support or inhibit the growth of specific microorganisms (Bais et al., 2006). Plant-microbe interactions can be classified into three groups: negative (pathogenic), neutral and positive interactions (Singh et al., 2004). The latter include those resulting in the mineralization of nutrients, nitrogen fixation, suppression of pathogens, production of plant-growth promoting compounds and increased acquisition of nutrients through mycorrhizal associations. The connected food web which develops around the rooting zone of plants can be divided into root, bacterial and fungal energy channels. Due to different turnover rates the bacterial energy channel has been classified as “fast cycle”, responsible for the degradation of labile detritus, while recalcitrant detritus is preferentially degraded by fungi and this has been classified as “slow cycle” (Moore et al., 2005).

Most plant–bacteria associations in the rhizosphere rely on biofilm formation which plays a key role in pathogenesis, symbiosis and commensal relationships (Ramey et al., 2004). Root associated biofilm forming pseudomonads have been studied

extensively and many of them promote growth of host plants and therefore have been used as biocontrol agents (Ramey et al., 2004). The mode of plant growth promotion by suppressing diseases, especially of fluorescent pseudomonads, is known as induced systemic resistance (ISR; Bakker et al., 2007). ISR is triggered by the release of jasmonic acid and ethylene (see review by Pieterse et al., 2002). The formation of bacterial cell aggregates on plant surfaces is often regulated by quorum sensing (QS). This cell - cell communication is based on the local density of bacteria via small weight molecules also known as autoinducers (Loh et al., 2002). For communication acylated homoserine lactones (AHL) are used by gram negative bacteria while gram positive bacteria use processed oligo-peptides to communicate (Miller and Bassler, 2001). Some plants are able to produce QS mimic compounds which may be an important element of control for rhizosphere interactions for establishing beneficial communities of bacteria (Hirsch et al., 2003).

1.3 Soil protozoa

In general, protozoa are classified as small unicellular eukaryotes with a maximum size of 50 µm. Forest soil samples typically harbour between 10^4-10^7 active protist individuals per gram dry weight soil (Adl et al., 2006). However, moisture and temperature affect the abundance of terrestrial protozoa directly but also indirectly by modifying the vegetation (Bamforth, 1980). From an ecological perspective protozoa are often classified into four groups: naked amoeba, flagellates, testate amoeba (testacea) and ciliates. The importance of protozoa in terrestrial ecosystems results mainly from their feeding activities (Bardgett, 2005). The direct effect relates to the enhancement of nutrient availability to plants by reducing the amount of nutrients bound in bacterial tissue (Bonkowski, 2003), whereas indirect effects relate to grazing-mediated shifts in the composition and activity of microbial communities.

From studies in freshwater ecosystems it is known that microbes often respond to the presence of predators with morphological shifts (Hahn and Hoefle, 2001; Pernthaler, 2005). The development of filaments and microcolonies are strategies to escape grazing pressure (Hahn and Hoefle, 2001). In terrestrial ecosystems virtually nothing is known whether protozoa selectively feed on certain bacterial species and the impact of selective grazing on rhizosphere bacteria for plant growth (Jjemba, 2001). Recent findings by Jousset et al. (2006, 2008) demonstrated an upregulation of secondary metabolites of *Pseudomonas fluorescens* in the presence of predators. Secondary metabolites, e.g. 2,4-diacetylphloroglucinol (DAPG), are toxic for protozoa and therefore bacteria may benefit from reducing protozoan grazing.

Amoebae colonize with the help of their pseudopodia the water film surrounding soil particles, dying roots, organic material and reach high densities in the rhizosphere of plants (Foster and Dormaar, 1991; Bouwman et al., 1993; Jjemba et al., 2001). At low soil moisture or low food availability they form resistant cysts awaiting more favourable conditions. Estimations of protozoan numbers are made by dilution methods based on the presence and absence of organisms (Darbyshire et al., 1974; Bamforth, 1980). Nonetheless, this method lacks the distinction between active and inactive (encysted) amoebae cells. The cultivation of free living amoebae is not demanding but cultures contain a variety of microorganisms as food source (Weekers et al., 1993). However, for setting up experiments on the effect of protozoa on soil processes and plant growth axenic cultures are needed (Schuster, 2002). Therefore, in the present study axenic cultures of the common bacterivorous soil amoeba *Acanthamoeba castellanii*, a naked amoeba belonging to the *Gymnamoebae* were established.

1.4 Interaction between plant, bacteria and protozoa

Beneficial effects of bacteria-protozoa interactions on plant growth are well documented (Clarholm, 1985; Kuikman et al., 1989, 1990; Jentschke et al., 1995; Bonkowski et al., 2002; Kreuzer et al., 2006) and were generally related to nutritional effects via the microbial loop (Clarholm, 1985). The microbial loop describes the links between nutrients bound in soil organic matter, microorganisms as primary producers, protozoa as major grazer of bacteria and plants (Fig.1). The model assumes that root exudates activate carbon limited microorganisms and induce microbial mobilization of nitrogen bound in soil organic matter. The increase in microbial populations prompts an increase in their consumers (Moore et al., 2003). By grazing on microbes protozoa release up to one-third of the nitrogen consumed by excretion thereby making it available for plant uptake.

Furthermore, a number of studies demonstrated that plants grown in presence of protozoa develop a bigger root system with increased lateral root growth. These effects are similar to effects caused by plant growth promoting hormones, such as auxins (Jentschke et al, 1995; Bonkowski and Brandt, 2002; Kreuzer et al., 2006). Based on these findings, it has been suggested that protozoa feed selectively on rhizosphere bacteria, thereby stimulating certain bacterial strains capable of promoting plant growth by releasing of hormonal substances such as auxin. The increased root volume enhances exudation rates which is assumed to further stimulate bacteria growth. In addition, it has been suggested that protozoan grazing favours nitrifying bacteria resulting in hot spots of nitrate concentrations which also act as a signal for lateral root elongation (Griffiths et al., 1989; Alphei et al., 1996; Zhang and Forde, 1998; Fig. 1).

To conclude, protozoan effects on plant growth are more complex than previously assumed with a combination of nutritional and non-nutritional responses to microbial grazing (Bonkowski, 2004).

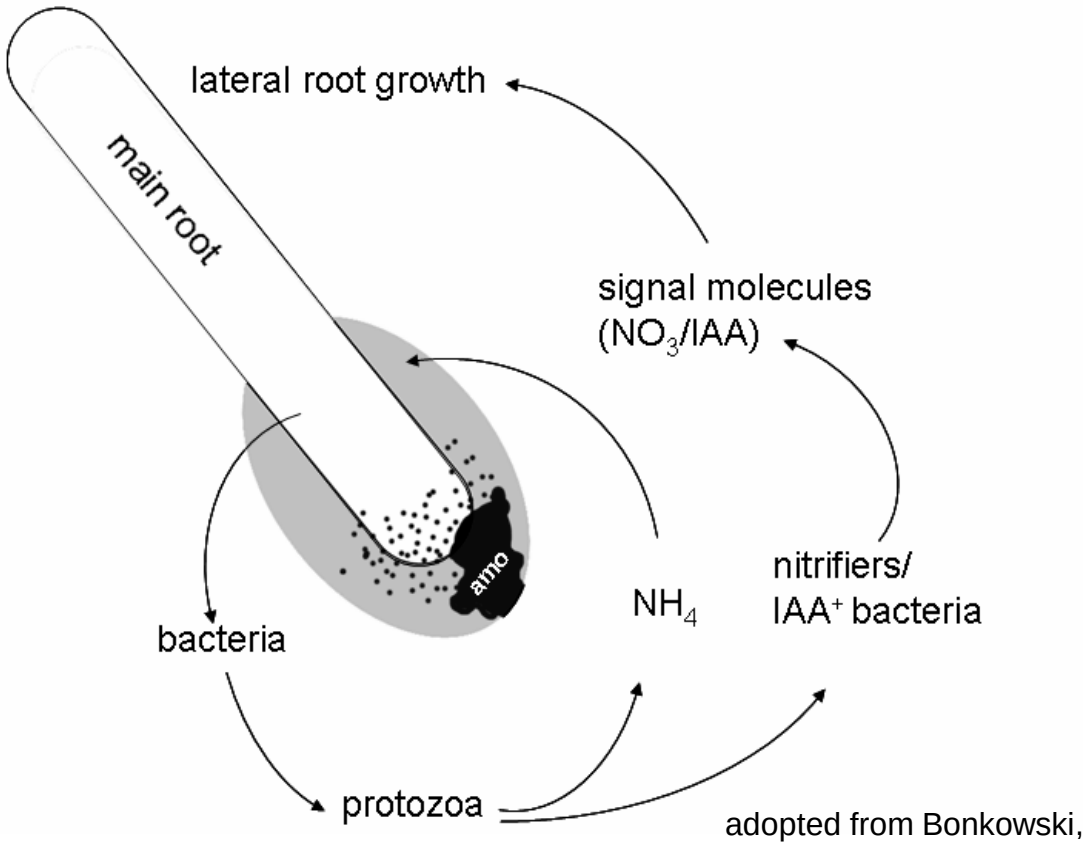

Figure 1 Schematic model of plant-bacteria-protozoa interactions. Plant root exudates soluble carbon and promote bacterial growth including utilization of nitrogen from soil organic matter. Protozoan grazing of the microbes mineralize nutrients which are now available for plant growth. Due to selective grazing by protozoa several bacteria become dominant in the community.

1.5 Methods for characterizing microbial community composition

Investigations on microbial community composition in soil can be structured into microbial, biochemical and molecular approaches (Spiegelman et al., 2005). Microbial or culture-based methods identify bacteria by cell counting, selective growth, and microscopic examinations to provide more general characteristics of the whole community, but only 1% of the microbes are cultivable (Torsvik et al., 1990).

Biochemical methods were based on gas chromatography and mass spectrometry to separate and precisely identify a range of biomolecules, like phospholipid fatty acid analysis (PLFA) of the living part of the microflora (Tunlid and White, 1992; Spiegelman et al., 2005). However, microbial culture-based and biochemical methods provide only information about changes in relative abundance of very broad taxonomic units.

Using the most common target of the 16S subunit of the rRNA cultivation independent molecular methods replaced classical methods in the last decades (Moter et al., 2000; Spiegelman et al., 2005; Schmid et al., 2006). The 16S rRNA harbors conserved as well as variable regions, which are essential for the phylogenetic characterization of different bacterial species. In addition to rRNA based analyses, the use of functional genes provides information about physiological groups of bacteria.

Molecular methods are based on the analysis of nucleic acids directly or indirectly extracted from soil samples. However, biases in extraction efficiencies and the debated reliability of the total soil bacteria diversity questions if results based on these methods are sound (Wintzingerode et al., 1997; Krsek et al., 1999, Niemi et al., 2001; Kirk et al., 2004; Singh et al., 2006). For the rapid, simultaneous and reproducible screening of spatial and temporal shifts in bacterial community compositions molecular fingerprinting methods have been developed. These methods are based on the separation of diverse PCR products amplified from DNA or RNA.

The separations can be assessed on different melting behaviors of double stranded PCR products due to differences in the primary structure of the target gene fragments (denaturing gradient gel electrophorese, DGGE; Oros-Sichler et al., 2006).

1.5.1 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular fingerprinting method which separates PCR-amplified gene fragments according to the differences in their sequence. The most prominent fragments for the screening are the eight different hypervariable (V) regions of the 16S rDNA gene (Yu et al., 2004). PCR-amplified DNA from a pool of taxonomic different genes is run on a polyacrylamide gel with a gradient of denaturing compounds, urea and formamide. DNA passes through an increasing concentration gradient of denaturant and is separated into single strands. The complete denaturing is prevented by a Guanin (G) and Cytosin (C) rich clamp, which is included in one of the primer for the PCR reaction. The mobility decreases with higher denaturant concentrations and DNA will rest when it is almost fully denatured. The position along the gradient is determined by the relative proportion of G+C and Adenin (A) + Thymin (T) in the amplified fragment since G-C bonds are more difficult to denature than A-T bonds (Spiegelmann et al., 2005). The method is widely used but suffers from some limitations. The typically used DNA fragment length is below 500bp which restricts the amount of information available for sequencing after the cut of interesting bands (Muyzer and Smalla, 1998). In addition, no quantitative information about species representation is provided because PCR amplification may lead to an under- or over-representation of the given taxa caused by amplification biases (Wintzigerode et al., 1997; Muyzer and Smalla, 1998). Direct sequencing of bands often fails because of co-migration from different gene fragments and requires clone libraries (Sekiguchi et al., 2001). Statistical analyses are possible with different methods (Fromin et al., 2002), but comparison of different runs is difficult because of individual variations of casted gels. Besides these limitations (see review of Spiegelman et al., 2005) DGGE is an efficient screening method for investigating shifts in bacterial communities and

allowing the identification of community members. Furthermore, the method can be extended to several phylogenetic and functional marker genes.

1.5.2 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) allows the culture-independent simultaneous visualization, identification, enumeration and localization of individual microbial cells (Moter et al., 2000). The method detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell (Moter et al., 2000). The protocol consists of fixation of the microbial cells, hybridization with fluorescently labeled nucleotides at the 5'-end and a washing step to remove unbound probes. Usually the FISH technique is simultaneously performed with up to three probes with different fluorescent labels in one sample. This allows the detection and identification of complex environmental samples due to mixed colors (Schmid et al., 2006). However, there are limitations in applying FISH to soil samples like autofluorescence of soil particles (see Review by Moter et al., 2000). Therefore, a separation of bacterial cells from soil particles with Nycodenz and subsequent immobilization on membranes is suggested by Bertaux et al. (2007) allowing to process a large number of samples with high precision.

1.6 Objectives

Positive effects of bacteria–protozoa interactions on plant growth are well documented (Clarholm, 1985; Kuikman et al., 1989; Bonkowski et al., 2001; Kreuzer al., 2006) and were generally assigned to the microbial loop. The microbial loop describes the release of nutrients from consumed bacterial biomass via protozoan grazing and subsequent nutrient uptake by plants (Clarholm, 1985). In addition to these nutrient based effects protozoa are also known to affect plant performance via

grazing induced shifts on soil bacteria communities (Jentschke et al., 2005; Bonkowski and Brandt, 2002; Kreuzer et al., 2006).

This study investigated the effect of protozoan grazing on soil microbial communities and on plant performance. We conducted several experiments to investigate whether improved plant growth is based on protozoa–mediated shifts in the bacterial community composition or on increased nutrient supply due to protozoan grazing. Therefore, a sand/litter Magenta system was established with full control of rhizosphere community composition of *Arabidopsis thaliana.* The rhizosphere community was represented by a diverse soil bacterial community (Chapter 3 and 4), single bacterial strains (Chapter 4) and a common soil amoeba as bacterial grazer (*Acanthamoeba castellanii*).

The second chapter deals with the impact of different DNA and RNA extraction protocols on denaturing gradient gel electrophoresis (DGGE) analyses since it is known that different extraction methods result in different community patterns of identical microbial communities (Singh et al., 2006). Therefore, a widely used commercial available extraction kit was compared with a method including phenol/chloroform purification. Subsequent data analyses were discussed whether the extraction method, time or protozoan grazing impact the visualization of complex bacterial communities.

The second experiment (Chapter 3) investigated the effect of protozoa–mediated shifts on bacterial communities and the impact on plant performance. We hypothesized that protozoan grazing positively affect plant growth by altering bacterial community composition. Therefore, the established molecular fingerprinting method was applied to analyse bacterial community composition in early stages of plant development of *Arabidopsis thaliana*. Fingerprinting pattern of DGGE were compared and, using fluorescence *in situ* hybridization (FISH), quantitative and

qualitative information on soil bacteria communities was obtained. To clarify whether the protozoan-mediated increase in plant growth is due to changes in bacteria–plant signaling, or by nutrient based effects, a follow-up experiment was conducted. Plants were inoculated with single bacteria strains for which we had indications from DGGE analyses that they may be involved in protozoa-mediated changes in plant growth. Analyses of plant tissue nitrogen and carbon concentration in systems with diverse bacterial communities as compared to single strains provided information about protozoa mediated changes in plant growth.

2 Optimization of DNA and RNA extraction methods from sand-filled microcosms for PCR-DGGE

2.1 Abstract

We investigated the effect of protozoan grazing on bacterial community composition with a molecular fingerprinting method. No differences between grazed and ungrazed communities could be detected with popular extraction methods of genomic DNA of soil bacteria. Therefore, the impact of different DNA and RNA extraction methods for analysing bacterial community composition was determined with denaturing gradient gel electrophoresis (DGGE). Phenol/chloroform extraction resulted in higher DNA yields. The following pattern analyses of the grazed und ungrazed bacterial community separated in two distinct clusters. For RNA extraction a combination of concentration and kit based method proved to be most efficient. The method resulted in high quality RNA with effective reverse transcription into cDNA. The results indicate that different extraction methods need to be tested to get high quality DNA and RNA for further fingerprinting methods of microbial communities. We propose for DNA extraction from a sand substrate the use of a direct method and an indirect method for RNA extraction.

2.2 Introduction

The knowledge of soil microbial diversity is limited in part due to the lack of easy to use molecular methodology for studying soil microbial communities (Kirk et al., 2004). Classical microbiological methods lack the resolution of whole terrestrial bacterial communities because approximately only 1% of soil bacteria can be cultured (Torsvik et al., 1990). In addition, cultivation based methods have further

limitations (Ritz et al., 2007). For instance, soil bacteria are very diverse and it is impossible to design a single growth medium for all bacterial groups (Oros-Sichler et al., 2006). Additionally, natural conditions like quorum sensing or growth competition were ignored.

Therefore, to get a more complete view on the composition of soil microbial communities molecular approaches are indispensable. Direct extraction of DNA or RNA from soil samples provides generally high yields of nucleic acids and a multitude of commercial kits are available. Additionally, lab and field specific studies exist describing improvements and optimizations of protocols (Felske et al., 1996; Niemi et al., 2001; Sessitsch, 2002; Lipthay et al., 2004; Costa et al., 2004).

Extraction methods are either indirect by separation of microorganism before cell lysis or direct, including cell lysis in the soil by chemical (SDS, phenol, enzymes) and physical disruption (sonication, beat beating or freeze-thawing). Liphtay et al. (2004) suggested for direct DNA extraction bead beating as method of choice, but efficiency of cell disruption depends on the energy input during beating, as well as on the type and speed of the beads (Bürgmann et al., 2001). After lysis different purification steps followed, like precipitations (ethanol, isopropanol, polyethylene glycol) and purifications (phenol/chloroform, spin columns) (Krsek and Wellington, 1999). Each of these steps can have an impact on the extracted quality and quantity of DNA and RNA.

Standardizing of extraction procedures is important since different protocols may result in dissimilar community patterns. Studies of terrestrial microbial diversity and composition often use PCR amplification of phylogenetic markers like the 16S rDNA region. To increase the sensitivity it is proposed to use more specific primers for the subclasses of bacteria (Milling et al., 2004). Another problem for investigation of soil

bacteria communities is the co-extraction of inhibitors like humic acids or other substances which often interfere with *Taq* polymerases.

DNA based community analyses detect bacteria irrespective of their viability or metabolic activity. To get insight into metabolically active bacteria, RNA based investigations need to be adopted. The rRNA content of bacteria represents a first approximation of bacterial activity (Duineveld et al., 2000). To obtain RNA more labour intensive extraction methods are needed (Sessitsch et al., 2002) to prevent degradation by enzymes which are omnipresent in soil as well as to the short life-time of mRNA (Costa et al., 2004).

The present study was performed to establish a reproducible and efficient procedure for the extraction of DNA and RNA from a sand/litter mixture with low cell density. As indicator of extraction efficiencies denaturing gradient gel electrophoresis (DGGE) was used. The method separates gene fragments based on different melting behaviour due to differences in the primary structure of the target 16S rDNA gene (Oros-Sichler et al., 2006). The obtained fingerprinting patterns were characterized using diversity indices (Shannon index). We focused our molecular analyses on the recovery of grazing induced shifts in the bacterial community composition due to protozoan grazing. In addition, the quantity of extracted DNA was analysed by measuring the nucleic acid concentration and DNA and RNA quality was checked on agarose gels.

2.3 Materials and methods

2.3.1 Magenta system

Magenta jars (Sigma-Aldrich, St. Louis, USA) were filled with 220 g dry weight of sand (grain size 1.0-1.2 mm) and amended with 0.5 g dry weight of *Lolium perenne* shoot material (45% C, 4% N; ground to a fine powder) to support bacterial growth.

Sand and grasspowder were thoroughly mixed and moistened by adding 6 ml sterile deionized water. The magenta jars were autoclaved three times; in between autoclaving the jars were incubated at room temperature for 48h. Subsequently, the sand substrate was checked for sterility by plating a sterile loop with adherent sand grains on nutrient broth agar (Merck, Darmstadt, Germany). The jars were inoculated with a protozoa-free filtrate of bacteria. The filtrate was obtained by suspending 20 g fresh weight of recently collected rhizosphere soil from a meadow (campus of the Biology Faculty, Darmstadt University of Technology) in 200 ml tap water and filtering the slurry through paper filters (Schleicher and Schuell, Dassel, Germany). Protozoa were subsequently excluded by filtering through 5 and then 1.2 µm filters (Millipore, Schwalbach, Germany). To check for contaminations by protozoa the filtrate was cultured in sterile nutrient broth (Merck) and Neff`s Modified Amoebae Saline (NMAS; Page, 1976) at 1:9 v/v prior to use for three days. For inoculation 1.5 ml of the protozoa-free inoculum was thoroughly mixed with the sand and 0.5 ml of washed axenic amoeba cultures (see Chapter 3) in half strength Hoagland (Sigma, Munich, Germany) were added.

2.3.2 Optimization of DNA extraction from sand

Three DNA extraction protocols were compared in order to optimize DNA extraction from the sand/litter substrate. In the first approach, the FastDNA® Spin Kit for soil (MP Biomedicals, Heidelberg, Germany) was used according to the instructions of the manufacturer. Briefly, 0.5 g sand was filled into multimix 2 tubes. Subsequently, 978 µl sodium phosphate buffer and 122 µl MT buffer were added. Cell lysis was processed in the Fast Prep instrument (MP Biomedicals) for 30 s at 5.5 m s^{-1}. Cell debris were subsequently precipitated by adding 250 µl protein precipitation solution (PPS). The supernatant including genomic DNA was purified from humic substances

with a GENECLEAN® procedure. Briefly, the supernatant was transferred to 1 ml binding matrix suspension and DNA was allowed to bind for 5 min. Subsequently, the matrix was transfered to Spin™Filter and centrifuged (12.200 rpm, 1 min). The resulting silica matrices with the bond DNA were washed with salt/ethanol wash solution (SEWS). The DNA was eluted with 100 µl preheated (60°C) DNA elution solution (DES).

The second protocol was similar to the approach above including a bead beating step, but with the use of different buffers and phenol/chloroform purification. The method is originally described as a combined DNA/RNA extraction from soil samples according to the lysis protocol of Lueders et al. (2004). Portions of sand/litter (0.6 g) were placed in screw-cap tubes containing Lysing Matrix D (MP Biomedicals). Subsequently, 500 µl of 120 mM sodium phosphate buffer and 250 µl TNS (500 mM Tris-HCl, 100 mM NaCl, 10% SDS; pH 8) were added. The cells were lysed by bead beating for 20 s at 6 m s^{-1}(Jossi et al., 2006). After centrifugation (10 min, 13.000 rpm, 4°C) the supernatant was collected, and the sand-bead mixture was extracted a second time by resuspending in 400 µl of 120 mM sodium phosphate buffer. The pooled supernatants were extracted with equal volumes of phenol-chloroform-isoamylalkohol (25:24:1 v/v/v) and chloroform-isoamylalkohol (24:1 v/v). For nucleic acid precipitation, two volumes of polyethylen glycol (PEG 6000; Merck, Darmstadt, Germany) were added and centrifuged (13.000 rpm, 4°C, 1 h). The pellets were washed with icecold 70% ethanol prepared with DEPC water and resuspended in 50 µl elution buffer (Qiagen, Hilden, Germany). Aliquots were checked on agarose gels (gel chamber on ice). Since no RNA was recovered, no further RNA removal steps were conducted for DNA-DGGEs.

With the sand substrate of a second experiment a third protocol was tested. The protocol was identical to the previous phenol/chloroform method but included a

concentration step of ectorhizospheric bacteria from a sand suspension on a membrane filter. Five gram fresh weight sand was suspended in 20 ml sodium phosphate buffer prepared with DEPC water and shaken for 5 min at 150 rpm on a rotary shaker. The supernatant was concentrated on a 0.22 µm filter (Millipore, Schwalbach, Germany) with a vacuum pump.

The yield and purity of the resulting DNA products were compared with a micro-photometer at 260 nm and 280 nm (NanoDrop Technologies, Wilmington, USA).

2.3.3 Optimization of RNA extraction

Previous to RNA extraction all plastic equipment was treated with 0.1 N NaOH in 1mM EDTA over night to denature RNAses, and subsequently rinsed twice with DEPC water before autoclaving. For RNA extraction we compared the FastRNA® Pro Soil-Direct Kit (MP Biomedicals), the Rneasy Plant Kit (Qiagen) and a phenol/chloroform extraction method. The first method was further optimized by concentrating extracted bacteria on 0.22 µm filter (Millipore) as described previously.

For RNA extraction with the FastRNA® Pro Soil-direct kit (MP Biomedicals), 0.6 g fresh weight sand were transferred to Lysing Matrix E tubes. Subsequently, 1 ml RNApro™ soil lysis solution was added. Cell lysis was done by bead beating (Fast Prep® Instrument) for 40 s at 6.0 m s^{-1}. After centrifugation the liquid supernatant was extracted with 750 µl phenol/chloroform (1:1, v/v). The upper aqueous phase was subsequently treated with 200 µl inhibitor removal solution. Genomic RNA was precipitated with 660 µl icecold 100% isopropanol over night at 4°C. The obtained RNA pellet was washed with 70% ethanol (in DEPC water) and air dried at room temperature. After elution in DEPC water cleaning steps followed by addition and subsequently spinning down of 600 µl of a matrix solution and 10 µl of matrix slurry.

The matrix with the bound RNA was washed with buffer and eluted again with 100 µl DEPC water.

RNA extraction with the Rneasy Plant Kit was done according to Jossi et al. (2006). Briefly, 0.5 g fresh weight sand were transferred into FastRNA™ tubes with green caps (MP Biomedicals) and cells lysed by bead beating (FastPrep® Instrument) with 450 µl RLT buffer (Qiagen) for 20 s at 6 m s^{-1} two times with a cooling step of 5 min in between. After centrifugation (13.000 rpm, 5 min) the supernatant was loaded on QIAshredder Spin Columns (Qiagen) and then processed as recommended by the manufacturer.

In a third approach, the RNA extraction was similar to the DNA extraction protocol described above including a phenol/chloroform purification step but with subsequent DNA digestion. For DNA digestion with Dnase I (Promega, Mannheim, Germany) 35 µl of the DNA/RNA extract, 40 µl Dnase I buffer and 10 µl of the enzyme to a final volume of 400 µl were incubated at 37°C for 30 min. Reaction was stopped after incubation with phenol/chloroform purification. Products were checked on agarose gel for remnant DNA and quality of RNA. For increasing the RNA extraction yields, bacteria in the sand were concentrated on a filter as described above. The yield and purity of the resulting RNA products were compared by eye on an Ethidiumbromide (EtBr) stained agarose gel.

2.3.4 Reverse transcription

RNA was reverse transcribed with the Access one-step reverse transcription system (Promega, Mannheim, Germany). Briefly, 2 µl RNA (original,1:3 and 1:5 diluted RNA in DEPC water) and 48 µl PCR Mix consisting of 1x *Tfl* reaction buffer, 0.2 mM dNTP Mix, 50 pM of each primer 630r (5`-cak aaa gga ggt gat cc-3`) and 616V (5`-aga gtt tga tym tgg ctc ag-3`), 1 mM $MgCl_2$, 5 U of AMV reverse transcriptase (Avian

Myeloblastosis Virus) and 5 U of *Tfl* (*Thermus flavus*) DNA polymerase were used in this reaction. Reverse transcription was carried out for 45 min at 48°C, followed immediately by 28 amplification cycles (30 s at 94°C, 45 s at 48°C, 1.5 min at 68°C) and a final extension step (5 min 68°C). PCR products were checked for fragment length on Ethidiumbromide stained agarose gels.

2.3.5 PCR amplification of 16S DNA gene fragments and DGGE analysis

The different extraction methods were compared by DGGE analysis of the PCR products. First, universal PCR amplifications of the 16S rDNA and cDNA were carried out with the primer pair 616V / 630r. The PCR reaction contained 5 µl DNA (1:5 dilution from the original genomic DNA and cDNA) and 45 µl PCR Mix consisting of 1xTaq buffer with KCl, 0.25 mM dNTP Mix, 2% DMSO, 1.2 µg BSA, 50 pM of each primer, 3.5 mM $MgCl_2$ and 0.5 µl Taq (Fermentas, St. Leon-Roth, Germany). The thermal cycling program contained an initial denaturating step at 94°C for 2 min followed by 30 cycles at 94°C for 1 min, at 50°C for 45 s, at 72°C for 90 s (at 72°C for 10 min for the last extention). The 40 bp GC-clamps (5`-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3` at the 5`end of the forward primer) were added using the Hot Start Mastermix (Qiagen) and a twenty-fold dilution of the adequate amplicon. The thermal cycling program contained an intitial denaturation step for 15 min followed by 10 cycles of amplification (at 94°C for 1 min, at 65°C for 30 s with a touchdown of 1.0°C every cycle) and 20 cycles of amplification (at 94°C for 1 min, at 55°C for 30 s, at 72°C for 1 min) and a final extension step (at 72°C for 10 min). PCR products were checked on (EtBr) stained agarose gels.

DGGE analysis of the 16S rDNA was conducted using the DCode™ system (Biorad Inc., Hercules, CA). Three µl of the PCR products were loaded on a 6%

polyacrylamide gel with a linear gradient from 45 to 65% denaturant; where 100% denaturant is defined as 7 M urea and 40% formamide. Gels were run at 60°C and 40 V over night in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) and stained in 0.01% Sybr Green I (Sigma, Munich, Germany) in 1x TAE at room temperature; images made with BDadig compact (Biometra, Göttingen, Germany) were analysed with the BioOne software package (Biorad).

2.3.6 Statistical analyses

For analysing whether the Fast prep and phenol/chloroform extraction affected the yield and purity of DNA as well as number of bands and Shannon diversity one factorial ANOVA was used (SAS 9.1, Cary, USA). Extracted DNA including concentration of bacterial cells on a filter was analysed separately since it was only done in the second experiment (see above). The number of bands was taken as a measure of extracted bacterial taxa. To compare the different extraction methods, the Shannon diversity index was calculated as $H` = - \sum p_i \cdot \ln p_i$, where p_i is the proportion of the total number of bands in the gel.

Effects of protozoan grazing on the two extraction methods were analysed by two-factor ANOVA considering whether protozoa affect DNA yields, band number, and Shannon diversity. The effect of the sampling time (day 0, 3 and 6) on the two extraction methods were analysed with a two factorial ANOVA considering whether extraction time affect DNA yield, band number and Shannon diversity. DGGE data of day 6 for each extraction method were imported into Excel Software (Microsoft Corp.). Matrices generated for principle component analyses (PCA) were structured with band intensities in columns and replicates as rows and analysed with CANOCO for windows (Version 4.5 Microcomputer Power; Ithaca, NY, USA). For statistical

analyses of the amoebae grazing effect on bacterial communities a two level factor discriminant function analysis (DFA) via multidimensional scaling (MDS) was applied.

2.4 Results

2.4.1 DNA yield and purity

Each of the methods yielded fragments with sizes >20 kb. With phenol/chloroform extraction slightly smaller fragements were obtained causing a smear on the agarose gel. DNA yields using the DNA Fast Prep® kit ranged between 7.0 ± 2.7 µg DNA g^{-1} sand fresh weight and the phenol/chloroform extraction between 22.8 ± 13.1 µg DNA g^{-1} sand fresh weight (Tab. 1). When bacteria were concentrated on a filter 40.55 ± 7.2 µg DNA corresponding to 4.12 ± 0.7 µg DNA g^{-1} sand fresh weight were extracted. Compared to the Fast Prep method the highest DNA yields were obtained with the phenol/chloroform extraction method, but this protocol had the highest variations (SD= 2.67 and SD= 16.13, respectively) in yield ($F_{1,91}$=89.54, $p < 0.0001$). The purity of DNA differed significantly as indicated by different A_{260}/A_{280} ratios. DNA extracted with the phenol/chloroform method was of lower purity than the DNA obtained with the customized kit ($F_{1,92}$= 10.17, $p < 0.01$).

Table 1 DNA yield, purity and diversity indices obtained with a kit based extraction method (DNA Fast Prep for soil) and a customized extraction protocol including phenol/chloroform purification. Both methods are based on a cell lysis step via bead beating.

	Fast Prep for soil	Phenol/chloroform
Extraction yield in µg g^{-1} sand fresh weight	7.04±2.7	22.8±13.1
Number of bands	11.5±2.4	15.2±3.9
Shannon index	1.34	1.30
A_{260}/A_{280} ratio	1.89	1.68

2.4.2 DGGE, band numbers and diversity indices

DNA banding pattern, i.e. bacterial community composition, was well reproducible with each of the methods. The number of bands differed between protocols without or with phenol/chloroform purification ($F_{1,76}$=26.85, p<0.0001; Fig. 2, Tab. 1). The calculated diversity indices of the DGGE profiles indicated that the method of extraction did not affect the genetic diversity of the bacteria as measured by the Shannon index (Fig. 2, Tab. 1).

2.4.3 Effect of protozoan grazing

With the extraction method used presence of the protozoan grazer *A. castellanii* did not affect the number of bands ($F_{3,79}$ < 0.01, p = 0.973) or the total DNA yield ($F_{3,89}$ = 0.01, p = 0.935). However, diversity indices were negatively affected by *A. castellanii* ($F_{1,184}$ = 6.06, p = 0.015). Using DNA from phenol/chloroform extraction method for DGGE analyses, the pattern indicate changes in genetic diversity of bacteria (Fig. 1, Tab. 2). The DFA method clearly separated the grazed from the ungrazed treatments only with the phenol/chloroform extraction method ($F_{7,4}$ = 268.8, p<0.0001).

Table 2 Two factor ANOVA table of F-values on the effect of the two extraction methods (Fast Prep and phenol/chloroform) and *Acanthamoeba castellanii* (Protozoa) on DNA extraction yield, number of bands and Shannon diversity

	Yield			Number of bands			Shannon diversity		
Factor	df	F	P	df	F	P	df	F	P
Method	3,89	86.99	**<0.0001**	3,79	27.91	**<0.0001**	3,256	0.84	0.36
Protozoa	3,89	0.01	0.935	3,79	0.00	0.9725	3,256	4.39	**0.037**
Method x Protozoa	3,89	0.04	0.849	3,79	1.30	0.258	3,256	5.35	**0.022**

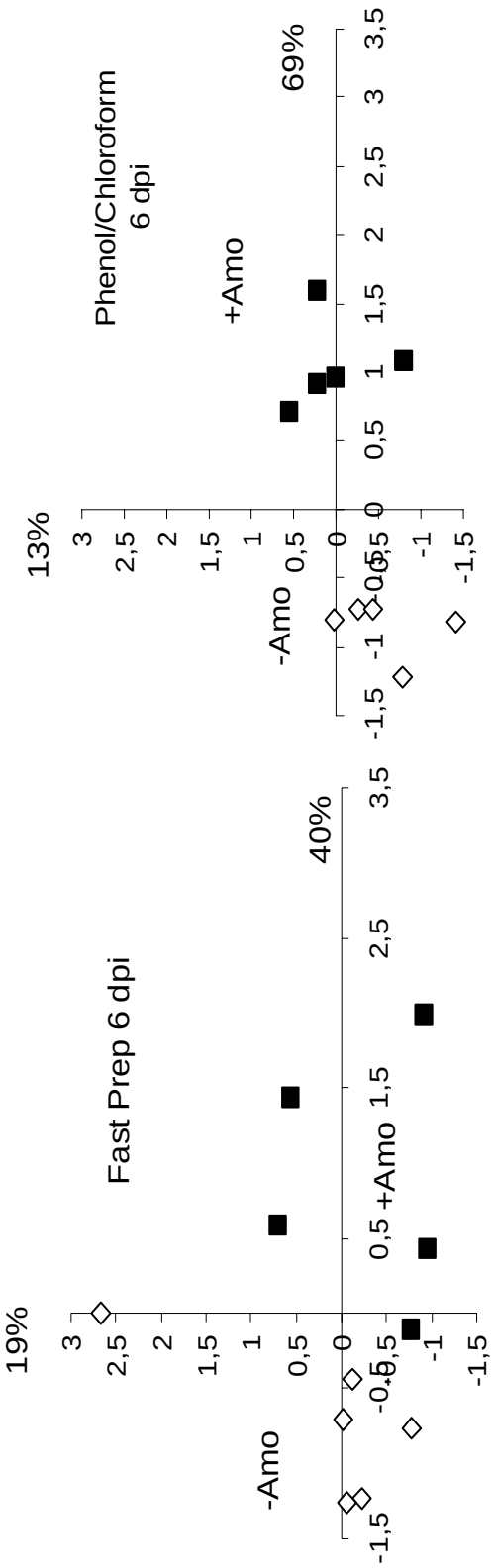

Figure 1 PCA ordination of grazing effects on bacterial communities analysed with DGGE 6 days past transferring of plants. The explained variation by the respective axes is given in %; squares: with amoeba, diamonds: without amoeba

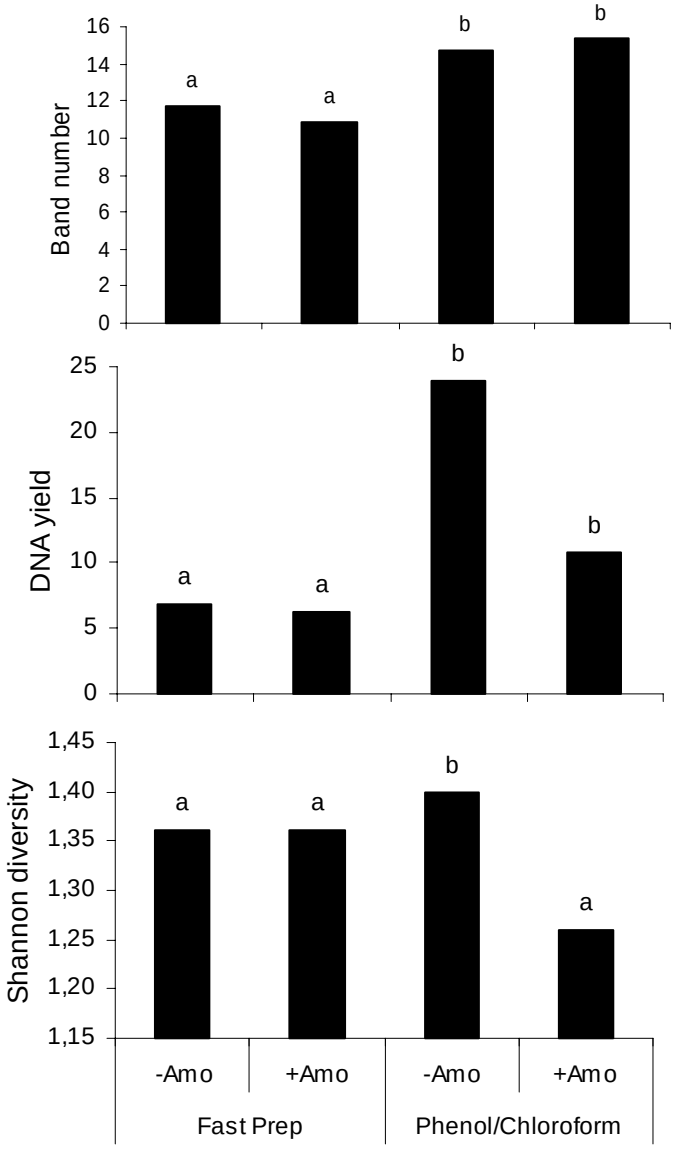

Figure 2 Effects of grazing by *Acanthamoeba castellanii* (-Amo, without amoebae; +Amo, with amoebae) on band numbers, DNA yield and Shannon diversity obtained by two extraction methods (Fast prep and phenol/chloroform). Bars with the same letters are not significantly different (Tukey's honestly significant difference, $P<0.05$)

2.4.4 Effect of sampling time

DNA extraction yields were constant at each of the sampling times obtained with the two extraction methods. As described above number of bands were higher with the phenol/chloroform protocol, but only at day 0 and day 3 with the phenol/chloroform method. Furthermore, the diversity indices were affected by the sampling time only at day 6, since the diversity of bacteria was reduced using the phenol/chloroform extraction procedure. The Shannon index decreased by 28% indicating an outgrowth of similar bacterial ribotypes due to protozoan grazing (Fig. 3, Tab. 3).

Table 3 Two-factor ANOVA table of F-values on the effect of sampling time (d0, d3 and d6) and DNA extraction method (Fast Prep for soil and phenol/chloroform) on DNA yield, number of bands and diversity indices

	Yield		Number of bands		Shannon diversity	
Factor	F	P	F	P	F	P
Method	84.44	**<0.0001**	121.01	**<0.0001**	0.98	0.32
Sampling time	0.26	0.77	50.23	**<0.0001**	8.28	**<0.001**
Method x Sampling time	0.14	0.87	67.64	**<0.0001**	2.14	0.12

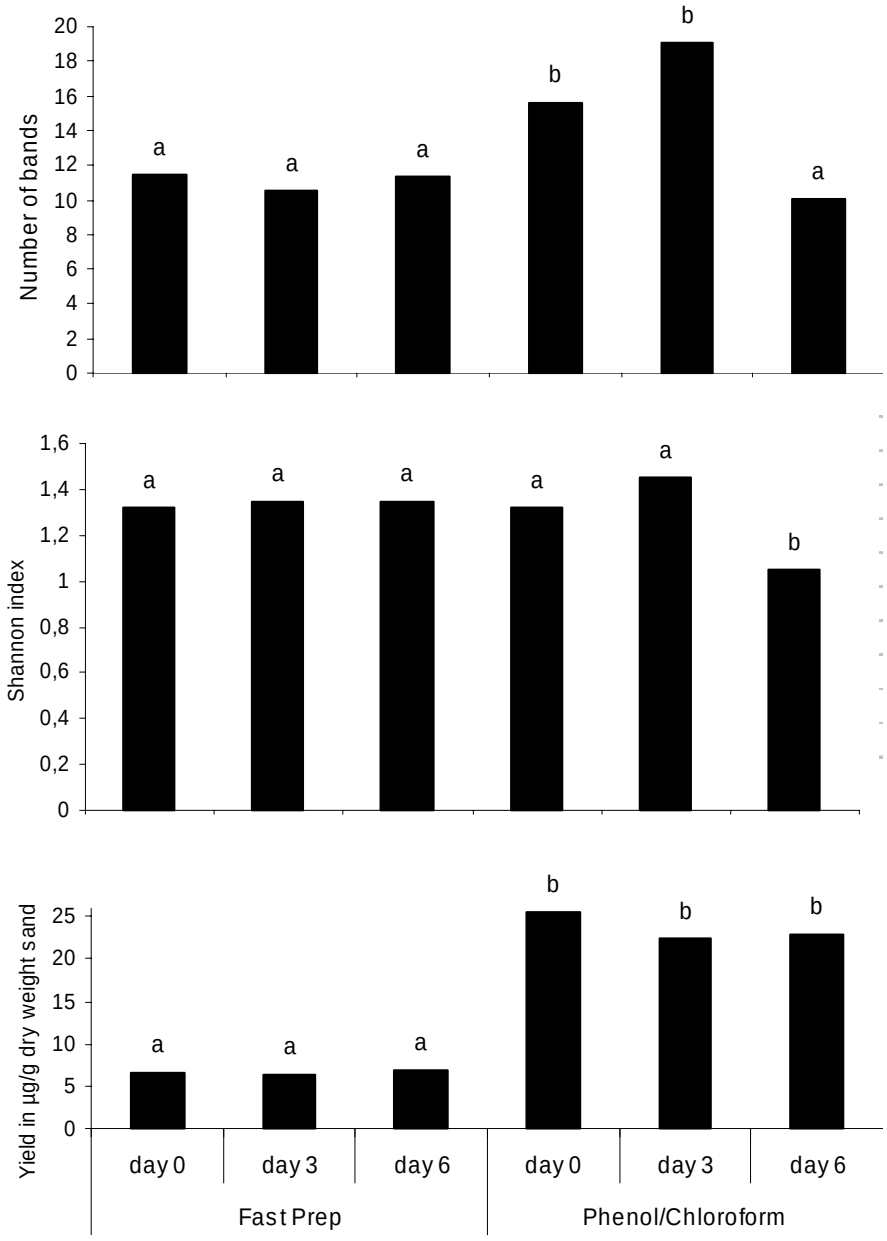

Figure 3 Effects of sampling time (d0, d3, d6) and DNA extraction method (Fast Prep for soil and phenol/chloroform) on the number of bands, Shannon diversity and DNA yield. Bars with the same letters are not significantly different (Tukey's honestly significant difference test, $P<0.05$)

2.4.5 RNA extraction

The Rneasy Plant kit and RNA Fast Prep® kit for soil and the phenol/chloroform purification protocol resulted in very low RNA yields and did not show two sharp bands characteristic for the 16S and 23S rRNA molecules (Fig. 4a, b). Instead, the RNA obtained was frequently degraded.

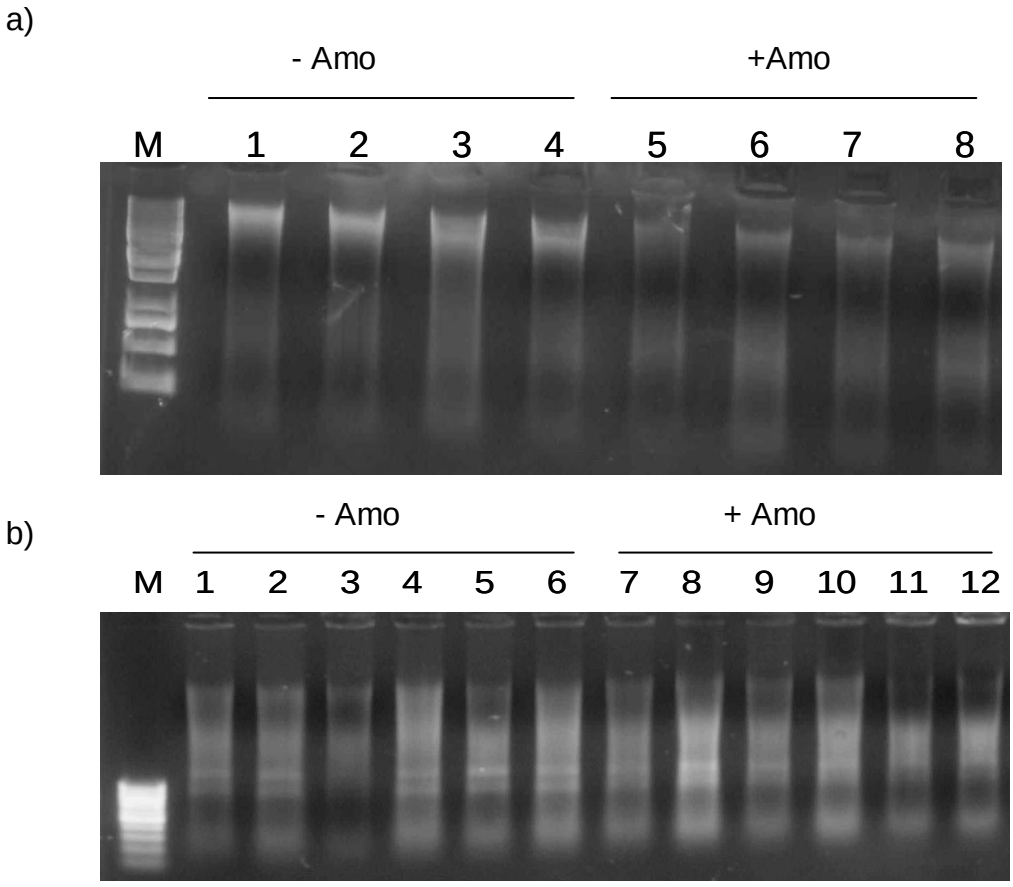

Figure 4 Ethidiumbromide stained 1% agarose gel showing nucleic acid yields extracted by DNA/RNA co-extraction including a phenol/chloroform purification step (a) and in combination with concentration on a filter (b); (a) lanes 1-4 without amoeba (-Amo), lanes 5-8 treatments with amoeba (+Amo), M-1kb Ladder; (b) lanes 1-6 without amoeba (-Amo), lanes 7-12 with amoeba (+Amo), M-low range ladder

In contrast, the RNA extraction with the Fast Prep® kit for soil in combination with concentration of bacterial cells on a filter disk resulted in distinct bands for 16S and 23S rRNA without any degraded fragments (Fig. 5). Furthermore, only RNA from the improved method could be reverse transcribed into cDNA indicating high purity of RNA for further PCR amplifications.

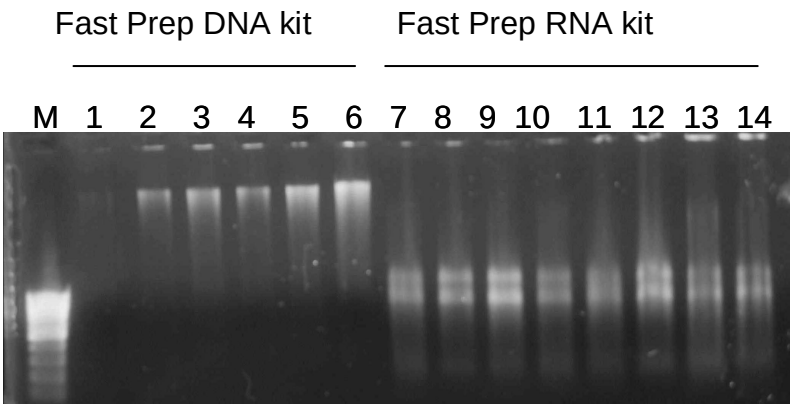

Figure 5 Ethidiumbromide stained 1% agarose gel showing nucleic acid yields extracted with the Fast Prep® kits in combination with concentration on a filter; lanes 1-3 DNA and 7-10 without amoeba, lanes 4-6 and 11-14 DNA with amoeba, M-low range ladder

2.4.6 Comparison of DNA and RNA extraction

To prevent any bias from the extraction methods, each filter with the concentrated sand supernatant was divided into two and analysed with Fast Prep® kits either for DNA or for RNA extraction, respectively (Fig. 5). For comparing the fraction of active bacteria with the total bacteria, PCR products from DNA and cDNA were analysed together on a DGGE gel (Fig. 6). All bands could be detected in both profiles indicating no differences between the active and total bacteria community.

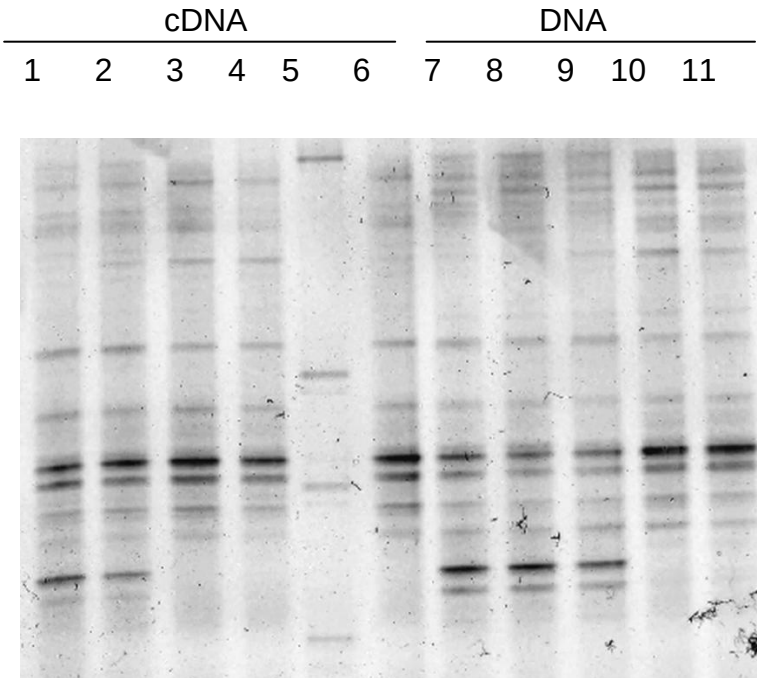

Figure 6 DGGE community analyses of bacterial cDNA and DNA from the sand substrate with 16S rDNA specific primer; lanes 1, 2, 7, 8, 9 without amoeba; lanes 3, 4, 6, 10, 11 with amoeba; lane 5 bacteria standard

2.5 Discussion

Studies on soil bacterial community compositions using DGGE are widely used because of easy handling, low acquisition and production costs. A large number of methods have been published for the extraction of total microbial community DNA from soil and reflects the heterogeneity of soils and subsequent analyses of DNA (Krsek and Wellington, 1999). The widely use of customized kits for soil DNA extraction (e.g. Fast Prep® kit) results in standardization of the protocol but lacks critical evaluation of the reliability of extraction procedure. It has been shown, e.g. that bacterial community composition in soil from acidic sites (Aguilera et al., 2005) or sandy substrates (this study) as indicated by DGGE patterns varies significantly with DNA extraction protocols. In the model plant–bacteria–protozoa test system a sand substrate was used to allow determination of rooting patterns and root biomass of *A. thaliana*. The tiny and fragile roots cannot be harvested undamaged from natural soil.

For analyzing bacterial community composition and grazing induced shifts due to bacterivorous protozoa in this system using DGGE a reliable extraction method was needed. The present study investigated the efficiency and reliability of existing protocols for DNA and RNA extraction applied to a sand/litter substrate with low cell density.

All tested protocols included a bead beating step with or without subsequent phenol/chloroform purification. The quantity and quality of DNA and RNA extracted from our sand/litter substrate varied with the extraction protocol. In general, the quantity of nucleic acid extraction is used as indicator of lysis efficiency of bacterial cells, while the quality determines the extent to which DNA can be used in molecular analysis (Krsek and Wellington, 1999).

Overall, the addition of a phenol/chloroform purification step resulted in the highest yield; however, the fraction of high molecular weight DNA changed towards smaller fragment sizes and the purity of the extracted DNA was decreased. High DNA yields are important detecting bacteria of low density and to ensure that the DNA sample representatively reflects the soil gene pool (Bürgmann et al., 2000). Fragmented DNA may lead to chimaeric PCR products in amplification reactions (von Winzingerode et al., 1997) and co-extracted humic acids (indicated by purity) can inhibit subsequent amplification reactions. Indeed, the banding pattern of DG-gels based on customized Fast Prep® kit and the phenol/chloroform extraction method differed. Contrary to findings by other authors (Aguilera et al., 2005), the phenol/chloroform extraction yielded a higher number of bands in DGGE gels indicating superior extraction efficiency. The findings in the present study confirm the hypotheses that methods, which produce the highest quantity of DNA do not necessarily contain the highest sequence diversity (Stach et al., 2001). In the present study, the diversity indices did not differ between the protocols suggesting that both

methods adequately reflected bacterial community compositions independently of extraction yield.

To verify the reliability of the used methods for our experimental setup we tested whether the DNA extraction method and fingerprinting analyses depend on the duration of the experiment. We analysed three different time points but neither d0 nor d3 affected DNA extraction yield and DGGE pattern as indicated by Shannon diversity, suggesting that the bacterial community of the sand substrate changed little with time. Presumably, the type of DNA extraction with bead beating combined with phenol/chloroform purification yielded representative molecular fingerprinting results allowing answering if protozoa affected bacterial community composition. Only with this extraction protocol significant changes in the community composition were detected. For this reason, all molecular analyses in the following studies on bacteria-protozoa interactions were carried out using the phenol/chloroform extraction method.

Compared to DNA, establishing suitable RNA extraction methods was more difficult. RNA is an indicator of metabolic activity and needs more labour intensive extraction methods. RNA is more susceptible to degrading enzymes and also fragmentation by bead beating may cause problems (Costa et al., 2004). We compared three different extraction methods, two kits from Qiagen and one from Qbiogene. The latter included a phenol/chloroform purification step. Each of the tested methods resulted in the degradation of RNA during bead beating and the nucleic acids were susceptible to degrading enzymes after cell lysis.

To improve the extraction efficiency, we concentrated bacterial cells obtained from our sand substrate on a filter prior to extraction. With this concentration method we obtained high yields of high quality RNA which could easily be reverse transcribed. *Ex situ* isolation has been widely used for extraction of nucleic acids because larger

volumes of soil can be examined (Krsek and Wellington, 1999) and the isolated DNA or RNA from the cell fraction is likely to be cleaner and of higher molecular weight than that obtained by *in situ* methods (Krsek and Wellington, 1999). However, in this study we did not include density gradient centrifugation with e.g. Percoll or Nycodenz to improve the recovery of bacterial cells from the sand/litter substrate. The advantage of the present RNA extraction protocol is the use of the same starting material for both DNA and RNA analyses, because the filter was split into two parts. One part was used for DNA extraction and the other for cDNA synthesis so that both extracted nucleic acids were directly comparable using customized Fast Prep extraction kits for DNA as well as for RNA. Surprisingly, no differences between the total and active community could be observed as indicated by the DGGE banding patterns. Therefore, further investigations on bacteria-protozoa interactions in our sand/litter substrate were limited to DNA based DGGE analyses.

In conclusion, we found a suitable DNA extraction to analyse shifts of bacterial communities due to protozoan grazing. We suggest testing at least two different extraction methods of DNA from soil for molecular fingerprinting analyzes to ensure reliability of the findings.

3 SOIL AMOEBA RAPIDLY CHANGE BACTERIAL COMMUNITY COMPOSITION IN THE RHIZOSPHERE OF *ARABIDOPSIS THALIANA*

3.1 Summary

The roots of growing plants are densely colonized by a diverse community of rhizosphere bacteria which significantly affect plant performance. However, little is known on the quantitative and taxonomic composition of bacterial assemblages in rhizosphere soil and most importantly, on the factors that structure these communities. Bacterivorous protozoa shape the structure of bacterial communities in aquatic and terrestrial ecosystems, and changes in bacterial rhizosphere communities likely are responsible for plant growth-promoting effects of protozoa.

We studied the effects of grazing by a common soil amoeba, *Acanthamoeba castellanii,* on the composition of bacterial communities in the rhizosphere of *Arabidopsis thaliana*. Shoot growth strongly increased in the amoeba treatment compared to sterile grown plants and the treatment with soil bacteria only. Denaturing gradient gel electrophoresis (DGGE) showed that the amoebae rapidly induced changes in the composition of the bacterial community, some bands already disappeared two days past inoculation of amoebae. In general, the DGGE banding pattern demonstrated specific and reproducible changes in bacterial community composition due to amoebal grazing. These changes were further investigated using fluorescence *in situ* hybridization (FISH) with probes for major bacterial phyla. While the number of metabolically active bacteria decreased in presence of amoeba their proportion increased strongly. The decrease in numbers was most pronounced in *Betaproteobacteria* and *Firmicutes*, however *Actinobacteria, Nitrospirae, Verrucomicrobiales* and *Planctomycetales* strongly increased. Other groups, such as

betaproteobacterial ammonia-oxidizers, *Gammaproteobacteria, Alphaproteobacteria* and *Cytophaga-Flexibacter-Bacteroides* did not change in abundance, but DGGE with specific primers for pseudomonads (*Gammaproteobacteria*) revealed both, specific changes in community composition and shifts in functional genes (*gacA*). This is the first study documenting significant and dynamic shifts in rhizosphere bacterial community composition due to protozoan grazing.

3.2 Introduction

Protozoa and bacteria form the oldest predator-prey system on earth, but apart from reports on morphological characters (Pernthaler, 2005) surprisingly little is known on the factors driving grazing resistance (Matz and Kjelleberg, 2006). Virtually nothing is known on the identity of bacterial species that are consumed and those that survive protozoan grazing in soil (Griffiths et al., 1999; Rønn et al., 2002; Kreuzer et al., 2004; Murase et al., 2006). Roughly estimated, one gram of rhizosphere soil may contain up to 10^9 bacteria and around 3000 to 14000 protozoa (Darbyshire, 1974; Bamforth, 1980). A number of studies with bacterial inocula demonstrate a strong coupling between the densities of bacteria and protozoa in soil. Numbers of bacteria generally decline in inverse proportion to the numbers of protozoa until a dynamic equilibrium with bacterial densities of 10^5 – 10^7 g^{-1} is reached (Danso et al. 1975, Habte and Alexander 1977, Acea et al. 1988, Clarholm 1981, 1989). Nevertheless the different bacterial taxa that constitute the rhizosphere bacterial community generally strongly differ in food quality for protozoa (Bjørnlund et al., 2005; Jousset et al., 2006).

Studies in freshwater ecosystems revealed a number of adaptations of bacteria against protozoan grazing, which either prevent ingestion or digestion by protozoa. For example, based on quorum sensing bacteria may form grazing resistant

filaments or built microcolonies (Pernthaler, 2005). In addition, they can escape predation by the formation of surface masking receptors or increasing their motility. Even inside the food vacuole, bacteria can either resist digestion or defend themselves by the release of toxins (Matz and Kjelleberg, 2006). In terrestrial ecosystems investigations on bacteria-protozoa interactions are much more difficult, because direct observations of shifts in morphology or abundance of bacteria are difficult to perform due to the opaqueness and autofluorescence of the soil substrate. Consequently, almost nothing is known on the identity of the bacteria that are consumed or rejected. Soil amoebae have been shown to graze preferentially on gram-negative bacteria (Foster and Dormaar, 1990; Andersen and Winding, 2004). Among gram-negative bacteria, pseudomonads are a particular important group of plant growth promoting rhizobacteria (Lugtenberg et al., 2002). These bacteria can control plant pathogens by e.g. producing antibiotics and inducing systemic resistance against eukaryotes in particular pathogenic fungi (van Loon et al, 1998; Pieterse et al., 2002; Bakker et al., 2007). Indeed, the *gacA* regulated toxin production of pseudomonads has been shown to play a significant role in bacterial defence against protozoan predators (Jousset et al., 2006).

To study the grazing effects of naked amoebae on soil bacteria composition denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH) was applied. Both methods provided culture-independent results about total bacterial populations in soil and proved to show dynamic shifts in terrestrial ecosystems (Duineveld et al., 2001). DGGE has been used extensively to study prokaryotic diversity. Sequencing of prominent bands provides information about the identity and phylogeny of bacterial species. The FISH technique gives quantitative and qualitative information about community composition.

Soil protozoa are known to promote plant growth. Positive effects of bacteria-protozoa interactions on plant growth are well documented (Kuikman et al., 1998; Bonkowski et al., 2002; Kreuzer et al., 2006). These effects are generally assigned to the microbial loop. The microbial loop describes the link between the nutrient turnover of soil organic matter through microbes, the transformation in microbial biomass, grazing by protozoa and transfer of nutrients to plants (Clarholm, 1985). Furthermore, grazing-induced changes in bacterial community composition leading to functional shifts have been previously shown (Bonkowski and Brandt, 2002; Kreuzer et al., 2006). Moreover, Bonkowski and Brandt (2002) and Phillips et al. (2003) suggested that predators of bacteria in the rhizosphere, such as protozoa and nematodes evolved mechanism to manipulate root carbon allocation belowground via grazing–induced shifts in bacterial community composition.

The aim of the present study was to monitor shifts in community composition of soil bacteria as a result of protozoan grazing in the early stages of plant development. We hypothesized that protozoa would significantly change the bacterial community composition. Therefore, we applied phenol/chloroform DNA extractions from the sand substrate and analyses based on PCR amplifications (see Chapter 2). The resulting molecular fingerprints of ungrazed and grazed bacterial communities were compared and distinctive bands were sequenced. These changes were further investigated using fluorescence *in situ* hybridization (FISH) with probes for major bacterial phyla and allowed comparison of grazing induced shifts of metabolic active and total bacteria composition.

3.3 Material and Methods

3.3.1 Magenta system

Magenta jars (Sigma-Aldrich, St.Louis, USA) were filled with 220 g dry weight of sand (grain size 1-1.2 mm) and amended with 0.5 g dry weight of *Lolium perenne* shoot material (45% C and 4% N), which had been grounded to a fine powder to support bacterial growth. Sand and grass powder were thoroughly mixed and moistened by adding 6 ml sterile, deionised water. The Magenta jars were autoclaved three times with incubation periods at room temperature of 48 h in between to kill sporulating bacteria and fungi. The Magenta jars were checked for sterility by plating a sterile loop with adherent sand grains on nutrient broth (NB, Merck, Darmstadt, Germany) agar. The jars were inoculated with a protozoa-free filtrate of a natural bacterial suspension. The bacterial filtrate was obtained by suspending 20 g fresh weight of recently collected rhizosphere soil from a meadow (campus of the faculty of Biology, Darmstadt University of Technology) in 100 ml tap water and filtering the soil slurry through paper filters (Schleicher & Schuell, Dassel, Germany). Protozoa were subsequently excluded by filtering through 5.0 and 1.2 µm Isopore filters (Millipore, Schwalbach, Germany), respectively. To check for protozoan contaminations, the filtrate was cultured for three days in sterile NB (Merck) and Neff`s Modified Amoebae Saline (NMAS) at 1:9 v/v prior to use (Page, 1976). For inoculation, 1.5 ml of the protozoa-free inoculum was thoroughly mixed with the sand, and 0.5 ml of an axenic amoebal culture of *Acanthamoeba castellanii* (as described later) washed in half strength Hoagland (Sigma-Aldrich) were added to the replicates receiving amoeba, resulting in a final density of approximately $1x10^3$ amoebae g^{-1} sand dry weight. The bacterial treatment received each 0.5 ml sterile half strength Hoagland solutions instead. Two days later *A. thaliana* seedlings were transplanted to the

Magenta jars in presence of bacteria, or bacteria plus axenic *A. castellanii* with 10 replicates each. Plants were watered every two days with 1 ml modified Gambourg B5-N containing 0.350 mg/l of ammonium nitrate as described by Zhang and Forde (1998).

3.3.2 Plants

Arabidopsis thaliana seeds were sterilized in 5% $Ca(ClO)_2$ solution (VWR, Darmstadt, Germany) containing 0.1% Tween 80 (VWR) for 10 min, followed by 5 min in 70% ethanol and 5 min in 5% NaOCl (VWR) containing 0.1% Tween 80 (VWR) and were subsequently washed three times with sterile deionised water. Seeds were dried on sterile filter disks and transferred to Square Petri dishes (VWR) with Gambourg medium (3.2 g l^{-1} Gambourg plus vitamins, 0.5% sucrose, 1% plant agar; Duchefa, Haarlem, Netherlands). An agar strip of 3 cm was removed and the Petri dishes were upright positioned. Ten seeds were equally spaced on the small cutting edge of the agar for germination. For vernalization of seeds, the agar plates were incubated at 4°C for 4 d in darkness and subsequently germinated upright in a growth chamber with a photoperiod of 10 h of light (150 µmol $m^{-2}s^{-1}$) at 24°C for 3 weeks before planting into Magenta jars.

3.3.3 Plant performance

Plant rosette diameters were monitored at 0, 3 and 6 days past plant inoculation (dpi), respectively. The mean rosette diameter of each plant was calculated from the average of three different vectors from tip to tip of opposite leaves. Day 0 was subtracted for further statistical analyses. Shoots and roots were dried (at 70°C for 3 days) for biomass determination.

3.3.4 Establishment of an axenic culture of *Acanthamoeba castellanii*

Acanthamoeba castellanii isolated from woodland soil (Göttinger Wald, Lower Saxony, Germany), were cultured with native bacteria in culture flask (Nunc A/C, Roskilde, Denmark) in NB-NMAS at room temperature. An axenic culture was established from this initially bacterized culture of amoeba by offering PGY medium (1% peptone, 1% glucose, 0.5% yeast-extract) containing the antibiotics streptomycin (final concentration of 10 µg ml^{-1}) and gentamycin (final concentration of 15 µg ml^{-1}) for killing the associated bacteria as described by Schuster (2002). The cultures were co-cultivated by subsequent dilution with PGY antibiotic solution every day for one week and one further week in PGY gentamycin solution until the cultures were bacteria-free. The axenic cultures were kept in PGY medium and refreshed every two weeks. Prior to the addition to the sand system amoebae were washed twice in 0.5 Hoagland (Sigma). Accordingly, 15 ml of the PGY solution were centrifuged (1000 rpm, 5 min) and the pellet was resuspended in 10 ml 0.5 Hoagland (Sigma). The centrifugation step was repeated once and the resulted amoeba pellet was resuspended in the required volume.

3.3.5 Enumeration of protozoa

Amoebae were enumerated with a modified most probable number (MPN) method (Darbyshire et al. 1974). Briefly, 5 g fresh weight sand were suspended in 20 ml sterile NB-NMAS and gently shaken for 20 min on a vertical shaker. Threefold dilution series with NB-NMAS were prepared in 96 well microtiter plates (VWR, Darmstadt, Germany) in quadruplicates. The plates were incubated at 15°C in darkness and after 3 and 5 days, respectively; the wells were inspected for presence of protozoa using an inverted microscope (100-320x magnification; Leitz, Wetzlar,

Germany). Densities of amoebae were calculated using automated analysis software as described by Hurley and Roscoe (1983).

3.3.6 DNA extraction from sand

For the extraction of nucleic acids, a combined DNA extraction was performed according to the lysis protocol of Lueders et al. (2004) including a phenol/chloroform purification step (see Chapter 2). Briefly, 0.6 g portions of sand were placed in screw-cap tubes containing Lysing Matrix D (MP Biomedicals, Heidelberg, Germany). Subsequently, 500 µl of 120 mM sodium phosphate buffer and 250 µl TNS (500mM Tris-HCl, 100 mM NaCl, 10%SDS; pH 8) were added. The cells were lysed by bead beating for 20 s and 6 m s^{-1} (Jossi et al., 2006). After centrifugation (13.000 rpm, 4°C, 10 min) the supernatant was collected, and the sand-bead mixture was extracted a second time by resuspending in 400 µl of 120 mM sodium phosphate buffer. The pooled supernatants were subsequently extracted with equal volumes of phenol-chloroform-isoamylalkohol (25:24:1 v/v/v) and chloroform-isoamylalkohol (24:1 v/v). For DNA precipitation, two volumes of polyethylen glycol (PEG 6000; VWR) were added and centrifuged (13.000 rpm, 4°C, 1h). The pellets were washed with icecold 70% ethanol prepared with DEPC water and resuspended in 100 µl elution buffer (Qiagen, Hilden, Germany). Aliquots were checked for presence and quality of DNA on agarose gels stained with Ethidiumbromide (EtBr).

3.3.7 PCR amplification

A nested PCR approach was used to amplify gene fragments with primer pairs as listed in Tab. 1 and Tab. 2 (adopted from Milling et al., 2004). First, universal PCR amplifications of the 16S rDNA were carried out with the primer pair 616V / 630R. The PCR reaction contained 5 µl DNA (1:5 dilution from the original genomic DNA) and 45 µl PCR Mix consisting of 1xTaq buffer with KCl, 0.25 mM dNTP Mix, 2%

DMSO, 1.2 µg BSA, 50 pM of each primer, 3.5 mM $MgCl_2$ and 0.5 µl Taq (Fermentas, St. Leon-Roth, Germany). The thermal cycling program contained an initial denaturating step at 94°C for 2 min, subsequently followed by 29 cycles at 94°C for 1 min, at 50°C for 45 s, and at 72°C for 90 s (at 72°C for 10 min for the last extention).

Table 1 Primersequences for the amplification of DNA for DGGE analysis of bacterial communities

Primer	Sequence 5´ to 3´
616V	aga gtt tga tym tgg ctc ag
630R	cak aaa gga ggt gat cc
F203α	ccg cat acg ccc tac ggg gga aag att tat`
R1492	tac ggy tac ctt gtt acg act t
F948ß	cgc aca agc ggt gga tga
R1492	tac ggy tac ctt gtt acg act t
F311Ps	ctg gtc tga gag gat gat cag t
R1459Ps	aat cac tcc gtg gta acc gt
338f	cct acg gga ggc agc ag
518r	att acc gcg gct gct gg
gacA2	mgy car ytc vac rtc rct gst gat
gacA-1f	tga tta ggg tgy tag tdg tcg a
gacA-1f-GC	gat tag ggt gct agt ggt cga
gacA- 2r	Ggt ttt cgg tga cag gca

GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Myzer et al. 2003) was added at the 5`end of the forward primer

Table 2 Annealing temperature for primer pairs used in DGGE analysis

Specifity	Primer	Annealing temperature in °C
16S rDNA	616V/630R	50
V3-Region 16S rDNA	338f/518r	56
Alphaproteobacteria	F203α/R1492	56
Betaproteobacteria	F948β/R1492	63
Pseudomonads	F311Ps/R1459Ps	63
gacA	gacA2/gacA- 1F	57
gacA-GC	gacA-1f-GC/gacA-2r	52

Different phylogenetic groups were amplified in a second PCR step using the primersystem described by Milling et al. (2004). Specific primer for the 16S rDNA V3-region, *Alphaproteobacteria*, *Betaproteobacteria* and pseudomonads (Tab. 1 and 2)

were amplified using the Hot Start Mastermix (Qiagen). Briefly, 2.5 µl of the purified 16S fragment were added to 12.5 µl Hot Start Mastermix, 1.5 mM $MgCl_2$, 3.125 pM of each primer with a final volume of 25 µl. Thermal cycling started with an initial denaturation step of 15 min, followed by 29 cycles of amplification (at 94°C for 1 min, for 30 s at different annealing temperatures as shown in Tab. 2, at 72°C for 1 min) and a final extension step (at 72°C for 10 min). The functional diversity of pseudomonads was characterized with *gacA* specific primers as described by Costa et al. (2007). Briefly, a nested PCR approach with gacA2/gacA-1f in a first and gacA-1f-GC/gacA-2R in a second amplification step was performed. Subsequently, the required 40–bp GC clamp for DGGE analyses was added using the Hot Start Mastermix (Qiagen) and a twenty-fold dilution of the adequate amplicon. The thermal cycling program contained an initial denaturation step of 15 min followed by 10 cycles of amplification (at 94°C for 1 min, at 65°C for 30 s with a touchdown of 1.0°C every cycle), 20 cycles of amplification (at 94°C for 1 min, at 55°C for 30 s, at 72°C for 1 min) and a final extension step (at 72°C for 10 min). PCR products were checked for fragment length on Ethidiumbromide stained agarose gels.

3.3.8 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rDNA was conducted using the DCode™ system (Biorad, Hercules, CA). Three µl of the PCR products were loaded on a 6% polyacrylamide gel with a linear gradient from 45 to 65% denaturant; where 100% denaturant is defined as 7 M urea and 40% formamide. Gels were run at 60°C and 40V over night in 1x TAE-buffer and stained in 0.01% Sybr Green I (Sigma-Aldrich) in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at room temperature; images made with BDadig compact (Biometra, Göttingen, Germany) were analysed with the BioOne software package (Biorad).

DGGE analysis of eubacterial 16S rDNA fragments amplified from the sand were compared by running 5 (0 dpi) or 6 (3 dpi and 6 dpi) replicates of each treatment with or without amoeba.

3.3.9 DGGE supported clone library

To obtain pure DNA sequences for interesting DG bands with a fragment length larger than 500 bp, a mixture of 16S rDNA fragments were cloned and sequenced. PCR products with the primer pair F948β/R1492 of five replicates per treatment were mixed and cloned into vectors as recommended by the manufacturer. Briefly, 2 µl 2x rapid ligation buffer, 1 µl pGEM® T easy vector, 2 µl PCR product and 1 µl T4 DNA ligase (Promega, Mannheim, Germany) with a final volume of 10 µl were mixed and incubated at room temperature for 1 h. For transformation 2 µl of the ligation mix were assorted with 50 µl of thawed JM109 competent cells (Promega). Tubes were incubated on ice for 20 min and flicked from time to time. Cells were heat-shocked for 45 s in a water bath (42°C) and immediately returned on ice for 2 min. Subsequently, 900 µl of pre-warmed SOC Medium were added and cells were incubated for 1.5 h (180 rpm, 37°C). One hundred µl of transformant cells were plated on $LB_{amp/IPTG/x\text{-}Gal}$ and incubated over night at 37°C. The resulting white colonies were PCR-amplified (with GC clamp) as described above and loaded on DG gels. Their melting behaviours were compared to those of bands present in the original DG gel.

3.3.10 Sequence analysis

PCR products from matched bands were selected for sequencing at Macrogene, Seoul, Korea with the standard primer M13r (5'cag gaa aca gct atg ac'3) and M13f (5'gta aaa cga cgg cca g'3). The nucleotide-nucleotide BLAST search tool (BLASTN) of the National Center for Biotechnology Information (NCBI, USA) was used for all sequences.

3.3.11 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was performed according to Bertaux et al. (2007). Three days past transfer of *A. thaliana* to Magenta jars, the whole root systems were collected and immersed in 2 ml 3% Paraformaldehyde (PFA; Merck) buffered with 1x PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.3). The root systems were vortexed to detach the ectorhizosphere. After removing the roots, the tubes containing the ectorhizosphere were vortexed and incubated at 4°C over night for fixation. The tubes were kept horizontal and the soil spread over the whole length of the tube in a thin layer to ensure good penetration of the fixative. A Nycodenz centrifugation step was performed to separate sand and litter particles from the bacterial community. The latter were subsequently immobilized on white Isopore GTTP membranes (pore size 0.2 µm, Ø 47 mm; Millipore), hybridized and DAPI-labelled as described in Bertaux et al. (2007). Briefly, a pinch of polyvinylpolypyrrolidone (PVPP; Sigma) was added to the fixed sample and shaken horizontally at 300 rpm for 20 min. The suspension was left to settle for 5 min and subsequently 1 ml of the supernatant was transferred to a new tube on top of 1 ml Nycodenz (1.3 g ml^{-1}; Gentaur, Brussels, Belgium). The tubes were centrifuged for 30 min at 13.000 rpm at ambient temperature and 1800 µl from the supernatant was finally collected. Fixed and separated microbes were immobilized on white Isopore GTTP membranes (Millipore) and washed with ca. 4 ml 1x PBS. A dehydration step was done on wet filter disks with ascending ethanol concentrations (50%, 80% and 96% ethanol) for 6 min each, subsequently air dried and conserved at room temperature until further use.

The probes used for hybridization, labelled with cy3, cy5 or fluorescein are listed in Tab. 3. DAPI (4,6-diamidino-2-phenylindoldihydrochloride) labelling enabled to detect all bacteria, including dead and inactive ones, while the EUB I,II,III probe showed all

the FISH detectable bacteria. The latter are alive and presumably active bacteria. To check for unspecific hybridization with each fluorochrome, negative controls were performed with the probes Apis2A-cy3, T-fluo, and U-cy5 specific for aphid endosymbionts not for soil bacteria. The hybridization was performed at 46°C for 1.5 h. The filter sections were washed thereafter individually in 1800 µl washing buffer at 48°C for 10 min, rinsed with distilled water and air dried at 46°C. The membranes were mounted with Citifluor AF1 antifading reagent or CitiDapi (Citifluor AF1 with DAPI) to label all dead, active and inactive bacteria on microscope slides.

Images were taken with a Confocal Laser Scanning Microscope (CLSM Biorad, Radiance 2100 rainbow, operated with LaserSharp 2000), built on a Nikon Eclipse TE2000-U, equipped with a Argon (457 nm, 488 nm) and a Helium Neon (543 nm) laser lines, and two diodes: blue (405 nm) for DAPI excitation and red (637 nm) for Cy5 excitation. A Plan-Apo 60x/1.4 (Nikon) oil immersion objective was used. DAPI and FISH/DAPI labelled bacteria were enumerated with Image J 1.3 by a macro-function to connect different steps automatically (Bertaux et al., 2007), the minimum and maximum parameters of each set of images being re-adjusted to remove the background.

Table 3 rRNA targeted oligonucleotide probes used for hybridization

Probe name	Position*	Sequence (5`-3`)	Theoretical stringency % Formamide	Specifity	Target	Reference
EUB I[a]	338-355	gctgcctccc gtaggagt	35	Eubacteria	16S rRNA	Amman et al., 1990
EUB II[a]	338-355	gcagccacc cgtaggtgt	35	*Planctomycetales*	16S rRNA	Daims et al., 1999
EUB III[a]	338-355	gcagccacc cgtaggtgt	35	*Verrucomicrobiales*	16S rRNA	Daims et al., 1999
LGC354A[b]	354-371	tggaagattcc ctactgc	35	*Firmicutes* (low GC content gram$^+$ bacteria)	16S rRNA	Meier et al., 1999
LGC354B[b]	354-371	cggaagattc cctactgc	35	*Firmicutes* (low GC content gram$^+$ bacteria)	16S rRNA	Meier et al., 1999
LGC354C[b]	354-371	ccgaagattc cctactgc	35	*Firmicutes* (low GC content gram$^+$ bacteria)	16S rRNA	Meier et al., 1999
HGC69a[c]	1901-1918	tatagttacca ccgccgt	25	*Actinobacteria* (high GC content gram$^+$ bacteria)	23S rRNA	Roller et al., 1994
ALF1b	19-35	cgttcgytctg agccag	20	*Alphaproteobacteria*, several members of *Deltaproteobacteria*, most spirochetes	16S rRNA	Manz et al., 1992
BET42a[c]	1027-1043	gccttcccact tcgttt	35	*Betaproteobacteria*	23S rRNA	Manz et al., 1992
GAM42a[c]	1027-1043	gccttcccac atcgttt	35	*Gammaproteobacteria*	23S rRNA	Manz et al., 1992
CFB560	560-575	wccctttaaa cccart	40	*Cytophaga-Flexibacter-Bacteroides*	16S rRNA	O`Sullivan et al., 2001
Ntspa712[c]	712-732	cgccttcgcc accggccttc c	50	most *Nitrospirae*	16S rRNA	Daims et al., 2001
Cya762	762-780	cgctccccta gctttcgtc	65	most *Cyanobacteria*	16S rRNA	Schönhuber et al., 1999
Nso1225	1224-1243	cgccattgtatt acgtgtga	35	betaproteobacterial ammonia-oxidizing bacteria	16S rRNA	Mobarry et al., 1996
Apis2a	nd	cctctttgggta gatcc	35	*Bucherna aphidicola* endosymbiont	16S rRNA	Moran et al., 2005
T16	nd	gccgacatga actcagtaaa	35	T-type endosymbiont	16S rRNA	Moran et al., 2005
U16	nd	gtagcaagct actccccgat	35	U-type endosymbiont	16S rRNA	Moran et al., 2005

* according to Brosius et al., 1981
[a,b] used in equimolar mixture
[c] used with the appropriate oligocompetitor

3.3.12 Statistical analyses

Statistical analyses of plant rosette diameters and amoeba abundance were performed with a three factor ANOVA (SAS 9.1, Cary, Florida, USA); means were compared using post-hoc Tukey tests at $p<0.05$.

DGGE data (band intensities, lane number, and band type) were imported into Excel Software (Microsoft Corp.) for each day separately. Matrices generated for PCA were structured with band intensities in columns and replicates as rows and analysed with CANOCO for windows (Version 4.5 Microcomputer Power (Ithaca NY, USA). The grazing effect of amoeba on bacterial communities was analysed with a two level factor discriminant function analysis (DFA) via multidimensional scaling (MDS).

Statistical analyses of FISH cell counts were performed with STATISTICA 7 (Statsoft, Hamburg, Germany). The experiment consisted of two treatments (plus / minus *A. castellanii*) with five replicates each. For each replicate the number of DAPI and FISH/DAPI labelled bacteria were summed up for five images. Proportions of FISH/DAPI labelled bacteria were calculated as a reference to the total number of DAPI labelled bacteria. To correct for artefactual unspecific hybridizations, the proportion of objects detected in the negative controls was subtracted from the numbers obtained. Prior to ANOVAs, homogeneity of variances was checked by Levene's test and data were log or Poisson transformed if necessary.

3.4 Results

3.4.1 Plant growth

Rosette diameter at 6 days past inoculation (dpi) of *A. thaliana* was significantly affected by the inoculation treatments ($F_{2,27}=30.66$, $p<0.0001$). In presence of amoebae rosette diameter significantly increased from 2.46 ± 0.64 to 3.19 ± 0.79

($F_{1,18}$=5.10; p<0.05). Shoot biomass at 6 dpi of *A. thaliana* was significantly affected by the inoculation treatments ($F_{2,27}$ = 124.64, p<0.0001). In presence of amoebae shoot biomass significantly increased from 1.03 ± 0.12 to 1.38 ± 0.12 ($F_{1,18}$ = 44.62; p<0.0001). Similarly, the root biomass at 6 dpi was significantly affected by the inoculation treatments ($F_{2,27}$ = 32.93, p<0.0001). In presence of amoebae root biomass significantly increased from 0.54 ± 0.06 to 0.65 ± 0.12 ($F_{1,18}$ = 6.82; p<0.05).

3.4.2 DGGE and cloning

High molecular weight DNA was recovered from all treatments. The fingerprints demonstrated good reproducibility and differences between the treatments with and without amoeba were clearly detectable by visual comparison of lanes. Amoeba rapidly changed the composition of the bacterial community. Some bands already disappeared at 0 dpi (i.e. 2 days past inoculation of amoebae). The pattern consisted of 16 main bands compared to 19 bands at 0 dpi and 3 dpi, respectively. At 0 dpi the banding pattern consisted of five stronger bands and a large number of less intense bands, indicating that few bacterial populations dominated while many populations were less abundant. At 3 dpi the number of strong bands had decreased, instead a higher number of weaker bands indicated a more equal abundance of ribotypes (Fig. 1).

At both sampling times, 3 dpi and 6 dpi, the treatments without and with inoculation of *A. castellanii* were clearly separated in a PCA ordination plot (Fig. 2). The separation occurred mainly along the first axis representing 61% and 69% of the overall variation in the dataset of 3 dpi and 6 dpi. The DFA method clearly separated the grazed from the ungrazed treatments at all time points (Tab. 4).

In amoeba treatments, some bands had disappeared, while others appeared instead in comparison to control treatments at both sampling dates, 3 and 6 dpi, respectively.

Table 4 Analysis of DGGE gels performed for 16S rDNA using discriminant function analysis (DFA) via multidimensional scaling (MDS) of grazed and ungrazed bacterial communities of three different time points 0, 3 and 6 dpi after transferring the plants.

Time point	df	F	P
0 dpi	8,1	245.1	**<0.05**
3 dpi	7,4	268.8	**<0.0001**
6 dpi	8,2	1091.9	**<0.001**

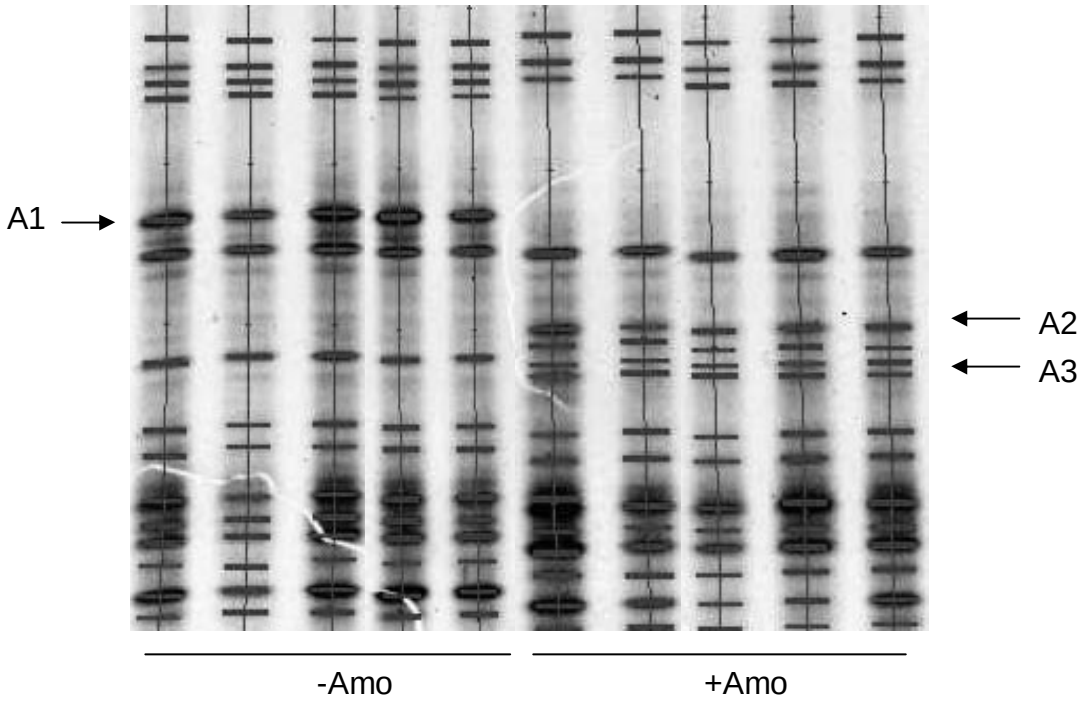

Figure 1 16S rDNA gene fragments specific DGGE fingerprints of 3 dpi representing treatments without amoebae (-Amo) and with amoebae (+Amo), bands were edited with the software; A1, A2 and A3 represents cloned and sequenced bands; A1 *Variovorax* sp.; A2 *Herbaspirillum* sp.; A3 uncultured bacterium

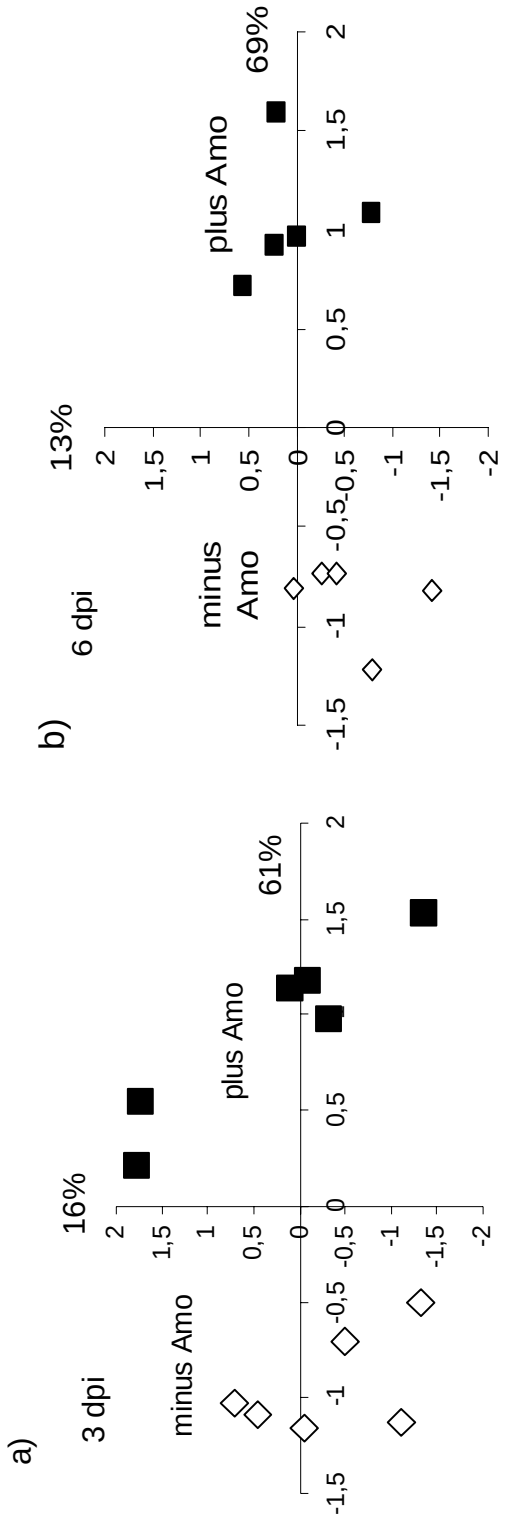

Figure 2 PCA ordination of grazed and ungrazed bacterial communities analysed with DGGE 3 dpi (a) and 6 dpi (b) past transferring of plants). The explained variation is given by the respective axes is given in %; squares: with amoeba; diamonds: without amoeba

After cloning and sequencing different bands at 3 dpi, sequences obtained for A1 (disappearing), A2 and A3, both appearing in amoebae treatments (Fig. 1) showed the highest similarity to *Variovorax* sp. KS2D-23 (99%, member of *Comamonadaceae*), *Herbaspirillum* sp. SE1 (99%) and an uncultured bacterium (95%), respectively.

In addition, for characterizing the established dominant bacterial community in our sand system a total of 60 clones were screened for matched bands in the community pattern and sequenced. Beside four uncultured bacteria, the clones showed highest similarity to *Alpha*-, *Beta*- and *Gammaproteobacteria*. Not all bands could be characterized indicating some bias in the cloning efficiency or insufficient screening of the library (Tab. 4).

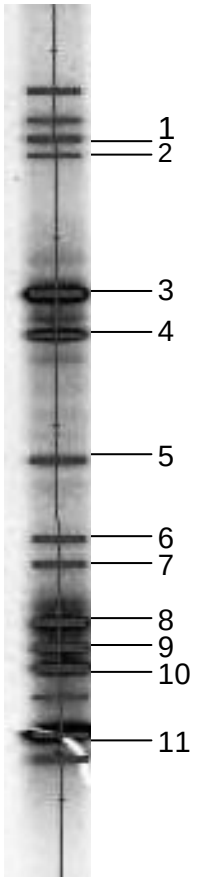

Table 4 Phylogenetic characterization of different clones belonging to bands in the bacterial community pattern at day 0, nd not defined; lateral banding pattern symbolize numbers for clone matching

clone	related bacterial sequence	similarity	Subgroup
1	uncultured bacterium,	99%	nd
2	*Comamonas* sp. KCTC 12005	99%	Beta
3	*Variovorax* sp. KS2D-23	89%	Beta
4	*Stenotrophomonas* sp.	99%	Gamma
5	*Delftia tsuruhatensis*	99%	Beta
6	Uncultured bacterium	99%	Gamma
7	Uncultured soil bacterium	99%	nd
8	Uncultured bacterium	90%	nd
9	*Rhizobium* sp*. H-4*	99%	Alpha
10	*Agrobacterium tumefaciens* strain HAMBI2405	99%	Alpha
11	*Aeromonas salmonicida* subsp*. salmonicida* A449	99%	Gamma

3.4.3 Group specific primers

To reduce the complexity of the banding pattern, specific primer for *Alpha-, Betaproteobacteria* and pseudomonads were used to analyse bacterial communities in samples without or with amoebae. The pattern obtained with *Betaproteobacteria*-specific primer was similar to that obtained with the universal proteobacterial 16S rDNA based DG gel. In contrast, with primers for the *Alphaproteobacteria* fewer bands with 3 strong and up to 8 weak bands were obtained. The pattern for pseudomonads consisted of 4 strong and 14 weak bands. The betaproteobacterial pattern differed strikingly between grazed and ungrazed treatments. However, also the analyses for pseudomonads and *Alphaproteobacteria* showed distinct and repeatable changes in the community composition, which were clearly separated into two different clusters without and with amoeba by UPGMA cluster analysis (data not shown).

3.4.4 *gacA* diversity

The richness of bands in the *gacA* compared to the pseudomonads pattern decreased with up to 15 bands in the pseudomonads specific gels to 3 stronger bands in the *gacA* genes (Fig. 3). Despite no changes in the number of bands were observed in the pseudomonads specific pattern, the pattern of the functional *gacA* gene changed strikingly due to protozoan grazing (Fig. 4) After checking the melting behaviour of 48 *gacA* clones obtained from gacA2/gacA-1F amplified DNA from 0, 3 and 6 dpi, 4 clones were selected for sequencing, which showed the same migration mobility as the bands G1 to G4 (Fig. 4). The gene sequence of G1, G2 and G3 showed similarity to *Pseudomonas fluorescens* PFO-1 (85%, 87%, and 84%). G4 shared 86% similarity with *Pseudomonas fluorescens* Pf-5.

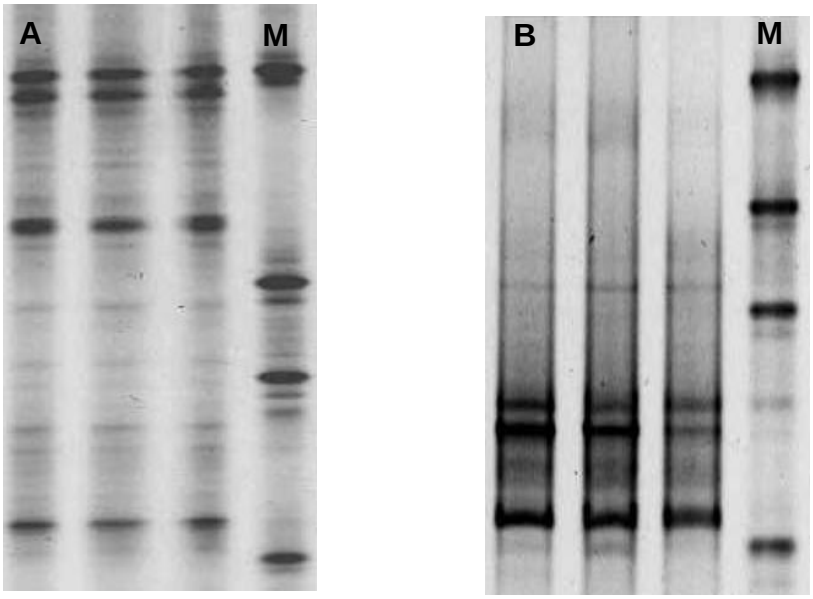

Figure 3 Comparing PCR- DG gels from pseudomonads (A) and *gacA* (B); differences in the marker lanes (M) were due to a 5% increased denaturing gradient in B

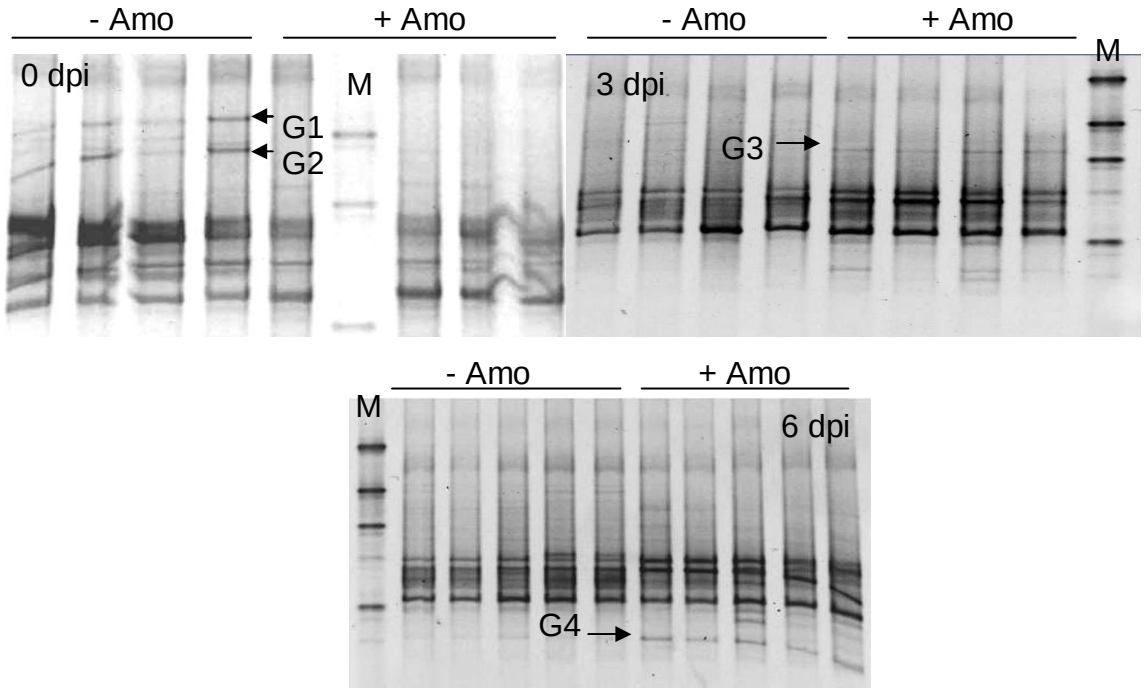

Figure 4 Comparison of *gacA* DGGE fingerprints from three different sampling dates (0, 3 and 6 dpi) of ectorhizospheric bacteria without amoeba (-Amo) and with amoeba (+Amo); M bacterial marker

3.4.5 FISH and Protozoa

After 5 days the numbers of amoeba had increased about 18-fold to $1.8x10^4$ amoeba g^{-1} sand dry weight, suggesting a significant consumption of protozoa. Compared to the control treatment, amoeba reduced the total numbers of bacteria (DAPI) at 3 dpi by 61% ($F_{1,8}$=22.44, p<0.01) and the numbers of active bacteria (EUB I,II,III) by 46% ($F_{1,8}$=11.22, p=0.01; Fig. 5). Despite these reductions the relative proportion of active bacteria increased by 24% ($F_{1,8}$=37.55, p<0.01) in presence of amoeba. The relative abundance of *Alpha*- ($F_{1,8}$=4.27, p=0.07), *Gammaproteobacteria* ($F_{1,8}$=0.48, p=0.50), *Cytophaga-Flexibacter-Bacteroides* ($F_{1,8}$=0.51, p=0.50), and betaproteobacterial ammonia-oxidizers ($F_{1,8}$=0.62, p=0.45) did not significantly change in presence of amoebae. In contrast, *Betaproteobacteria* ($F_{1,8}$=6.01, p=0.04) and *Firmicutes* ($F_{1,8}$=5.27, p=0.04) decreased by half whereas the relative abundance of *Nitrospirae* ($F_{1,8}$=18.93 p<0.01), *Verrucomicrobiales* ($F_{1,8}$=13.07, p<0.01), *Planctomycetales* ($F_{1,8}$=9.03, p<0.05) and *Actinobacteria* ($F_{1,8}$=23.38, p<0.01) increased significantly. Filamentous bacteria belonging to *Verrucomicrobiales*, *Planctomycetales* and *Actinobacteria* were only present in the amoeba treatment (Fig. 5,6).

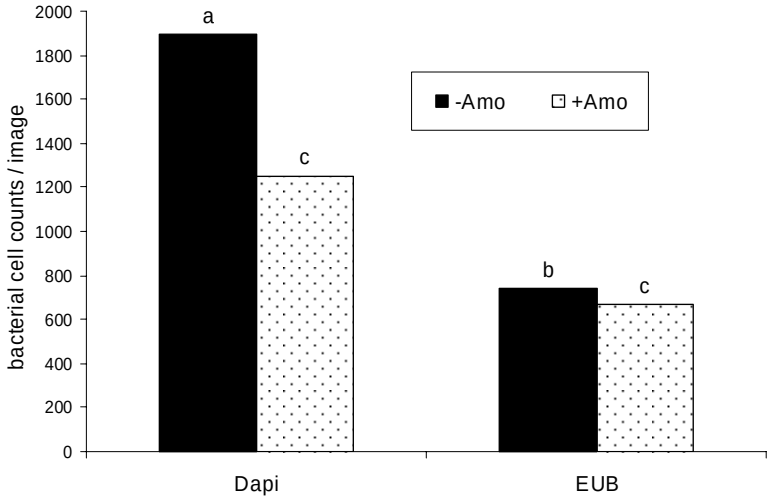

Figure 5 Effects of *Acanthamoeba castellanii* on bacterial populations stained with DAPI (total cell number), EUBI,II,III mix for active bacterial cell; Bars sharing the same letter are not significant different (Tukey's honestly difference, p<0.05)

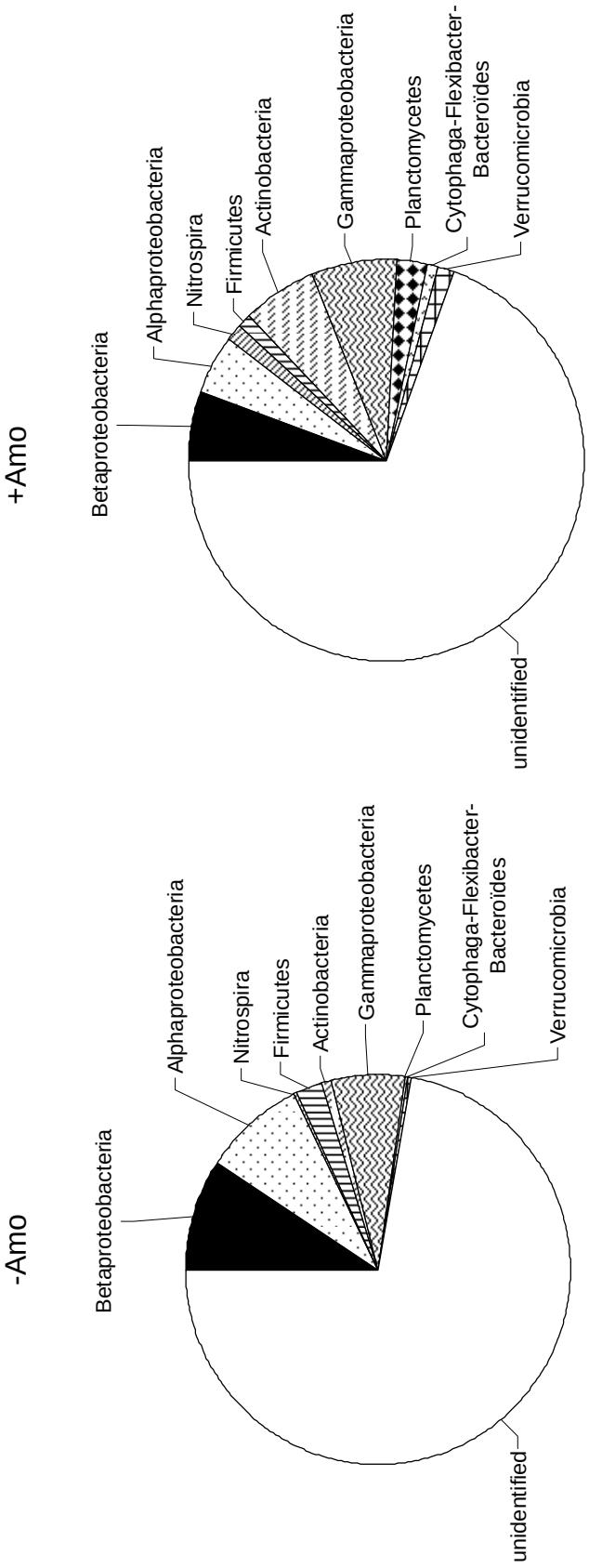

Figure 6 Grazing effects of *Acanthamoeba castellanii* on bacterial populations 3 dpi analysed with Fluorescence *in situ* hybridization (FISH) using probes for dominant bacterial taxa in soil; left without amoeba (-Amo), right with amoeba (+Amo)

3.5 Discussion

The DGGE profiles demonstrated that the inoculation procedure re-established a diverse bacterial community in our Magenta systems, containing all major rhizosphere bacteria (Zul et al., 2007). Cloning and matching of the sequences to excised bands proved the presence of a diverse range of phylogenetic groups and uncultured bacteria. This indicated a successful establishment of natural microbial communities in our experimental system.

DGGE with universal primers showed the loss of bands in amoeba treatments, and simultaneously an appearance of new bands, indicating that certain bacterial taxa were consumed while others gained competitive advantage in presence of protozoan grazers. The repeatable, treatment-specific pattern demonstrated grazing preferences of amoebae for distinct bacterial taxa which were replaced by grazing tolerant taxa in a deterministic way. Remarkably, the bacterial community composition changed very rapidly; some bands has already disappeared two days after addition of amoeba.

Both DGGE and FISH demonstrated that *A. castellanii* most strongly affected *Betaproteobacteria*. Also Kreuzer et al. (2006), using FISH found Acanthamoebae strongly affected *Betaproteobacteria* in the rhizosphere of rice on an agar medium. Similarly, using terminal restriction fragment length polymorphism (T-RFLP) analyses Murase and colleagues (2006) found *Betaproteobacteria* to be preferentially ingested by protozoa in an anoxic rice field soil.

Variovorax sp., a member of the *Comamonadaceae* (*Betaproteobacteria*) had virtually disappeared three days after the addition of the protozoan grazers. In contrast to our findings, *Comamonadaceae* have been described as being grazing resistant in aquatic systems (Hahn and Höfle, 1998; Matz and Kjelleberg, 2005). *Comamonadaceae* are known for their high metabolic versatility in degrading organic

substances (Lu et al., 2006), such as homoserine lactones which play a key role in quorum sensing among gram negative bacteria (Uroz et al., 2003). Similarly, FISH analyses performed at day three testified rapid and significant shifts in the relative abundances for six out of ten dominant taxonomic groups of soil bacteria. In congruence with previous knowledge, amoebae significantly reduced the total cell number by 61%, caused by predation on soil bacteria. As expected too, the fraction of presumably active bacteria increased in the amoeba treatment by 24% indicating that the loss in bacterial numbers due to predation was in part compensated by an increased activity. An increase in energy metabolism of grazed bacterial communities was previously described and may result from selective grazing on dormant bacteria increasing the proportion of younger strains with high metabolism rates (Alphei et al., 1996). In particular, the two phyla most abundant in soil, *Betaproteobacteria* and gram-positive bacteria were strongly affected by protozoan grazing. For gram-positive bacteria, FISH showed an increased relative abundance for Actinobacteria, and a decreased one for *Firmicutes.* The results for *Firmicutes* were surprising, because all gram-positive bacteria are believed to be less preferred by protozoa because of their cell-wall and thus to benefit from protozoan grazing (Griffiths et al., 1999; Rønn et al., 2002; Murase et al., 2006).

Changes in the banding pattern of *Alphaproteobacteria* and *Gammaproteobacteria* did not respond to protozoan grazing and could be confirmed with FISH. Similarly, Kreuzer et al. (2006) also did not find effects of Acanthamoebae on *Gammaproteobacteria*. However, DGGE with specific primers for pseudomonads documented a shift among these *Gammaproteobacteria*, a result consistent with findings of Rønn et al. (2002) studying effects of protozoa on bacterial communities in organic patches. Using the response regulator gene *gacA* in *Pseudomonadaceae* (Sekiguchi et al., 2001; de Souza, 2005; Costa et al., 2007) which is required for the

production of secondary metabolites, such as antibiotics, exoproteases and HCN the functional response of bacteria to protozoan grazing was investigated. In our sand system *gacA* diversity changed with time. Six days past plant inoculation some bands disappeared in the amoeba treatment indicating prey preference for certain Pseudomonad strains. However, one new band occurred after 6 days in presence of amoebae, suggesting a gradual shift to grazing resistant bacteria which became more dominant. Indeed, the *gacA* regulated toxin production of pseudomonads has been shown to play a significant role in bacterial defence against protozoan predators (Jousset et al., 2006).

We clearly demonstrated changes in bacterial community due to grazing, which may be associated with plant growth promoting rhizobacteria. Shoot and root biomass of *A. thaliana* increased significantly in the presence of amoeba. Positive effects of bacteria-protozoa interactions on plant growth are well documented (Kuikman et al., 1989; Bonkowski et al., 2001; Kreuzer et al., 2006). These effects are generally assigned to the microbial loop, which describes the release of locked up nitrogen from consumed bacterial biomass, which is then available for plants (Clarholm, 1985). In addition, rhizosphere bacteria can positively influence plant growth (Bais et al., 2006). It has been repeatedly suggested that protozoa influence plant growth via grazing-mediated changes in bacteria community composition (Bonkowski, 2004). We propose that changes in composition are responsible for plant growth promotion. A variety of mechanisms related to changes in bacterial community composition may be responsible for the stimulation of plant growth and the release of signal molecules, such as hormones, toxins or other metabolites (Kreuzer et al., 2006).

Protozoa-mediated changes in bacterial populations at all three sampling dates suggest that protozoan grazing rapidly structures bacterial community composition as shown with DGGE analyses.

Our sand/litter substrate allows a reliable detection of the fast turnover rates of bacteria due to protozoan grazing including short generation times of both. The rapid shift in the bacterial community two days after inoculation of amoeba was not intensified with extended incubation time. To our knowledge, this is the first study describing constant and rapid changes in soil bacterial community composition due to grazing of *A. castellanii*. Further and more specific investigations are now needed elucidate the coupling between bacterial community shifts and plant growth promotion by protozoa.

4 The effect of protozoa on plant growth: the role of bacterial diversity and identity

4.1 Summary

We investigated if protozoan-mediated changes in plant performance depend on the composition of the rhizosphere bacterial community. *Arabidopsis thaliana* was grown in a sand/litter substrate inoculated with both bacteria (*Comamonas testosteronii* or *Pseudomonas fluorescens* or a diverse bacterial soil filtrate) and *Acanthamoeba castellanii*. In addition to treatments with bacteria and with bacteria plus protozoa sterile control plants were investigated. Plant growth was measured by analysing rosette diameter, shoot and root biomass, and plant tissue nitrogen concentration. Plant rosette diameter was generally increased in the presence of amoebae, whereas root biomass was reduced in both single strain bacterial treatments. Further amoebae increased plant tissue nitrogen concentration resulting in lower C-to-N ratio. Amoeba-mediated changes in rosette diameter and plant tissue C-to-N ratio were not significantly altered between the single strain *Comamonas testosteronii* and diverse bacterial systems suggesting that the effects were independent of bacterial community composition. The results suggest that the amoeba-mediated changes in plant growth were due to an acceleration of nutrient mobilization caused by grazing of the amoeba on bacterial biomass thereby mobilizing nutrients locked in bacterial tissue, i.e. the microbial loop in soil.

4.2 Introduction

It has been widely documented that the presence of bacterivorous soil protozoa in the rhizosphere increases plant growth (Clarholm, 1984; Kuikman et al., 1989, 1990; Alphei et al., 1996; Bonkowski et al., 2000, Kreuzer et al., 2004). The suggested mechanism known as the microbial loop in soil describes the stimulation of plant growth caused by the release of nutrients locked up in bacterial biomass due to protozoan grazing (Clarholm, 1994; Bonkowski, 2004). Soil microbes are strongly carbon limited and profit from carbon resources in root exudates (Wardle, 2002); however, nutrients, like nitrogen and phosphorus, necessary for microbial growth have to be mobilized from soil organic matter. By building up microbial biomass nutrients get locked up in microbial tissue. Grazing on soil bacteria by protozoa release up to one third of the nitrogen locked up in bacterial biomass, thereby making it available for plant root uptake (Clarholm, 1985, 1994; Bonkowski et al., 2000). In addition to increasing nutrient availability, protozoa have been suggested to affect plant growth by changing rhizosphere signaling (Alphei et al., 1996; Bonkowski et al., 2001; Bonkowski and Brandt, 2002; Bonkowski, 2004).

Protozoa change the morphological, taxonomical and functional composition of bacterial communities, thereby altering bacteria-plant relationships which likely feed back to plant growth. Taxonomically, protozoan grazing appears to generally favour *Beta*- and *Gammaproteobacteria* (Rønn et al., 2002; Kreuzer et al., 2006). From a functional perspective, Bonkowski and Brandt (2002) suggested that protozoan grazing favours auxin producing bacteria resulting in increased lateral root growth. Furthermore, nitrogen oxides produced by rhizosphere bacteria may also act as signalling compound initiating specific plant responses (Lamattina et al., 2003). Enhanced nitrogen availability in the rhizosphere due to protozoan grazing may lead

to increased bacterial nitrogen metabolism including dissimilatory nitrate reduction and the production of nitrous oxide (NO). Nitrous oxide has been demonstrated to cause plant growth promoting and root growth modulating effects in rhizobacteria-root interactions (Creus et al., 2005). Altered plant growth, in particular that of roots, is likely to feed back to the bacterial community via changes in root exudation and these effects may foster protozoa as major bacterial grazer.

In a previous experiment we analysed the bacterial community composition in a sand/litter system with *Arabidopsis thaliana* affected by *Acanthamoeba castellanii*. DGGE profiles from ungrazed and grazed bacterial communities resulted in specific and reproducible changes in the banding pattern of Proteobacteria. Bands which disappeared in the presence of protozoa were related to *Variovorax* sp. (*Comamonadaceae*) a member of *Betaproteobacteria*. Additionally, changes occurred in the functional screening of the *gacA*-gene fragment, a phylogenetic marker for pseudomonads (*Gammaproteobacteria*). The bacterial community composition shifted rapidly since some bands already disappeared two days after the inoculation of amoebae.

To prove if increased plant growth in the presence of protozoa is caused by protozoan mediated shifts in the bacterial community composition or based on increased nutrient mineralization due to protozoan grazing we used a recently established sand/litter system with *A. thaliana* which allows investigation of plant-bacteria-protozoa interactions at semi-natural conditions with full control of the rhizosphere community composition (Krome et al., submitted). The system was inoculated with single bacterial strains for which we had indications that they may be involved in protozoa-mediated changes in plant growth, i.e. *Comamonas testosteronii (Comamonadaceae)* and *Pseudomonas fluorescens*. *Pseudomonas fluorescens* and certain other pseudomonads are known as pathogenic as well as plant growth

promoting for cultivated plants. Since microarray results showed a decrease in plant stress response when growing with diverse bacteria and *A. castellanii* (Krome et al., in prep.) and DGGE showed that increased plant growth in presence of *A. castellanii* is associated with specific shifts in bacterial community composition we hypothesized that protozoan-mediated increase in plant growth is due to changes in bacteria-plant signaling, not only by nutrient based effects (microbial loop). We proved this assumption by investigating protozoa-mediated changes in plant growth and nutrient uptake in systems with diverse bacterial communities as compared to single species of bacteria of different phylogenetic affiliation (*Beta*- and *Gammproteobacteria*).

4.3 Material and methods

4.3.1 Magenta system

Magenta jars (Sigma - Aldrich, St. Louis, USA) were filled with 220 g dry weight of sand (grain size 1-1.2 mm) and amended with 0.5 g dry weight of *Lolium perenne* shoot material (45% C, 4% N) which had been grounded to powder to support growth of bacteria and of amoeba feeding on these bacterial populations. Sand and grass powder were thoroughly mixed and watered by adding 6 ml sterile distilled water. For sterilization the Magenta jars were autoclaved three times; in between autoclaving the jars were incubated for 48 h at room temperature. The jars were checked for sterility by plating a sterile loop with adherent sand grains on nutrient broth agar (NB with 1.5% agarose; Merck, Darmstadt, Germany).

4.3.2 Bacterial inoculum

For establishment of a diverse rhizosphere bacterial community the sand/litter system was inoculated with soil bacterial filtrate. The filtrate was obtained by suspending 20 g fresh weight of recently collected rhizosphere soil from a meadow

(campus of the Faculty of Biology, Darmstadt University of Technology) in 200 ml tap water and filtering the soil slurry through paper filters (Schleicher and Schuell, Dassel, Germany). Protozoa were subsequently excluded by filtering first through 5.0 and then through 1.2 µm Isopore filters (Millipore, Schwalbach, Germany). To check for protozoan contaminations, the filtrate was cultured for three days in sterile nutrient broth (NB, Merck) and Neff's Modified Amoebae Saline (NMAS) at 1:9 v/v prior to use (NB-NMAS; Page, 1976). Single colonies from two bacteria strains, *Comamonas testosteronii* (ATCC 17454) and *Pseudomonas fluorescens* WCS 417r (kindly provided by Corne Pieterse, Institute of Environmental Biology, University of Utrecht) were inoculated in 5 ml NB media (Merck) and incubated over night. One ml of the culture medium was transferred to preheated 50 ml NB media and incubated again (180 rpm, 30°C, 4 h). Bacterial cells were pelletized (4500 rpm, 2 min) and washed two times with 0.5 Hoagland solution (Sigma-Aldrich, St. Louis, USA). Optical density (OD) was measured at 580 nm and adjusted to 0.1 by subsequent dilution.

4.3.3 Experimental setup

The experiment was setup in a four factorial design with the factors Amoeba (with and without), Soil filtrate (with and without), *C. testosteronii* (with and without) and *P. fluorescens* (with and without). All two and three factor combinations but not the four factor combination was included. Ten replicates were set up per treatment except for the Soil filtrate x *P. fluorescens* and the Soil filtrate x *C. testosteronii* (with and without amoebae) which were set up with five replicates only as a result of restricted size of the growth chamber.

For bacterial inoculation 1 ml of the bacterial soil filtrate or 200 µl (OD = 0.1) single strain solution were added to the sand/litter substrate. Mixtures of bacterial inoculations consisted of 1 ml bacterial soil filtrate and 200 µl of the single strains (*C.*

testosteronii, P. fluorescens) or both strains mixed in equal shares (2 x 200 µl) and were thoroughly mixed with the sand.

Cell densities of single bacteria strains in the sand/litter substrate were checked by the plate count method on NB agar by plating 100 µl of a 1:10 dilution (sand/0.5 Hoagland). After reaching a density of 10^6 cfu g^{-1} fresh weight sand in the single strain treatments 0.5 ml of a solution from axenically cultured *A. castellanii* were added to amoeba treatments. Two days later *A. thaliana* plants were transferred to the Magenta jars (see above). The jars were watered every two days with 1 ml modified Gambourg B5-N with 0.350 mg l^{-1} of ammonium nitrate as described in Zhang and Forde (1998).

4.3.4 Plants

Seeds of *A. thaliana* were sterilized by immersing into 1% CaOCl for 5 min and for another 5 min in 70% ethanol. Then the seeds were washed three times with sterile deonized water. Seeds were dried on sterile filter disks and transferred to square Petri dishes (VWR, Darmstadt, Germany) with Gambourg medium (3.2 g l^{-1} Gambourg plus vitamins, 0.5% sucrose, 1% plant agar; Duchefa, Haarlem, Netherlands). From one side of the Petri dishes an agar strip of 3 cm was cut off and the Petri dishes positioned upright. Ten sterile seeds of *A. thaliana* were placed equally spaced on the edge of the agar (see above) for germination. For vernalization the agar plates were incubated at 4°C for 4 d in darkness and subsequently for germination placed upright in a growth chamber with a photoperiod of 10 h of light (150 µmol $m^{-2}s^{-1}$) at 24°C for 3 weeks before planting into Magenta jars. The Magenta jars were incubated at 22°C/19°C day/night regime of 10/14h and randomized every other day.

4.3.5 Analyses

Plant rosette diameter was monitored after 0 and 7 days. The mean rosette diameter of each plant was calculated from the average of three different vectors from tip to tip of opposite leaves; measurements from day 0 were subtracted to give growth increments. At harvest shoots and roots were dried (70°C, 3 d) for biomass determination. Carbon and nitrogen concentration in plant tissue (pooled leaves and roots) were measured from dried plant material using an element analyser (Carlo Erba 1400, Milan, Italy).

Amoebae were enumerated at day 0 and 7 with a modified most probable number method (MPN; Darbyshire et al., 1974). The sand was suspended by adding 20 ml of sterile NB-NMAS to 5 g fresh weight sand. The suspension was gently shaken at 160 rpm for 20 min on a vertical shaker. Threefold dilution series with NB-NMAS were prepared in 96 well microtiter plates (VWR, Darmstadt, Germany) in quadruplicates. The plates were incubated at 15°C in the darkness and wells were inspected for presence of protozoa using an inverted microscope (100-320x magnification; Leitz, Wetzlar, Germany) after 3 and 5 days. Densities of amoeba were calculated using automated analysis software as described by Hurley and Roscoe (1983). Bacterial cell numbers were enumerated by spreading out dilution series from 10^{-6} to 10^{-8} on 1/10 TSB-Agar (VWR, Darmstadt, Germany) in three x three replicates per dilution and treatment. The initial dilution was prepared by suspending 1g fresh weight sand with 9 ml Hoagland (Sigma-Aldrich, St.Louis, USA).

4.3.6 Statistical analyses

Bacterial cell numbers and amoebae cell densities were analysed using GLM (SAS 9.1 Institute, Cary, USA). To investigate whether *A. castelanii* or the treatment specific bacterial inoculum impact plant growth a complete factorial design with the

factors amoeba (with and without), soil filtrate (with and without), *C. testosteronii* (with and without) and *P. fluorescens* (with and without) excluding four factor interactions was conducted. As dependent variables individual root and shoot biomass, shoot diameter and nutrient concentration (carbon, nitrogen, C-to-N ratio) were analyzed. Statistical analyses were performed using the GLM procedure in SAS 9.1. Differences between means were inspected using Tukey's honestly significant difference test at $p<0.05$.

4.4 Results

4.4.1 Density of *Acanthamoeba castellanii* and cell numbers of single bacterial strains

The initial cell density of *A. castellanii* ranged from 1000 to 15000 amobae g^{-1} dry weight sand. At both sampling dates the density significantly varied between treatments ($F_{5,24} = 6.98$; $p = 0.0004$ and $F_{5,24} = 10.40$, $p < 0.0001$ for day 0 and 7, respectively). Except for treatments with the diverse bacterial community of the soil filtrate, where the number of amoebae remained similar to the initial density, the number of *A. castellanii* increased in each of the treatments. At day 0 densities of amoebae were at a maximum in treatments with diverse bacterial community (Fig.1). In contrast, at day 7 the numbers of amoebae were at a maximum in the *P. fluorescens* only treatment (Fig.1).

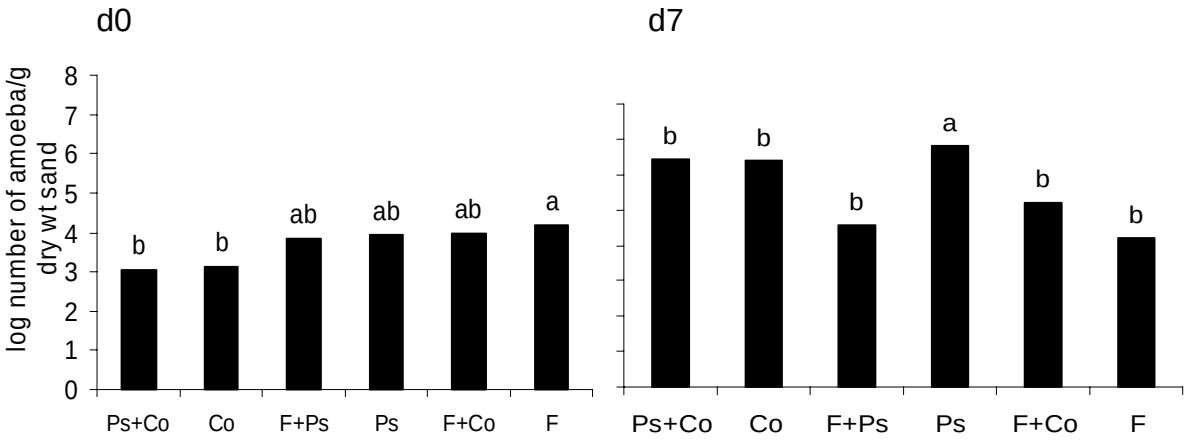

Figure 1 Numbers of amoebae in sand/litter substrate inoculated with *Pseudomonas fluorescens* (Ps), *Comamonas testosteronii* (Co) and bacterial soil filtrate (F) at the start (day 0) and the end (day 7) of the experiment; bars sharing the same letter are not significantly different (Tukey's honestly difference, $p<0.05$

Bacterial cell numbers were counted at day 7 from the *C. testosteronii* and *P. fluorescens* only treatments; without protozoa they reached densities of 7.32 x 10^8 and 5.23 x 10^8 cfu g^{-1} dry weight sand, respectively. In presence of amoebae the density of both *C. testosteronii* ($F_{1,4}$ = 9.64, $p<0.0361$) and *P. fluorescens* ($F_{1,4}$ = 142.1, $p<0.0003$) were significantly decreased to 2.73 x 10^7 and 4.42 x 10^7 cfu g^{-1} dry weight sand, respectively.

4.4.2 Rosette diameter and plant biomass

Generally, amoebae significantly increased the rosette diameter of *A. thaliana*. The bacterial soil filtrate (Amo x Filtrate; Tab. 1, Fig. 2), *P. fluorescens* (Amo x *P. fluorescens*; Tab. 1, Fig. 2), and *C. testosteronii* (Amo x *C. testosteronii,* Tab. 1; Fig. 2) significantly increased rosette diameter of *A. thaliana* but only if amoebae were also present, by 11%, 11% and 15%, respectively. Additionally, *C. testosteronii* significantly increased rosette diameter but only in the single strain treatments (significant Filtrate x *C. testosteronii* interaction, $F_{12,65}$ = 7.37, p = 0.009).

Shoot biomass was significantly increased by amoebae but only in the single strain treatments (significant Amo x Filtrate interaction, Tab. 1, Fig. 3). The bacterial soil

filtrate alone significantly decreased shoot biomass by 15%. In addition, shoot biomass depended on both *C. testosteronii* and *P. fluorescens*. In single strain treatments both species increased shoot biomass (increase in shoot biomass with *P. fluorescens* by 26% and with *C. testosteronii* by 23%, respectively) whereas in presence of both shoot biomass remained unaffected (significant *P. fluorescens* x *C. testosteronii* interaction; Tab. 1).

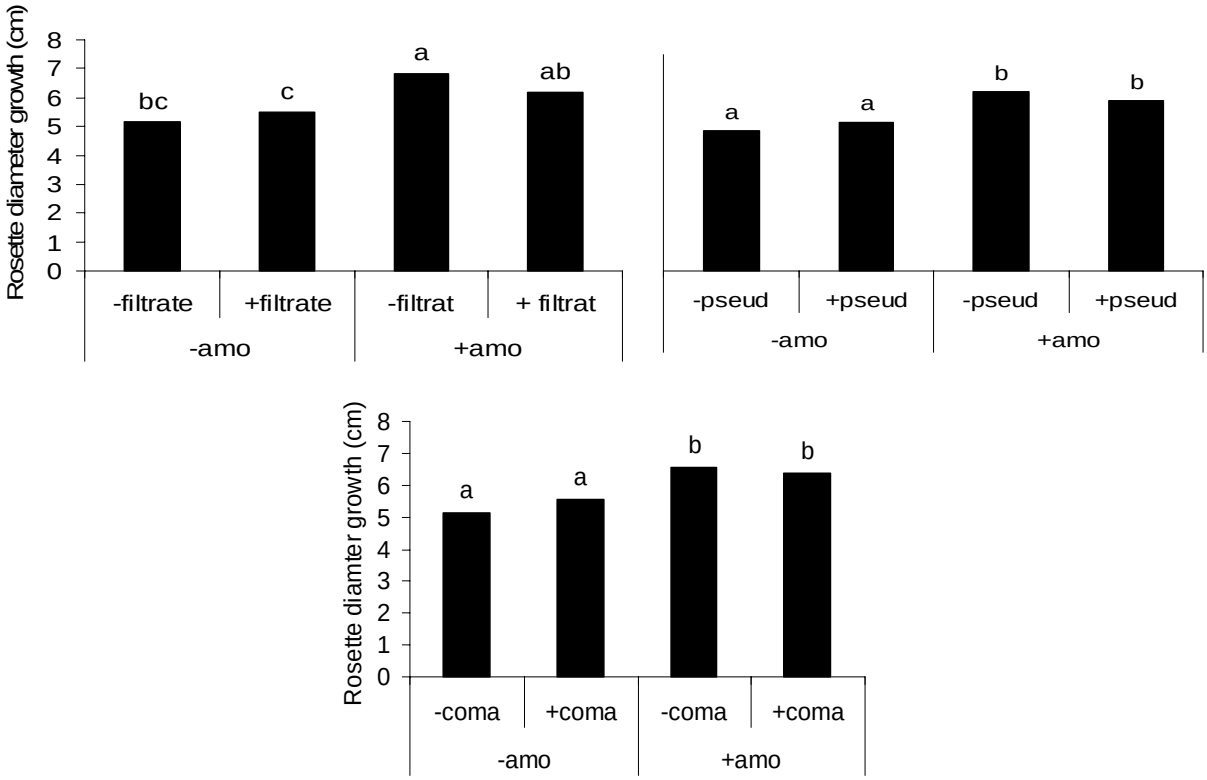

Figure 2 Effect of amoebae (+/- Amo) on rosette diameter of *Arabidopsis thaliana* inoculated with bacterial soil filtrate, *Pseudomonas fluorescens* (+/- pseud) and *Comamonas testosteronii* (+/- coma). Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, $p<0.05$)

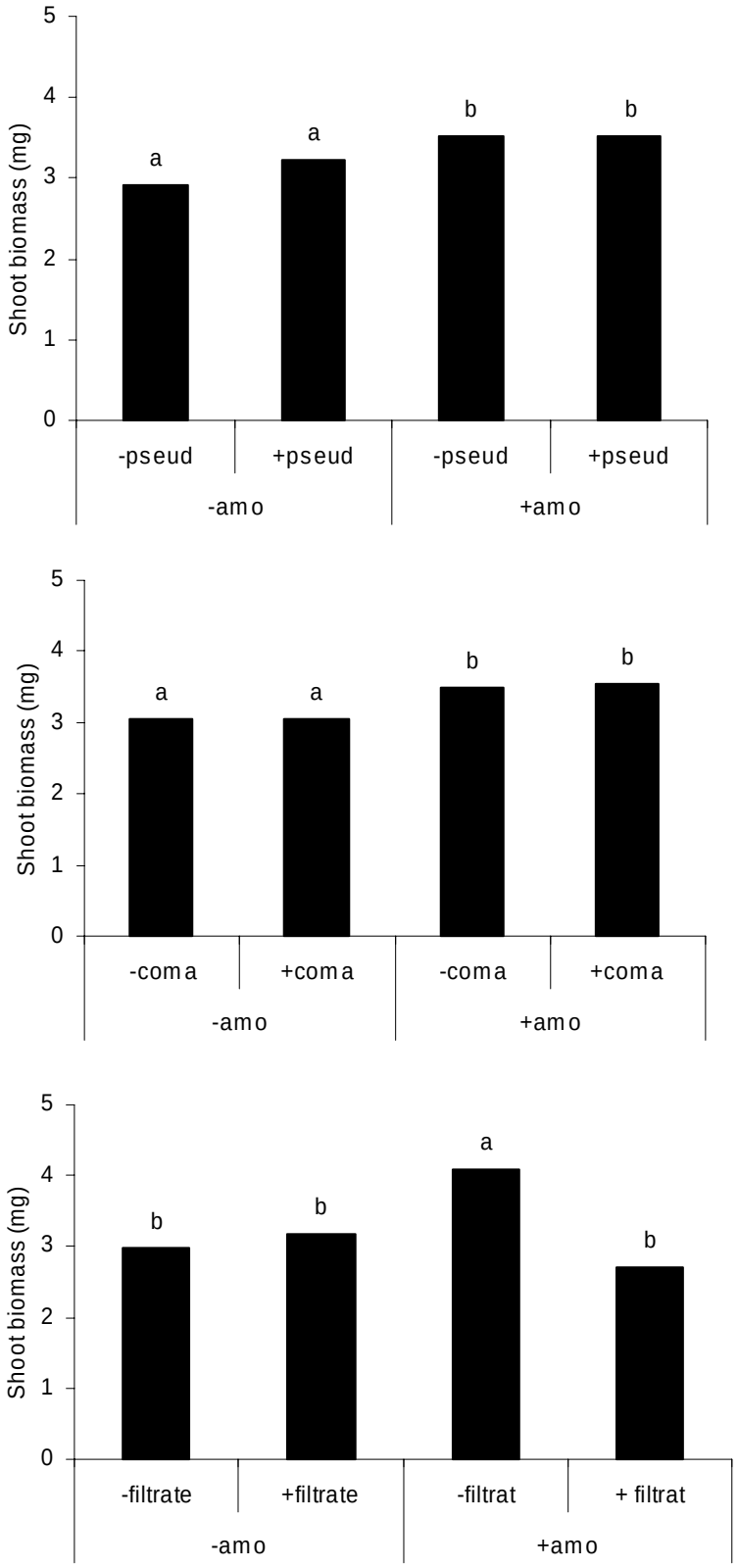

Figure 3 Effect of amoebae (+/- amo) on shoot biomass of *Arabidopsis thaliana* inoculated with bacterial soil filtrate (-/+ filtrate), *Pseudomonas fluorescens* (-/+ pseud) and *Comamonas testosteronii* (-/+ coma). Bars sharing the same letter are not significantly different (Tukey's honestly significant difference, $p<0.05$)

Both bacterial species significantly increased root biomass but only in absence of amoebae ($F_{12,79}$ = 24.05, p < 0.0001 for the interaction between amoebae and *C. testosteronii* and $F_{12,79}$ = 6.98, p = 0.0099 for the interaction between amoebae and *P. fluorescens;* Fig. 5). However, the effect of both bacteria species was not additive, i.e. the effect in the single species treatments resembled that in two species treatment (significant *P. fluorescens* x *C. testosteronii* interaction; Tab. 1, Fig. 6).

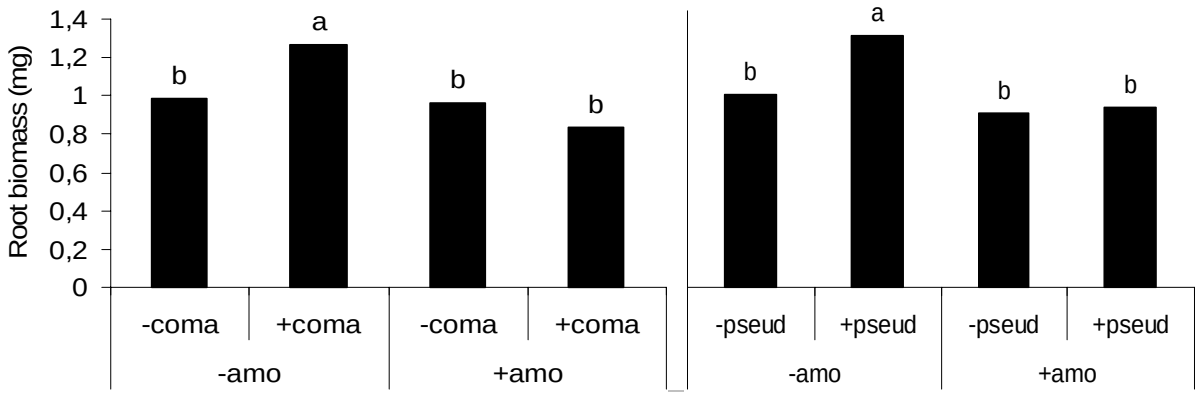

Figure 5 Effect of *Comamonas testosteronii* (+/- coma) and *Pseudomonas fluorescens* (+/- pseud) on root biomass of *Arabidopsis thaliana* in presence and absence of amoeba (-/+ Amo). Bars sharing the same letter are not significantly different (Tukey's honestly difference, p<0.05)

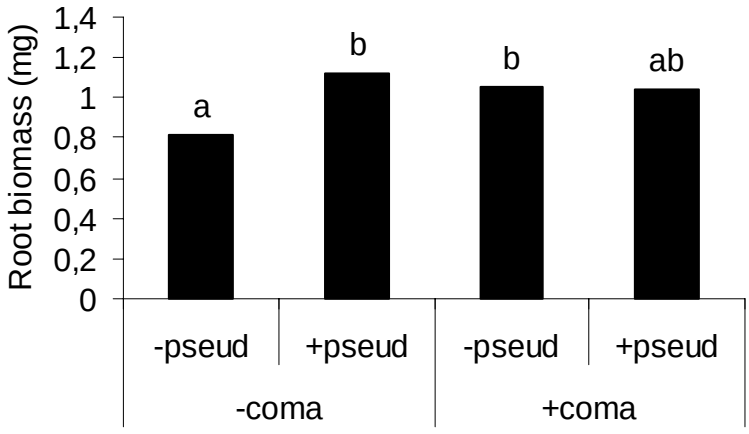

Figure 6 Effect of *Comamonas testosteronii* (+/- coma) on root biomass of *Arabidopsis thaliana* in presence and absence of *Pseudomonas fluorescens* (+/- pseud); Bars sharing the same letter are not significant different (Tukey's honestly significant difference, p<0.05)

Table 1 F-values of a four-factor ANOVA on the effects of amoeba (Amo), diverse bacterial soil filtrate, *Pseudomonas fluorescens* and *Comamonas testosteronii* on rosette diameter, root and shoot biomass of *Arabidopsis thaliana*

		Rosette diameter		Root biomass		Shoot biomass	
Factor	df	F	P	F	P	F	P
Amo	1	**55.95**	**<0.0001**	**13.28**	**0.0005**	**4.22**	**0.0433**
Filtrate	1	0.65	0.422	1.75	0.189	**5.39**	**0.0228**
P. fluorescens	1	0.06	0.804	**10.54**	**0.0017**	0.02	0.893
C. testosteronii	1	0.71	0.403	1.81	0.182	0.33	0.570
Amo x Filtrate	1	**8.66**	**0.0045**	0.85	0.361	**12.01**	**0.0009**
Amo x *P. fluorescens*	1	**8.94**	**0.0039**	**6.98**	**0.0099**	**4.94**	**0.0291**
Amo x *C. testosteronii*	1	**22.12**	**<0.0001**	**24.05**	**<0.0001**	**4.72**	**0.0328**
Filtrate x *P. fluorescens*	1	2.99	0.089	0.19	0.661	0.49	0.488
Filtrate x *C. testosteronii*	1	**7.37**	**0.009**	0.22	0.639	0.00	0.967
P. fluorescens x *C. testosteronii*	1	1.69	0.198	**15.27**	**0.0002**	**4.95**	**0.0289**
Amo x Filtrate x *C. testosteronii*	1	0.31	0.579	0.15	0.704	0.12	0.735
Amo x Filtrate x *P. fluorescens*	1	1.31	0.257	0.28	0.597	0.47	0.493

4.4.3 Plant tissue carbon and nitrogen concentration

Plant tissue carbon concentration of *A. thaliana* was not affected by any of the treatments but plant tissue nitrogen concentration significantly varied with experimental treatments. *Comamonas testosteronii* significantly increased plant tissue nitrogen concentration with the increase being more pronounced when amoeba were also present (significant Amo x *C. testosteronii* interaction, Tab. 2, Fig. 7). *Pseudomonas fluorescens* alone significantly increased plant tissue nitrogen concentration ($F_{12,79} = 5.57$, $p = 0.021$).

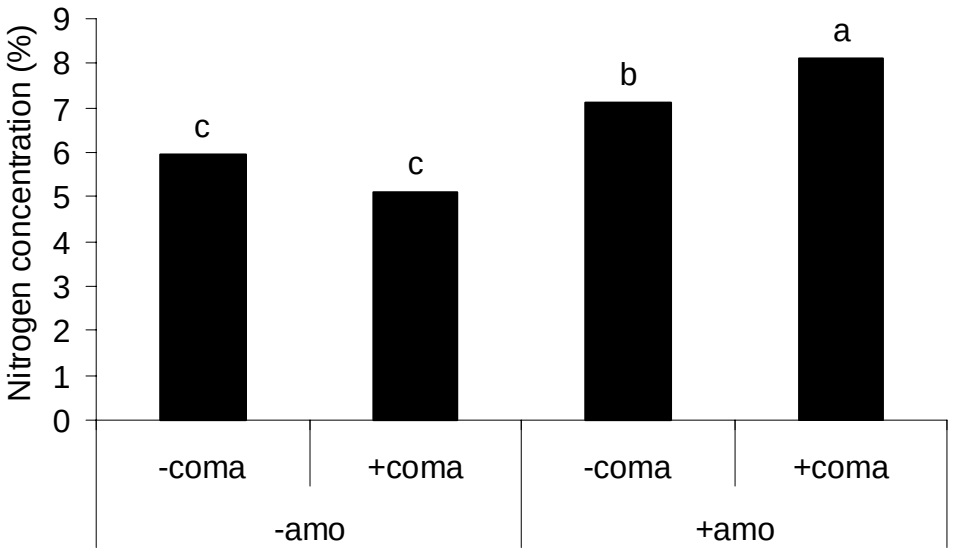

Figure 7 Effect of *Comamonas testosteronii* (+/-coma) on plant tissue nitrogen concentration in *Arabidopsis thaliana* in presence and absence of amoeba (+/- amo). Bars sharing the same letter are not significant different (Tukey's honestly significant difference, $p<0.05$)

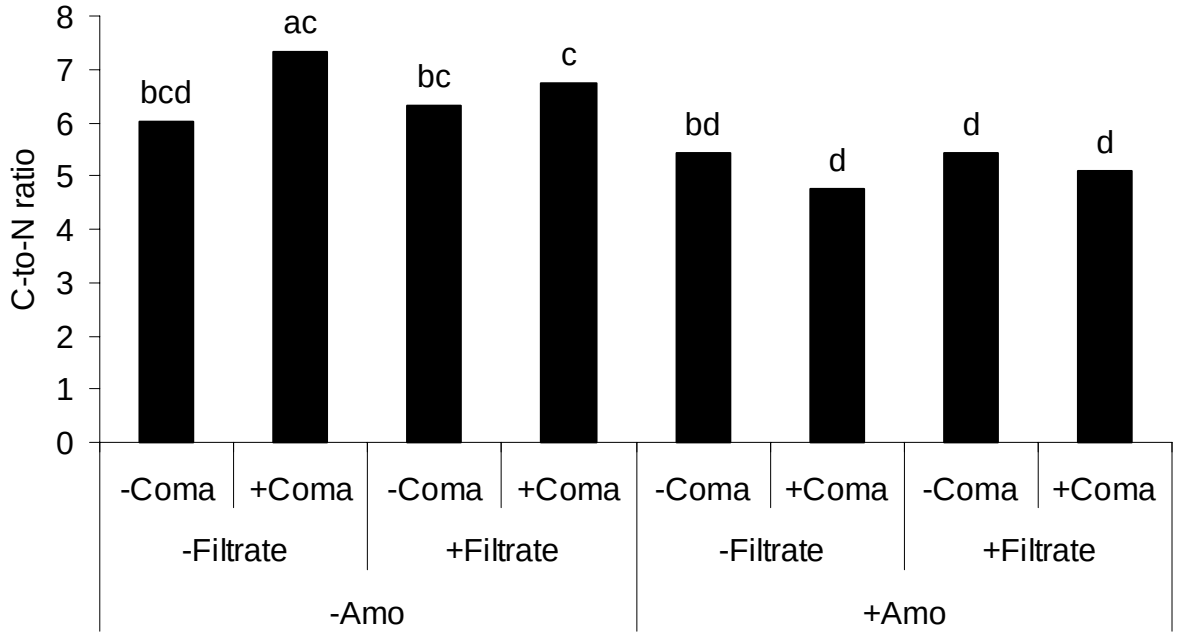

Figure 8 Effect of bacterial filtrate (+/- Filtrate) and *Comamonas testosteronii* on plant tissue C-to-N ratio of *Arabidopsis thaliana* in presence and absence of amoebae (+/- Amo); bars sharing the same letter are not significant different (tukey's honestly difference, $p<0.05$)

Furthermore, in the presence of amoebae both bacterial soil filtrate and *C. testosteronii* significantly decreased plant tissue C-to-N ratio with the decrease being more pronounced when *Comamonas* sp. were also present (significant Amo x Filtrate x *C. testosteronii* interaction; Tab. 2, Fig. 8).

P. fluorescens increased plant tissue C-to-N ratio with the increase being more pronounced when *C. testosteronii* was also present (significant *P. fluorescens* x *C. testosteronii* interaction, Tab. 2).

Table 2 F-Values of a four-factor ANOVA on the effects of amoeba (Amo), diverse bacterial soil filtrate, *Pseudomonas fluorescens* and *Comamonas testosteronii* on plant tissue carbon and nitrogen concentration as well as C-to-N ratio of *Arabidopsis thaliana*

		Carbon		Nitrogen		C-to-N ratio	
Factor	Df	F	P	F	P	F	P
Amo	1	2.25	0.139	**74.35**	**<0.0001**	**202.94**	**<0.0001**
Filtrate	1	0.87	0.355	0.02	0.891	0.51	0.478
P. fluorescens	1	1.09	0.299	**5.57**	**0.021**	**10.28**	**0.0021**
C. testosteronii	1	0.01	0.922	0.05	0.831	**9.60**	**0.0029**
Amo x Filtrate	1	1.82	0.183	0.06	0.804	**5.38**	**0.0235**
Amo x *P. fluorescens*	1	1.15	0.288	0.57	0.454	0.26	0.609
Amo x *C. testosteronii*	1	1.70	0.197	**13.35**	**0.0005**	**33.07**	**<0.0001**
Filtrate x *P. fluorescens*	1	0.23	0.635	0.38	0.538	3.55	0.064
Filtrate x *C. testosteronii*	1	1.56	0.217	0.38	0.540	0.00	0.999
P. fluorescens x C. testosteronii	1	0.31	0.580	3.45	0.068	**9.37**	**0.0032**
Amo x Filtrate x *C. testosteronii*	1	0.30	0.588	3.14	0.081	**6.89**	**0.011**
Amo x Filtrate x *P. fluorescens*	1	0.12	0.728	0.10	0.752	0.00	0.948

4.5 *Discussion*

In the previous experiment we investigated shifts in soil bacterial community composition due to protozoan grazing. As indicated by DGGE and FISH protozoan grazing strongly impacted the density of *Beta*- and *Gammaproteobacteria*. Based on these results we focused in the present study on plant-bacteria-protozoa interactions including mixed bacterial community with single strains such as *Comamonas testosteronii* (ATCC 17454) and *P. fluorescens* (WCS 417r). *Comamonas testosteronii* is phylogenetically related to plant pathogens and *P. fluorescens* functions as plant growth promoting rhizobacterium (PGPR). We hypothesized that the protozoa mediated changes in plant growth due to increased nutrient mobilization (microbial loop in soil, Clarholm, 1985) is modulated by changes in bacteria-plant-signalling.

Previous studies investigating protozoa-plant interactions have been performed exclusively in systems containing diverse bacterial communities. In such experiments it has been shown that protozoan grazing significantly affects bacterial community composition (Griffiths et al., 1999; Rønn et al., 2002; Bonkowski and Brandt, 2002; Kreuzer et al., 2006). Protozoa exert top down pressure on bacterial populations and this selects for bacterial species with certain traits, e.g. the ability to produce toxins (Bonkowski 2004; Jousset et al., 2006). Therefore, in addition to increasing nutrient supply, protozoa are likely to affect plant growth via changing bacteria-plant signalling, e.g. by changing plant hormone production by bacteria resulting in increased lateral root growth or NO-signalling (Bonkowski and Brandt, 2002; Lamattina et al., 2003, Krome et al., submitted).

We investigated whether protozoan-mediated increase in plant growth is associated with specific shifts in bacterial community composition or with nutrient based effects

due to the release of nutrients by setting up combined treatments of single bacteria and diverse bacteria. Additionally, we investigated if single bacterial species effect on plants and their modulation by protozoa also hold if co-inoculated with diverse soil bacteria, i.e. in rhizosphere communities resembling those in the field.

Both of the bacterial species improved plant performance suggesting that both function as PGPR. *C. testosteronii* and *P. fluorescens* alone increased root and shoot biomass, but not either in combination of both or with the bacterial soil filtrate. This may have been caused by unsuccessful establishment of the inoculated beneficial microorganism associated with loss of activity in combination with the natural soil bacteria which usually act as a buffer against incoming microorganisms (van Veen et al., 1997; Björklof et al., 2002). *Pseudomonas fluorescens* WCS417r has been shown before to beneficially affect plant growth before (Pieterse et al., 2001). However, this has been assumed to be due to the induction of systemic resistance, i.e. the reduction of pathogen infection, which in our single species treatments cannot have been responsible for the increased plant performance. In other fluorescent pseudomonads the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (*acdS*) was detected (Blaha et al., 2006) which is known to lower plant ethylene levels and thereby stimulating root and shoot growth (see review by Glick et al., 2007). In addition, the intermediate nitrous oxide (NO) of dissimilatory nitrogen reduction pathway has been demonstrated to cause plant growth promoting and root growth modulating effects in rhizobacteria-root interactions (Creus et al., 2005).

Protozoa have been shown repeatedly to increase the biomass of a number of plant species which was almost uniformly ascribed to an increase in nutrient supply to plants due to protozoan grazing (Clarholm, 1984, 1985, 2004; Kuikman et al., 1989, 1990). Confirming these results, rosette diameter of *A. thaliana* growing in presence of *A. castellanii* was significantly increased in each of the bacterial treatments,

whereas shoot biomass was only increased in the single strain treatments. Furthermore, the reduction in bacterial numbers in the presence of protozoa and the increase in density of protozoa in presence of both *C. testosteronii* and *P. fluorescens* suggest that the protozoa-mediated increase in plant performance was due to the liberation of nutrients locked up in bacterial biomass, i.e the microbial loop in soil. The fact that effects of protozoa on plant growth were less pronounced in the diverse bacteria treatment (increase in rosette diameter but decrease in shoot biomass) suggests that the diverse bacterial community may have contained bacterial species antagonistic to *A. thaliana*, potentially plant pathogens, and these bacteria attenuated the beneficial protozoa effects despite being grazed by *A. castellanii*.

Supporting the conclusion that the protozoan-mediated increase in plant performance was due to improved plant nutrient supply, tissue nitrogen concentration of *A. thaliana* were significantly increased in presence of amoebae. However, the effect of amoebae varied with the bacterial species applied. In the treatment with *C. testosteronii* and amoeba tissue nitrogen concentration was increased. Parallel to the increase in nitrogen concentration, the tissue C-to-N ratio of *A. thaliana* grown with *A. castellanii* was strongly reduced in the treatments with bacterial soil filtrate and *C. testosteronii*, respectively. *Arabidopsis thaliana* plants fixed more carbon in presence of both bacterial inoculum indicating decreased nitrogen availability in the sand/litter substrate due to incorporation of nitrogen in bacterial biomass. In presence of amoebae C-to-N ratio decreased due to improved nitrogen availability as described in the microbial loop with the effect strongly represented by *C. testosteronii*.

In contrast to previous studies on protozoa-plant interactions (Jentschke et al., 1995; Bonkowski et al., 2001; Bonkowski and Brandt, 2002; Kreuzer et al., 2006; Krome et al., submitted), protozoa did not stimulate root growth in the present experiments.

Rather, root biomass decreased in the single bacterial strain treatments. Presumably, reduced investment into roots reflects the protozoa-mediated increase in plant nutrient availability. In fact, using the same sand/litter model system as in the present study Krome et al. (submitted) found amoebae to increase ammonium concentrations in sand and increased concentrations of ammonium are known to result in reduced plant investments into roots (Davidson, 1968).

In conclusion, the used *C. testosteronii* strain proved to be the best choice to show the microbial loop effect in our system, since the plant reacted strongly in growth and nutrition status. The decreased root biomass is related to higher ammonium concentration in the sand/litter substrate due to protozoan grazing on the bacterial single strain.

In the previous experiment we demonstrated, that increased plant growth in presence of protozoa is independent of protozoan mediated shifts in the bacterial community composition by comparing diverse bacterial communities with single strains of two different phyla. The functional diversity of soil and rhizosphere bacterial communities may be so high that even selective grazing does not affect the overall community function in this respect. We suggest that nutrient based effects (i.e. microbial loop) are more important than changes in plant-bacteria signalling. However, this cannot be separated completely, because alterations in the nutrient status of the microhabitat may influence also general bacterial activities (see previous chapter) and thus its capacity to produce signalling substances of relevance for plant development (Creus et al., 2005; Schuhegger et al., 2006).

5 General discussion

In the present work I investigated the effect of protozoan grazing (*Acanthamoeba castellanii*) on microbial community composition and on plant growth in a sand/litter Magenta system with full control of rhizosphere community composition of *Arabidopsis thaliana*. Soil bacteria community composition was assessed with denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridisation (FISH). Therefore, DNA extraction methods suitable for the system were established. Experiments were conducted based on results from culture independent molecular analyses of microbial communities investigating whether protozoan-mediated changes in plant growth are caused by shifts in bacteria-plant signalling or increased nutrient mineralization.

Protozoa and bacteria form the oldest predator-prey system on earth, but apart from reports on morphological characters (Pernthaler, 2005) surprisingly little is known on factors that drive grazing resistance (Matz and Kjelleberg, 2006). Furthermore, only few studies exist on protozoan-mediated bacterial shifts in bacterial community composition in soil (Griffiths et al., 1999; Rønn et al, 2002; Kreuzer et al., 2006; Murase et al., 2006). To uncover the structure of bacterial communities which are consumed and those that survive protozoan grazing in soil, denaturing gradient gel electrophoresis (DGGE) was applied. DGGE is suitable for rapid screening of PCR amplified products from complex microbial communities and became an important tool in microbial molecular ecology (Singh et al., 2006). Previous studies reported that different extraction methods resulted in different community patterns of identical microbial communities (Felske et al., 1996; Niemi et al., 2001; Sessitsch et al., 2002; Lipthay et al., 2004; Costa et al., 2004; Singh et al., 2006); this was confirmed in the experiment presented in the first chapter. I investigated the effect of different DNA

extraction protocols on DGGE fingerprinting and subsequent detection of bacterial shifts due to protozoan grazing. Not all tested extraction methods provide reliable and reproducible information about protozoa – mediated shifts in bacteria community composition. When comparing a customized Fast Prep® kit with a protocol including phenol/chloroform purification differences occurred in the extraction yield and purity as indicated by $A_{260/280}$ ratio. Less DNA was extracted with the kit based protocol but products were of higher purity. Fingerprinting of both extraction methods differed in band number but not in diversity. The Shannon-index was similar but only the method including phenol/chloroform purification obtained significant protozoa-mediated shifts in DGGE fingerprints. Comparing DNA with RNA based DGGE community pattern no significant differences appeared. The increase in presumably active bacteria could only be demonstrated by a second culture independent approach as described in chapter two. Fluorescence *in situ* hybridisation (FISH) provided qualitative and quantitative information about bacterial populations in soil. The enumeration of bacteria by FISH revealed a significant reduction of total cell number caused by predation. The detected increase in active bacteria confirmed previous studies (Alphei et al., 1996; Kreuzer et al., 2006) indicating that the loss in bacterial numbers due to predation was in part compensated by enhanced activity. Subsequent data analyses of community fingerprinting demonstrated that shifts in bacterial communities occurred very rapidly. Treatment-specific fingerprinting demonstrate grazing preferences for distinct bacterial groups and an outgrowth of grazing tolerant taxa. Remarkably, the bacterial community composition changed very rapidly; some bands had already disappeared two days after the addition of amoeba.

Adaptations of bacteria against protozoan grazing are well known. They can escape ingestion and digestion by forming grazing resistant filaments or microcolonies

(Pernthaler, 2005) or even defend themselves by releasing toxins (Matz and Kjelleberg, 2006).

In our experimental setup, DGGE and FISH proved that protozoan grazing strongly impact *Betaproteobacteria, Firmicutes* and *Gammaproteobacteria* composition. Using DGGE and sequencing of prominent bands more precise information were obtained. Among *Betaproteobacteria* protozoan grazing predominantly affected the *Comamonadaceae*.

Grazing induced changes in rhizosphere bacterial community composition leading to functional shifts have been reported (Jentschke et al., 2005; Bonkowski and Brandt, 2002; Kreuzer et al., 2006) and could be confirmed in my present work with DGGE using the *gacA* marker gene for pseudomonads. Strong and highly reproducible changes in the *gacA*-banding pattern indicated a major shift in secondary metabolite production of pseudomonads (*Gammaproteobacteria*) in response to protozoan predators as previously described by Jousset at al. (2006). By upregulation of these secondary metabolites, e.g. cyanhidric acids, DAPG and exoproteases (Haas and Keel, 2003), pseudomonads can escape protozoan predation.

The strong increase in plant performance to protozoan grazing could be demonstrated in the two experiments described in Chapter 3 and 4. Positive effects of bacteria-protozoa interactions on plant growth are well documented (Clarholm, 1985; Kuikman et al., 1989; Bonkowski et al., 2001; Kreuzer al., 2006). These effects were generally assigned to the microbial loop, i.e. the release of nutrients from consumed microbial biomass via protozoan grazing and subsequent nutrient uptake by plants (Clarholm, 1985). In addition to that, changes in plant-bacteria signalling and subsequent increased lateral root growth due to hormonal substances, e.g. auxin are well documented (Bonkowski and Brandt, 2002; Krome et al., submitted). By analysing bacterial community compositions in absence and presence of amoebae I

was able to show, that increased plant growth was associated with changes in the bacterial composition. In Chapter 3 shoot and root biomass of *Arabidopsis thaliana* increased significantly in the presence of amoebae suggesting an outgrowth of plant growth promoting rhizobacteria. However, increased nutrient supply due to grazing on bacterial and reducing bacterial fixed nutrients as indicated by reduced cell numbers could not be excluded.

A follow-up experiment was conducted to clarify if increased nutrient supply or protozoa-mediated shifts in bacterial community composition were responsible for increased plant growth. The sand/litter substrate of the experimental systems was inoculated with single bacterial strains for which we had indications that they may be involved in protozoa – mediated changes in plant growth. For inoculation, we selected a natural bacterial soil community and members of *Beta*- and *Gammaproteobacteria, Comamonas testosteronii* and *Pseudomonas fluorescens*, respectively. *C. testosteronii* is phylogenetically related to plant pathogens whereas *P. fluorescens* function as plant growth promoting bacteria. We expected an increase in plant growth due to nutrient liberation via grazing and protozoa-mediated changes in plant-bacteria signalling. Rosette diameter of *A. thaliana* growing in presence of protozoa was significantly increased in each of the bacterial treatments, whereas shoot biomass was only increased in the single strain treatments. Contrary to findings of the experiment presented in Chapter 3, root growth in the single strain treatments was not increased reflecting the protozoa-mediated increase in plant nutrient availability.

Supporting this conclusion, tissue nitrogen concentration of *A. thaliana* was significantly increased in the presence of amoebae. Additionally, plants fixed more carbon in the presence of *C. testosteronii* and bacterial soil filtrate indicating that the plants incorporated nitrogen from bacterial biomass. Parallel to increased tissue

nitrogen concentration, tissue C-to-N ratio in the presence of amoebae was strongly reduced in both treatments with the effect being most pronounced in presence of *C. testosteronii*. The use of single strains proved that increased plant growth in presence of protozoa is not necessarily based on shifts in the bacterial community composition. Rather, nutrient based effects (i.e. the microbial loop in soil) alone may result in increased plant growth. Depending on the nutrient status of the plants nutrient based or plant-bacteria signalling based changes in plant growth by protozoa may predominate.

5.1 Conclusion

In conclusion, *Acanthamoeba castellanii* affects plant growth by grazing on bacterial communities. Changes in bacterial communities occur very rapid as demonstrated by two complementary molecular techniques. Using single bacterial strains I could demonstrate that the rapid shifts in bacterial community compositions are not essential for increased plant growth in presence of protozoa. Rather, increased nutrient supply to plants due to protozoan grazing is sufficient for increasing plant growth and this may override effects based on microbial-plant signalling.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation ohne fremde Hilfe angefertigt und mich keiner anderen als die von mir angegebenen Schriften und Hilfsmittel bedient habe. Die Etablierung des Magenta Systems erfolgte zu gleichen Teilen von mir und Kristin Krome. Alle Arbeiten zur Fluoreszenz *in situ* Hybridisierung (FISH) im Kapitel 3 wurden von Joanne Bertaux durchgeführt.

Ich habe noch keinen weiteren Promotionsversuch unternommen.

Mainz, den 31. Januar 2008