Interactions in the rhizosphere:

Plant responses to bacterivorous soil protozoa

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ZUSAMMENFASSUNG

Die Förderung von Pflanzenwachstum durch bakterivore Bodenprotozoen wird meist mit einer Mobilisierung von festgelegtem Stickstoff aus bakterieller Biomasse erklärt. Es existieren jedoch auch Hinweise dafür, dass das Pflanzenhormon Auxin (Indol-3-Essigsäure; IAA) an der Pflanzenwachstumsförderung durch Protozoen beteiligt ist. In der vorliegenden Doktorarbeit wurden morphologische, physiologische und transkriptionale Pflanzenreaktionen auf bakterivore Bodenprotozoen untersucht.

In einem ersten Experiment (Kapitel 2) wurde die Wirkung von Bodenbakterien und der Bodenamöbe *Acanthamoeba castellanii* auf die Wurzelmorphologie und den Auxinmetabolismus von *Lepidium sativum* und *Arabidopsis thaliana* analysiert. Bodenbakterien erhöhten die Konzentration an konjugiertem IAA ohne die Wurzelmorphologie zu beeinflussen. Die zusätzliche Anwesenheit von *A. castellanii* hingegen führte zu einer erhöhten Konzentration an freiem IAA sowie zu einer vermehrten Bildung von Lateralwurzeln. Bodenprotozoen steigern demnach die Ausdehnung des Wurzelsystems durch Veränderungen des pflanzlichen Auxin-metabolismus und ermöglichen so eine verbesserte Ausbeutung von Nährstoffen. Obwohl *A. castellanii* ebenfalls eine erhöhte Lateralwurzelbildung in *A. thaliana* induzierte, reagierten die Reporterpflanzen ARR5::GUS und DR5::GUS nicht auf Auxin, jedoch auf den Auxinantagonisten Cytokinin. Möglicherweise war hierfür eine erhöhte Nitratverfügbarkeit verantwortlich, da Nitrat zu einer Akkumulation von Cytokinin führt.

Zur Durchführung des zweiten Experiments (Kapitel 3) wurde ein definiertes Laborsystem mit *A. thaliana* entwickelt, welches die detaillierte Untersuchung von

Interaktionen zwischen Bodenbakterien und *A. castellanii* auf das Pflanzenwachstum erlaubt. Bodenbakterien sowie *A. castellanni* steigerten das Pflanzenwachstum bereits drei Tage nach der Inokulation, wobei der Einfluss von *A. castellanii* denjenigen der Bodenbakterien übertraf. Die Wachstumssteigerung ging mit einer erhöhten Kohlenstoff-, aber nicht Stickstoffaufnahme einher. Später erhöhten die Bodenprotozoen jedoch die Ammoniumverfügbarkeit, was vermutlich zu einer Verlängerung der vegetativen Wachstumsphase und erhöhten Reproduktion von *A. thaliana* führte. Die Ergebnisse legen nahe, dass *A. thaliana* die bevorstehende Stickstoffmobilisierung antizipiert und mit einer Erhöhung des Spross- und Wurzelwachstums reagiert. Die damit verbundene Vergrößerung der Wurzel ermöglicht später die vermehrte Aufnahme von Stickstoff, welches eine erhöhte Reproduktion bedingt.

In dem dritten Experiment (Kapitel 4) wurde der Einfluss von *A. castellanii* auf transkriptionale Veränderungen in *A. thaliana* mittels eines DNA arrays und quantitativer real time PCR untersucht. Die Initiierung einer Wachstumssteigerung durch *A. castellanii* war zunächst nicht mit einer Veränderung der Genexpression von stickstoffinduzierbaren Genen verbunden. Später wurden jedoch Gene der Ammoniumassimilation hoch reguliert, welches die Ergebnisse aus Kapitel 3 bestätigt. Die Transkriptionsanalyse zeigte weiterhin, dass durch Bodenprotozoen Abwehrmechanismen in der Wurzel reduziert, im Spross jedoch induziert werden. Möglicherweise reduziert *A. castellannii* die Besiedlung der Wurzeln mit pflanzenschädigenden Bodenbakterien durch Beweidung, welches eine verminderte Abwehr und somit eine Reduktion der Investitionen in den pflanzlichen Sekundärmetabolismus in der Wurzel ermöglicht. Der allgemein verbesserte Nährstoff- und Energiestatus der Pflanze könnte dafür verantwortlich sein, dass sowohl Wachstum als auch Abwehr im Spross gesteigert wurden.

Zusammenfassend deuten die Ergebnisse daraufhin, dass der positive Effekt von Bodenprotozoen auf das Pflanzenwachstum nicht nur durch eine erhöhte Stickstoffverfügbarkeit verursacht wird. Vielmehr scheinen die Pflanzen die Stickstoffmobilisierung über Signalstoffe in der Rhizosphere wahrzunehmen und steigern bereits vorher Spross- und Wurzelwachstum, was die Nährstoffaufnahme verbessert und das Pflanzenwachstum sowie die Reproduktion erhöhen. Die Reduktion von schädlichen Bodenbakterien durch Bodenprotozoen und die damit verbundene Reduktion von induzierter Abwehr in den Wurzeln haben vermutlich zu dem gesteigerten Pflanzenwachstum beigetragen. Die Abwehrinduktion im Spross führte nicht zu einer Reduktion von Pflanzenwachstum, was darauf hindeutet, dass die verbesserte Stickstoff- und Energieversorgung eine gleichzeitige Investition in Abwehr sowie in Wachstum ermöglicht.

SUMMARY

Plant growth promotion by bacterivorous soil protozoa is generally assigned to an improved nitrogen supply due to the mobilisation of nitrogen fixed in bacterial biomass. However, there is evidence that protozoa may also stimulate plant growth by non-nutrient effects with the phytohormone auxin (indole-3-acetic acid; IAA) being likely involved. This PhD Thesis was performed to investigate morphological, physiological and transcriptional responses of plants to soil protozoa and to assess the involvement of nitrogen and plant hormones in the protozoa-induced plant growth promotion.

In the first experiment (Chapter 2) modifications of root architecture and internal auxin metabolism of *Lepidium sativum* and *Arabidopsis thaliana* due to the presence of a diverse soil bacterial community and the protozoan species *Acanthamoeba castellanii* were analysed. Soil bacteria enhanced concentrations of conjugated IAA in *L. sativum* shoots without affecting root architecture, whereas soil bacteria plus *A. castellanii* increased free bioactive IAA concentrations and root branching. The results indicate that soil protozoa stimulate root foraging via affecting plant internal modifications of auxin metabolism and thus enable enhanced nutrient capture and plant growth. However, despite increased root branching, *A. thaliana* reporter plants for auxin and cytokinin did not respond to auxin but to cytokinin. Since soil protozoa increased nitrate concentrations in the rhizosphere the results suggest that nitrate caused an accumulation of cytokinin in the plant and interacted with its hormonal antagonist auxin, which finally induced increased root branching.

In the second experiment (Chapter 3) a defined laboratory system using *A. thaliana* as model plant was designed allowing to investigate effects of interactions between a diverse soil bacterial community and *A. castellanii* on plant performance in detail. Soil

bacteria and protozoa increased growth of *A. thaliana* already three days past plant inoculation (dpi) with the effects of protozoa exceeding those of bacteria only. The immediate growth response was accompanied by an increased carbon but not nitrogen allocation. However, three days later protozoa enhanced ammonium availability and plant uptake of nitrogen from organic material, which presumably was responsible for prolonged vegetative growth and increased seed production. The results suggest that *A. thaliana* sensed the upcoming mobilization of nitrogen presumably by changes in rhizosphere signalling and initiated carbon fixation and root carbon allocation which payed off later by increased nutrient capture and strongly increased plant reproduction.

In the third experiment (Chapter 4) transcriptional changes of *A. thaliana* genes involved in plant signalling and stress response as well as nitrogen responsive genes were investigated by performing a DNA array and quantitative real time PCR. Nitrogen responsive genes were not immediately regulated by soil protozoa, but later ammonium responsive genes were up-regulated supporting the results obtained in the experiment reported in Chapter 3. Transcription analysis further demonstrated that soil protozoa down-regulate defence mechanisms in plant roots, but induce plant defence in plant shoots. This suggests that soil protozoa inhibit detrimental soil bacteria by selective grazing leading to a reduced defence in roots and thus reduced investment in secondary metabolite production. Improved nutrient and energy status of *A. thaliana* may be responsible for increased shoot growth in presence of protozoa despite plant defence concurrently being enhanced.

Overall, the results suggest that the effect of protozoa on plant growth in fact initially may not be caused by increased nitrogen availability. Rather, the plants appear to anticipate the subsequent up-coming nitrogen mobilization due to changes in rhizosphere signalling and increase carbon assimilation and allocation to roots

resulting in strongly increased plant growth and seed production, i.e. plant fitness. Further, protozoa-mediated reduction in detrimental bacteria may have contributed to increased plant growth by saving costs for secondary metabolite production. Notably, the induction of plant defence in shoots by protozoa was not associated with reduced plant growth but rather the opposite, suggesting that due to increasing nitrogen supply protozoa enable plants to invest in defence in shoots and in parallel increase plant growth and reproduction.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Rhizosphere

Plant roots are essential for the development of the plant part above the ground and thus for shoot growth and later plant reproduction (Torrey, 1976; McCully, 1999). Besides plant stabilization, one of the key functions of roots is the uptake of water and nutrients and the transport into the shoot above the ground (Aiken & Smucker, 1996; Malamy, 2005). In addition, roots synthesize plant hormones which are translocated to the above ground parts of the plant and influence shoot development and performance (Aiken & Smucker, 1996; Haberer & Kieber, 2002; Aloni et al., 2005; Ljung et al., 2005). However, root metabolism and thus performance of the whole plant is not independent of physical, chemical and biological cues in the soil surrounding the root (Barea et al., 2005; Giri et al., 2005; Malamy, 2005, Hodge, 2006). The part of soil near and influenced by roots is named rhizosphere (Hiltner, 1904) and its biotic composition plays an essential role for root metabolism and development (Barea et al., 2005). The rhizosphere is characterized by high density, activity and diversity of soil microorganisms with bacteria being more important than fungi because of the supply of easily decomposable organic matter by plant roots (Alphei et al., 1996; Wardle, 2002). In comparison to the bulk soil, which harbours less than 10^8 bacterial cells g^{-1} soil, concentrations in rhizosphere soil can reach 10^{12} g^{-1} soil (Foster, 1988) with the highest concentrations in the zone of root elongation (Jaeger et al., 1999). The structure and activity of microbial populations play a crucial role for soil functions, since microorganisms are driving forces for fundamental metabolic processes, such as decomposition of organic matter and mineralization of

nutrients (Nannipieri et al., 2003; Hopkins & Gregorich, 2005). The composition of the rhizosphere bacterial community derives from the indigenous bulk soil community and develops in concert with the growing plant root (Hawkes et al., 2007). The vast majority of bacteria are heterotrophs which rely on photosynthetically fixed carbon by plant shoots leading to an accumulation of soil bacteria around roots (Cheng & Gershenson, 2007). A substantial part of the fixed carbon, up to 40 % (Lynch & Whipps, 1990; Bonkowski, 2002), is translocated into the roots and from the roots. Carbon rich root exudates are released into the surrounding soil where they are used by microorganisms (Milchunas et al., 1985; Semenov et al., 1999). There is evidence that soil bacteria have the ability to increase root respiration and exudation (Sarig et al., 1992; Vedder-Weiss et al., 1999; Joseph & Phillips, 2003; Phillips et al., 2004); and it was demonstrated that lumichrome is involved in the enhancement of root respiration induced by the soil bacterium *Sinorhizobium meliloti* (Phillips et al., 1999). The plant itself is able to manipulate the structure of the bacterial community that develops around the root via adjusting the composition of root exudates released; in addition to simply acting as resources, exudates can influence biotic interactions by attracting beneficial and pathogenic microorganisms (Nehl et al., 1997; Tesfaye et al., 2003).

1.2 Soil Bacteria–Plant Interactions

Interactions between plants and soil bacteria are either detrimental, neutral or beneficial (Ping & Boland, 2004; Singh et al., 2004). Effects of detrimental, pathogenic bacteria on plants are obvious, they act as a sink for plant carbon, damage root tissue, reduce water and nutrient uptake, and thus decrease plant growth and reproduction (Weste & Ashton, 1994; Packer & Clay, 2003). Effects of

beneficial soil bacteria are more concealed but Plant Growth Promoting Rhizobacteria (PGPR) are receiving increased attention in the last years. PGPRs are defined as rhizosphere bacteria which colonize the root environment and promote plant growth either directly or indirectly (Bashan & Holguin, 1998; Persello-Cartieaux et al., 2003; Vessey, 2003; Barea et al., 2004). The direct plant growth promotion usually entails either facilitating the uptake of nutrients from the environment or providing the plant with compounds synthesized by the bacterium like plant hormones. Indirect promotion of plant growth implies the reduction or prevention of deleterious effects of phytopathogenic organisms. A particular strain of PGPRs may affect plant growth and development by one or more of these mechanisms (Holguin et al., 1999; Dobbelaere et al., 2001).

1.2.1 Plant Hormones

The potential to produce or degrade plant hormones like auxin, ethylene and cytokinin is widespread among rhizobacteria and is suggested to be responsible for a large part of positive effects of rhizobacteria on plant growth (Patten & Glick, 1996; Bashan & Holguin, 1997; Lambrecht et al., 2000; Dobbelaere et al., 2001; Ashgar et al., 2002; Patten & Glick, 2002; Persello-Cartieaux et al., 2003; Vessey, 2003; Ryu et al., 2005; Tsavkelova et al., 2006).

The plant hormone auxin, indole-3-acetic acid (IAA), is known to be the key signal in initiation and growth of lateral roots (Celenza et al., 1995; Malamy & Benfey, 1997; Casimiro et al., 2001; Himanen et al., 2002; Casimiro et al., 2003; Laskowski et al., 2006). The release of auxins by particular rhizobacterial strains is assumed to stimulate the elongation of primary or the formation and elongation of lateral roots (Barbieri et al., 1986; Barbieri & Galli, 1993; Frankenberger & Arshad, 1995;

Lambrecht et al., 2000; Dobbelaere et al., 2001; Patten & Glick, 2002; Vessey, 2003). Lower concentrations of IAA synthesized by bacteria are assumed to cause root growth by stimulation of plant cell elongation or cell division, whereas high concentrations of IAA induce the formation of lateral root primordia and thus increases root branching (Patten & Glick, 2002). The formation of the root system may also be indirectly influenced by modulating the ethylene metabolism in plants via the release of bacterial synthesized auxin (Glick et al., 1998; Patten & Glick, 2002). A number of PGPRs are known to contain the 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) which interferes with the biosynthesis of ethylene in plants. It is suggested that bacterial auxins stimulate the synthesis of the ethylene precursor ACC in plants which is taken up by the bacteria attached to the roots and hydrolyzed to ammonia and α-ketobutyrate. The decrease of ethylene concentrations in plants is assumed to prevent ethylene inhibition of root elongation (Glick et al., 1998).

Therefore, the synthesis of auxins and/or the degradation of plant ethylene by PGPRs triggers the formation of an elongated, highly branched root system and thus root proliferation. This enhances the exploitation of soil by plants with increased water and nutrient capture explaining plant growth promotion by rhizobacterial strains (Dobbelaere et al., 2001; Ashgar et al., 2002; Khalid et al., 2004; Persello-Cartieaux et al., 2003; Vessey, 2003; Ryu et al., 2005). Thus, bacteria-mediated phytostimulation results in an alteration of the complex and balanced network of plant hormones responsible for root formation and plant growth (Ping & Boland, 2004).

1.2.2 Biocontrol of Plant Diseases

The accumulation of particular strains of PGPRs in the rhizosphere is known to reduce detrimental effects of root and leaf pathogens in plants. The reduction of plant diseases by PGPRs is assumed to result either from the growth reduction of the pathogen due to microbial competition or antibiosis, or from the activation of an induced systemic resistance (ISR) in plants (Compant et al., 2005; Mercado-Blanco & Bakker, 2007).

The elicitation of ISR is widespread and occurs in a variety of non-pathogenic soil microorganisms (Van Loon et al., 1998; Bakker et al., 2007; Mercado-Blanco & Bakker, 2007). The induction of ISR by PGPRs systemically suppresses the development of diseases in above- and below ground plant parts (Pieterse et al., 2002). Phenotypically, ISR resembles a systemic aquired resistance (SAR), which is induced by an inoculation with a pathogen and which refers subsequent resistance to challenging pathogens (Sticher et al., 1997). Unlike in the SAR (Sticher et al., 1997), salicylic acid is not required for the activation of ISR induced by *Pseudomonas fluorescens* (Pieterse et al., 1996). From experiments with mutants of *Arabidopsis thaliana* which are non- or less responsive to ethylene or jasmonic acid, it was concluded that response to these plant hormones is required for expression of ISR (Pieterse et al., 1998; Knoester et al., 1999). ISR is effective against a different spectrum of pathogens than SAR; it predominantly confers resistance to pathogens that are sensitive to jasmonate and ethylene induced defence mechanisms (Ton et al., 2002). However, ISR induced by *Pseudomonas aeruginosa* depends on salicylic acid (Audenaert et al., 2002) indicating diverse activation mechanisms of ISR by PGPRs. For long it was assumed that ISR is based on the up-regulation of defence related genes but this could not be confirmed (Van Loon et al., 1998). Instead, plants

expressing ISR exhibit a faster and stronger activation of defence responses after they have been infected with a pathogen (Van Wees et al., 1999; Conrath et al., 2002, Verhagen et al., 2004). This capacity for enhanced defence expression is called ‘priming’ (Conrath et al., 2002) and entails the advantage of enhanced disease protection and low costs (Van Hulten et al, 2006).

1.3 Soil Protozoa

The constant release of easily degradable root exudates into the rhizosphere favors a microflora consisting of fast growing bacteria leading to increased microbial biomass and activity around roots (Van Veen et al., 1989; Alphei et al., 1996, Wardle, 2002). These bacterial populations are top-down controlled by the grazing of bacterivorous nematodes and protozoa (Ingham et al., 1986; Moore et al., 1988; Wardle, 2002). Bacterivorous soil protozoa in soils accumulate in ‘hotspots’ of microbial activity as in the humus region or closed to plant roots attracted by its prey (Griffiths, 1990; Hausmann & Hülsmann, 1996; Scheu et al., 2005) reaching densities of 10^4-10^7 active protist individuals per gram dry weight forest soil (Adl et al., 2006). Bacterivorous protozoa are more important than nematodes since they have access to bacteria living in small pore spaces unavailable to nematodes (Bamforth, 1997).

Soil protozoa are unicellular eukaryotic organisms with a size of 5-50 µm which comprise flagellates, ciliates and amoebae classified by their way of moving and feeding (Bamforth, 1997; Adl et al., 2005; Clarholm et al., 2006). Ciliates and flagellates have distinct and constant cell shapes and feed on free swimming soil bacteria, whereas amoebae have more plastic bodies that continually change shape and ingest organisms attached to soil particles (Hausmann & Hülsmann, 1996;

Clarholm et al., 2006). Due to the high biomass and particular feeding mode naked amoebae are the most important bacterivorous protozoa in soil (Clarholm, 1994). With their pseudopodia amoebae reach bacterial colonies in soil pores inaccessible to other protozoa and have access to the majority of bacteria in soil (Ekelund & Rønn, 1994). The high number and reproduction of protozoa (Coleman, 1994) enables them to control microbial growth and turnover efficiently (Bonkowski et al., 2000a). In terrestrial ecosystems protozoa are particularly important for nutrient mineralization which is crucial for plant growth and development (Scheu et al., 2005).

1.4 Soil Bacteria – Protozoa – Plant Interactions

The high amount of carbon released by plant roots suggests that high abundance and activity of soil bacteria is of fundamental importance for plants because otherwise this carbon may better be used for plant tissue production (Griffiths et al., 2007). The promotion of soil bacteria by the release of root exudates rich in carbon appears contradictory since soil bacteria are known to compete with plant roots for nutrients (Hodge et al., 2000). However, nutrients are only temporarily locked up in the bacterial biomass and are successively mobilized by grazing of bacterivorous protozoa on bacterial populations in the rhizosphere (Bonkowski et al., 2000b; Scheu et al., 2005). This re-mobilization process in soil has traditionally been assigned to be responsible for observed plant growth promotion in presence of soil protozoa (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Clarholm, 2005). The assumed mechanism, the so-called ‘microbial loop in soil’ (Fig. 1.1), is triggered by the release of carbon rich root exudates which increase bacterial growth in the rhizosphere. Nitrogen mineralized from soil organic matter is sequestered and locked up in the growing bacterial biomass and thus is not available to plants. Due to

grazing of protozoa on bacteria, nitrogen is remobilized and released into the rhizosphere and can be taken up by the plant roots resulting in an improved plant nitrogen supply and thus plant growth. About 60 % of the nitrogen ingested by protozoa is suggested to be in excess of structural needs and is excreted into the rhizosphere predominantly as ammonium (Griffiths & Bardgett, 1997).

However, later studies questioned whether enhanced nitrogen availability is the sole reason for protozoan-mediated plant growth promotion. For example, protozoa have been found to increase plant biomass independently of increased nitrogen concentrations in plant tissue (Jentschke et al., 1995; Alphei et al., 1996) and additions of nitrogen did not result in the same growth promoting effect as the addition of protozoa (Clarholm, 1985; 2005). Furthermore, it was calculated that protozoa are unlikely to release sufficient amounts of nitrogen to explain the positive effects on plant growth (Robinson et al., 1989; Griffiths & Robinson, 1992). These observations indicate that it is not sufficient to explain the plant promoting effects induced by protozoa by increased nitrogen availability (Bonkowski, 2004).

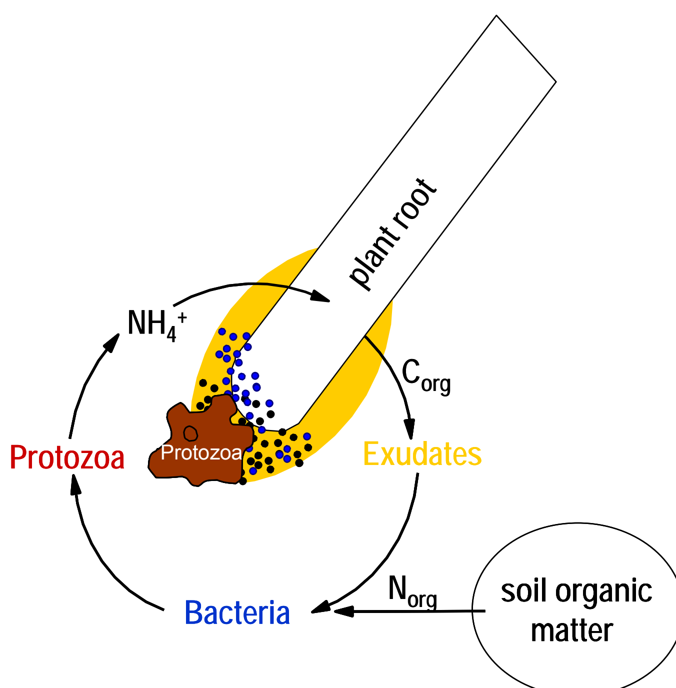

Figure 1.1: The ‚microbial loop' according to Clarholm (1985): Root exudation (C_{org}) stimulates the growth of soil bacteria, which sequester nitrogen from organic matter (N_{org}) in the bacterial biomass. Grazing of bacterivorous protozoa on bacterial biomass releases excess nitrogen as ammonia (NH_4^+) into the soil, which then is available for plant uptake.

Investigations on soil protozoa – plant interactions revealed that protozoa induce the production of lateral roots leading to an extensive highly branched root system with more fine roots (Jentschke et al., 1995; Bonkowski et al., 2000a; Bonkowski et al., 2001a; Bonkowski & Brandt, 2002) resembling hormonal effects on root growth induced by PGPRs. These changes indicate that in addition to improved nitrogen supply hormonal effects are involved in protozoa plant interactions since the plant hormone auxin is responsible for the induction of lateral root primordia (Torrey, 1976). Indeed, Bonkowski & Brandt (2002) demonstrated that, in addition to increased numbers of lateral roots, grazing of soil protozoa on bacteria shift the composition of the microbial community towards auxin producing bacteria. Several investigations from freshwater (Pernthaler et al., 1997; Jürgens et al., 1999; Posch et al., 1999) but also from soil systems show that protozoa do not indiscriminately ingest bacteria, but selectively feed on certain bacterial strains (Griffiths et al., 1989; 1999;; Rønn et al., 2002; Rosenberg, 2008).

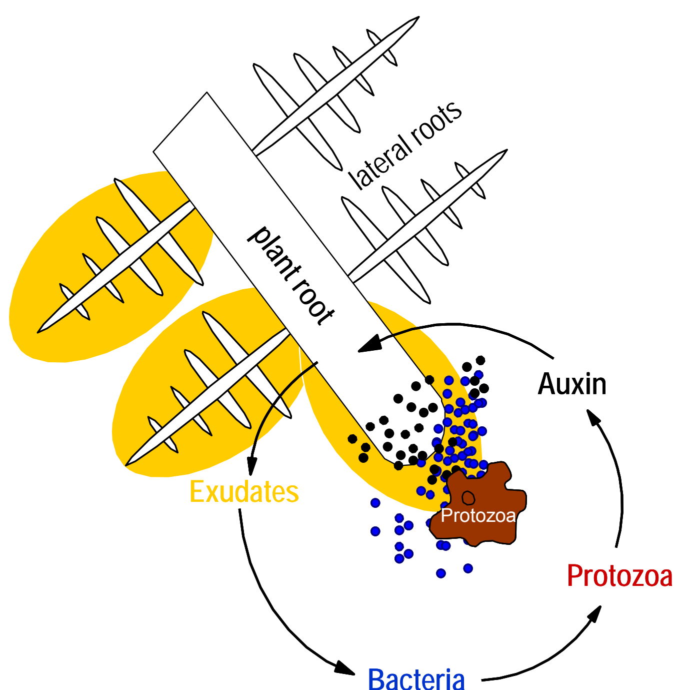

Figure 1.2: The ‚hormonal loop' modified after Brandt & Bonkowski (2002):
Root exudation stimulates the growth of a diverse bacterial community and subsequently of bacterivorous protozoa. Selective grazing by protozoa favours indole-3-acetic acid producing bacteria. The release of IAA induces lateral root growth, leading to release of more exudates and subsequent bacterial growth.

The stimulation of auxin producing bacteria suggests that protozoa affect root morphology and thus plant growth through hormonal effects via selective grazing on rhizosphere bacteria (Fig. 1.2). Exudates released by the roots stimulate bacterial and subsequent protozoan growth as in the microbial loop. Due to the selective grazing of protozoa on soil bacteria certain bacterial strains capable to release auxins are stimulated. Accordingly, growth of the root system is enhanced, which increases nutrient uptake but also increases rates of exudation, thereby further stimulating bacterial-protozoan interactions.

1.5 Objectives

As detailed above, plant growth promotion by bacterivorous soil protozoa was traditionally assigned to an increase in nitrogen availability in the rhizosphere caused by the release of nitrogen locked-up in bacterial biomass by protozoan grazing (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Clarholm, 2005). However, later studies questioned if the enhanced nitrogen supply is solely responsible for plant growth promotion (Robinson et al., 1989; Griffith & Robinson, 1992; Jentschke et al., 1995; Alphei et al., 1996). Additionally, it was assumed that soil protozoa modify the hormonal balance in plants via selective grazing on microorganisms thereby promoting auxin producing rhizobacteria (Bonkowski & Brandt, 2002; Bonkowski, 2004). This PhD thesis was conducted to further elucidate and assess the involvement of nitrogen and the plant hormone auxin in plant – bacteria-protozoa interactions by investigating morphological, physiological and transcriptional responses of *Lepidium sativum* and *Arabidopsis thaliana* to the presence of soil bacteria as well as to the presence of the bacterivorous naked soil amoeba *Acanthamoeba castellanii*.

Bonkowski & Brandt (2002) assumed that via selective grazing of *A. castellanii* the rhizobacterial community composition changes towards increased proportions of IAA producing bacteria, which subsequently results in increased plant uptake of IAA and thus triggers lateral root formation. However, although an increase in root branching associated with a stimulation of auxin producing bacteria by protozoa was demonstrated, information about modifications of endogenous auxin levels in plants due to the presence of soil bacteria and protozoa is still lacking. In **Chapter 2** we hypothesized that the presence of soil bacteria as well as the presence of *A. castellanii* affect the plant internal IAA metabolism and assumed that *A. castellanii* intensifies the hormonal plant response induced by soil bacteria. First, by using the same experimental design as Bonkowski & Brandt (2002) we analysed if soil bacteria and the naked amoeba *A. castellanii* induce shifts in the concentrations of free and conjugated IAA in *L. sativum* plants using gas chromatography–mass spectrometry. Additionally, the transgenic lines of *Arabidopsis thaliana* DR5::GUS and ARR5::GUS were used to visualize expression patterns of the plant hormones auxin and the auxin-antagonist cytokinin in rhizobacterial and protozoan treatments.

Bigger plants support a greater root system which releases increased amounts of carbon rich root exudates into the rhizosphere resulting in increased bacterial biomass and therefore prey for bacterivores (Phillips et al. 2003). Since soil bacteria as well as *A. castellanii* may benefit from increased plant growth we hypothesized that both rapidly promote performance and development of *A. thaliana*. **Chapter 3** describes the establishment of a defined laboratory system using *A. thaliana* as model plant which allows investigations of immediate effects of soil bacteria – *A. castellanii* interactions on *A. thaliana* in detail. To proof the suggested plant growth promotion by soil bacteria and *A. castellanii* we analysed growth and reproduction, as

well as tissue carbon and nitrogen contents of *A. thaliana.* Further, by using ^{15}N labelling we traced the incorporation of nitrogen from organic matter into the plants as affected by bacteria and protozoa.

The results obtained in Chapter 3 suggest that the immediate growth promotion of *A. thaliana* induced by *A. castellanii* is not based on nitrogen effects. Rather, changes in rhizosphere signalling induced the plant growth promotion. However, later throughout the experiment soil protozoa improved the nitrogen supply of *A. thaliana*. In **Chapter 4** we further elucidated to what extend the immediate growth promotion of *A. thaliana* by protozoa results from changes in rhizosphere signalling or nutrient effects. We analysed transcriptional regulation of nitrogen responsive genes and genes involved in plant signalling and stress response of *A. thaliana.* We assumed that nitrogen responsive genes are not immediately induced by the presence of *A. castellanii*, but are up-regulated later in the experiment. Additionally, we hypothesized that genes involved in plant signalling response are regulated by *A. castellanii*.

CHAPTER 2

SOIL BACTERIA AND PROTOZOA AFFECT ROOT BRANCHING VIA EFFECTS ON THE HORMONAL BALANCE IN PLANTS

2. 1 Abstract

Lateral roots are crucial for the plasticity of root responses to environmental conditions in soil. Bacterivorous soil amoebae were shown to increase root branching and to foster auxin producing soil bacteria. However, information about modifications of internal auxin levels by soil bacteria and amoebae is missing. We investigated the effects of a soil bacterial community and amoebae (*Acanthamoeba castellanii*) on root branching and on auxin metabolism in *Lepidium sativum* and *Arabidopsis thaliana*.

In a first experiment, soil bacteria increased conjugated auxin concentrations in *L. sativum* shoots, but did not affect free bioactive auxin and root branching. In contrast, free auxin concentrations in shoots and root branching were increased in presence of soil bacteria plus amoebae. The results confirm that soil bacteria as well as soil amoebae strongly modify auxin metabolism in plants, but indicates that the regulation of increased root branching relies on plant internal modifications of auxin metabolism rather than on the bacterial synthesis of auxin in the rhizosphere.

In a second experiment 2, *A. thaliana* reporter plants for auxin (DR5) and cytokinin (ARR5) also responded with increased root branching in presence of amoebae. Surprisingly, reporter plants did not respond to auxin but to cytokinin. We hypothesize that root growth promotion was caused by interactions between amoebae and bacteria resulting in increased concentrations of nitrate in the

rhizosphere, thus leading to the accumulation of cytokinin and interactions with free auxin in plants, which finally lead to increased root growth.

Overall, the results indicate that mutual control mechanisms exist between plant hormone metabolism and microbial signalling, and that the fine tuning of the hormonal interactions of plants with free-living bacteria is influenced by the presence of bacterial grazers, such as amoebae.

2.2 Introduction

Plant roots operate in an environment which is extremely heterogeneous, both spatially and temporally (Hodge, 2006). The formation of lateral roots from the primary root plays a crucial role for the plasticity of root responses to external factors since it is the main determinant of root architecture throughout plant development (Malamy, 2005; Lucas et al., 2008). The plant hormone auxin, i.e. indole-3-acetic acid (IAA), is known to be the key signal for the initiation and regulation of lateral roots (Celenza et al., 1995; Himanen et al., 2002; Casimiro et al., 2003; Laskowski et al., 2006). Active transport mechanisms maintain local auxin gradients in the plant and cellular auxin levels are controlled by interactions between auxin synthesis, degradation and conjugation (Blilou et al., 2005).

The formation of lateral roots and the resulting iterative construction of root branching largely defines the plant's ability to forage for water and nutrients (Malamy & Benfey, 1997; Malamy, 2005), but also poses an important sink for photosynthates which is determined by the numbers of root tips – the major sites of root exudation (Farrar et al., 2003, Henry et al., 2005). The exudation of photosynthates display a crucial energy source fuelling the bacterial energy channel (Paterson, 2003), suggesting that root branching is prone to microbial manipulation (Bonkowski, 2004).

Plant Growth Promoting Rhizobacteria (PGPR) have been shown to release IAA and are assumed to modify plant auxin levels resulting in an elongated, highly branched root system (Barbieri & Galli, 1993; Lambrecht et al., 2000; Dobbelaere et al., 2001; Asghar et al., 2002; Patten & Glick, 2002). An increased root branching promotes soil bacterial growth by the enhanced release of carbon rich root exudates. However, natural bacterial communities in the rhizosphere are subject to predation by bacterivores, mainly protozoa and nematodes (Griffiths et al. 2007, Rosenberg, 2008). The presence of nematodes and protozoa in the rhizosphere was shown to cause an increase in the formation of lateral roots resembling effects of auxin producing PGPR (Jentschke et al. 1995, Kreuzer et al., 2006; Mao et al., 2007). Bonkowski & Brandt (2002) demonstrated the proportion of IAA producing bacteria in the rhizosphere of *Lepidium sativum* to be increased in presence of the widespread and abundant naked amoebae species *Acanthamoeba castellanii*. The parallel increase in the production of lateral roots suggested that selective grazing of *A. castellanii* on rhizobacteria enhanced bacterial effects on root growth via fostering IAA producing bacteria. An induction of an increased root branching by soil bacteria leads to a positive feedback for the bacteria and subsequent bacterivores via increased root carbon exudation (Alphei et al., 1996; Kreuzer et al., 2006). Furthermore, the promotion of bacterivores feeds back to the plant by increasing plant nitrogen supply originating from consumed bacterial biomass indicating mutualistic interactions between soil protozoa and plants (Kuikman et al., 1991; Bonkowski et al. 2000a,b; Bonkowski et al. 2001a).

Although there is evidence that root architecture is controlled by external signals released by rhizobacteria and protozoa virtually nothing is known on internal changes in plant signalling, such as shifts in the hormonal balance of plants. If protozoa indeed amplify effects of IAA-producing rhizobacteria, an increase of bacteria-

mediated plant responses could be expected in presence of protozoa. Since the plant hormone auxin plays a central role in the initiation and regulation of lateral root growth, we first analysed root branching as well as internal free and conjugated auxin concentrations in *Lepidium sativum* (garden cress) after inoculation with rhizobacteria or bacteria plus *A. castellanii*. Second, we used transgenic lines of *Arabidopsis thaliana* to visualize expression patterns of the plant hormones auxin and the auxin-antagonist cytokinin in treatments with soil bacteria and in treatments with soil bacteria plus amoebae.

2.3 Material and Methods

To investigate effects of rhizobacteria and *A. castellanii* on root growth, sterile grown plants, plants inoculated with a diverse soil bacterial community and plants inoculated with a diverse soil bacterial community plus *A. castellanii* were set up. The bacterial inoculum was obtained by suspending 20 g fresh weight of recently collected rhizosphere soil from a meadow (campus of the Biology Faculty, University of Technology, Darmstadt, Germany) in 200 ml distilled water and filtering the soil slurry through folded paper filters (Schleicher & Schuell, Dassel, Germany). Subsequently, the filtrate was filtered through sterile 5 and 1.2 µm isopore filters (Millipore, Schwalbach, Germany) to exclude soil protozoa. To check for protozoan contaminations, the bacterial filtrate was cultured for three days in sterile nutrient broth (Oxoid Ltd., Cambridge, UK) in Neff`s Modified Amobae Saline (Page, 1976) at 1:9 v/v (NB-NMAS) prior to use.

Protozoa were taken from axenic cultures of *A. castellanii.* Cultures were established from specimens isolated from woodland soil (Göttinger Wald, Lower Saxony, Germany) and cultured axenically in sterile PGY medium (1 % peptone, 1 % glucose,

0.5 % yeast; VWR, Darmstadt, Germany). Prior to inoculation, *A. castellanii* cells were washed twice in NB-NMAS at 1000 rpm for 5 min.

Experiment 1

The first experiment was carried out under strictly controlled conditions to measure *in planta* changes in bioactive free and conjugated concentrations of IAA in addition to effects on root growth. We used *L. sativum* (garden cress; Carl Kämpf GmbH, Mainz, Germany) as experimental model plant to relate the results to a previous investigation (Bonkowski & Brandt, 2002).

Lepidium sativum seeds were sterilised for 10 min in 0.5 % NaOCl (VWR, Darmstadt, Germany) and subsequently, for 5 min in 70 % ethanol. After sterilisation, the seeds were washed 3 times with sterile deionised water and transferred to square Petri dishes (VWR) containing 0.5 % plant agar (Duchefa, Haarlem, The Netherlands) in half-strength Hoagland medium (Hoagland's No. 2 Basal Salt Mixture; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). In the square Petri dishes, a strip of 3 cm agar was removed and Petri dishes were upright positioned. Seeds were equally spaced on the small cutting edge of the agar for germination. The following treatments were set up: plants were grown on agar either completely sterile (Sterile), in presence of bacteria (Bacteria), or bacteria plus axenic *A. castellanii* (Amoebae) with 9 replicates, each.

Treatments with bacteria were inoculated with ca. 10^{10} CFUs in 100 µl NB-NMAS; which were evenly spread on the agar surface. Amoebae treatments were inoculated in addition with 50 µl of a suspension of axenic *A. castellanii* (ca. 5000 ind.). Corresponding amounts of sterile NB-NMAS were added to Sterile and Bacteria treatments, respectively. The experiment was performed in a growth chamber with a day / night regime of 14 / 10 h at 24 °C / 20 °C and a photon flux density of

450 µMol m^{-2} s^{-1} for 14 days. The roots were scanned (Regent 1600+ scanner, Regent Instruments Inc., Ottawa, Canada) and root morphology analysed with WinRhizo software (Winrhizo 5.0a, Regent Instruments Inc.). Subsequently, roots and shoots were immediately frozen in liquid nitrogen and kept at -80 °C for auxin measurements.

Determination of free and bound auxins

Free and bound IAA was determined from shoots and roots after pooling 3 replicate plants, leaving 3 replicates of each treatment in total. For the quantitative determination of free and total bound IAA in roots and shoots, the pesteled frozen plant material (approximately 10 mg dry wt) was extracted with isopropanol/glacial acetic acid (95:5 v/v) by incubating at 4 °C in the dark for 1 h. As internal standard, 100 ng (free) and 200 ng (total) $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, USA) was added (Cohen et al., 1986). For each sample three independent extractions were performed.

After centrifugation (13.000 g, 10 min) the samples were evaporated to the aqueous phase. For the determination of free IAA the aqueous phase was adjusted to pH 3.0 and twice extracted with ethyl acetate; then the organic phases were pooled and evaporated to dryness. The extracts were methylated with diazomethane (Cohen, 1984) and resuspended in ethyl acetate for GC-MS analysis. Conjugated IAA was hydrolyzed with 7 M NaOH at 100 °C under N_2 for 3 h. The hydrolysates were cooled to room temperature adjusted to pH 2.5, and subsequently purified on C18-columns (J.T. Baker, Phillipsburg, NJ, USA) by elution with acetonitrile (Chen et al., 1988). The extracts were evaporated to dryness, methylated with diazomethane (Cohen, 1984) and resuspended in ethyl acetate for GC-MS analysis. The GC–MS analysis was performed on a Varian Saturn 2100 Ion Trap MS system using electron impact

ionization at 70 eV, coupled to a Varian CP-3900 GC equipped with a Varian CP-8400 autosampler (Varian, Walnut Creek, USA). For the analysis 2.5 µl of the methylated sample dissolved in 20 µl ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column (length 30 m, diameter 0.25; Phenomenex, Aschaffenburg, Germany) using Helium as carrier gas at 1 ml min^{-1}. Injector temperature was 250 °C and the temperature program started with 70 °C for 1 min, followed by an increase of 20 °C min^{-1} to 280 °C, then 5 min isothermically at 280 °C. The methyl ester of IAA eluted under these conditions at 10 minutes. The settings of the mass spectrometer were as described in Campanella et al. (2003a). For higher sensitivity the µSIS mode (Varian Manual) was used to monitor the diagnostic ions. The endogenous amounts of free auxin were calculated by the isotope dilution equation (Cohen et al., 1986). For the determination of IAA, the molecular and quinolinium ions of the methylated substance at m/z 189 / 195 and 130 / 136, respectively, were monitored (ions deriving from endogenous and $^{13}C_6$-IAA). The concentrations for conjugated IAA were calculated by subtracting the amount of free IAA from the amount of total IAA.

Experiment 2

The second experiment was performed in soil containing a diverse bacterial community, which was planted with transgenic lines of *A. thaliana* to monitor the *in situ* changes of plant hormone production in absence (Bacteria treatment as control) or presence of *A. castellanii* (Amoebae treatment).

Soil and culture flasks

A sandy soil (10 % silt, 0.6 % C_{org}, 0.05 % N, C / N 12, $pH_{[0.1\ M\ CaCl]}$ 7.0), collected at the abandoned August-Euler-airfield located west of Darmstadt, Germany

(49°51’ N; 8°35’ E), was sieved (2 mm) and autoclaved (30 min). A total of 135 g dry wt of autoclaved soil with a water content of 10 % were filled into sterile 250 ml Nunclon filter cap flasks (Nunc, Wiesbaden, Germany). The flasks were kept upright, leaving a headspace of 150 ml for plant growth.

Plant material

To monitor auxin *in situ*, we used the *A. thaliana* Columbia-0 DR5::GUS transgenic line, which contained a highly active synthetic auxin-response element (DR5), a minimum promoter, and a β-glucuronidase (GUS) reporter gene (Ulmasov et al., 1997). To measure changes in plant cytokinin concentrations, we used the *A. thaliana* Wassilewskija ARR5::GUS transformant, which contained a cytokinin-responsive *Arabidopsis* response regulator (ARR) 5 gene, fused to a GUS reporter gene (D’Agostino et al., 2000; Werner et al., 2003). The ARR5 gene belongs to the cytokinin primary response genes of the *Arabidopsis* type A family of two-component response homologues (Deruère & Kieber, 2002). The GUS activity in the reporter lines coincides with endogenous hormone concentrations and therefore can be used to monitor the contents and distribution of auxins (DR5) or cytokinins (ARR5) in *A. thaliana* (Ulmasov et al., 1997; Sabatini et al., 1999; D'Agostino et al., 2000; Casimiro et al., 2001).

The *Arabidopsis* seeds were surface sterilized by washing with 70 % ethanol for 3 min, followed by 3 min in 10 ml 5 % NaOCl (VWR) with two drops of Brij 35 (BDH, Poole, UK) under vacuum (-90 hPa). Subsequently the seeds were washed ten times with sterile tap water and directly sown into soil of the culture flasks. After sowing, the seeds were cold treated at 4 °C for 4 d in darkness to promote and synchronize germination. The plants were subsequently germinated in a growth chamber with a

photoperiod of 10 h of light (150 µmol m^{-2} s^{-1}) at 24 °C. After germination the plants received a higher light intensity (250 µmol m^{-2} s^{-1}) at 20 °C.

The plants were harvested 2 and 6 weeks after germination. After separating roots and shoots, shoot dry weight was determined (24 h, 70 °C). With a corer (2 cm diameter) a subsample of roots was removed and washed from adhering soil for histochemical GUS assays. The roots from 6-week old plants were scanned (Regent 1600+ scanner) and their root morphology analysed with WinRhizo software (Winrhizo 5.0a, Regent Instruments Inc).

Histochemical β-glucuronidase (GUS) assay

For histochemical staining for GUS activity the washed roots of seven replicates of each treatment were incubated for 2 h with a staining solution containing 1 mM 5–bromo-4-chloro-3-indoly-β-D-glucuronide at pH 7.0 (X - Gluc; Molecular Probes, Leiden, The Netherlands), and further incubated at 37 °C for 24 h according to Jefferson (1987). Subsequently the roots were incubated for 24 h in a clearing solution of 100 % chloral hydrate: 90 % lactic acid (2:1, v/v) at 4 °C. The cleared root samples were viewed in 90 % lactic acid with an Aristoplan microscope (Leica, Bensheim, Germany), and pictures of ten root tips per sample were taken at 100x magnification with a Nikon Coolpix 990 (Tokio, Japan) digital camera with fixed aperture. The average blue colour intensity of the root tips was quantified using the programme ImageJ (NIH, Bethesda, USA) after extracting the blue and green colour information of the RGB-picture with a red-filter function and correction for the background.

Determination of available nitrate and ammonia in soil

At the first harvest after two weeks, 5 g fresh weight of soil were suspended in 20 ml distilled water and subsequently shaken for 20 min at 60 rpm on a rotary shaker and centrifuged at 1000 g for 10 min. The concentrations of ammonium and nitrate in the supernatant of the soil suspension were determined with respective NH_4^+ and NO_3^- electrodes (Windaus Labortechnik, Clausthal-Zellerfeld, Germany). After drying of the soil, the concentrations of NH_4^+ and NO_3^- per unit dry weight of soil were calculated.

Statistical analysis

Data which were not independent of each other, e.g. concentrations of free and conjugated IAA in shoots and roots (Experiment 1) and data on shoot biomass and root architecture (Experiment 2) were analysed by multivariate analysis of variance (MANOVA, Roy's Greatest Root). In case of significant MANOVA results we proceeded with analysis of variance (ANOVA) (i.e. 'protected ANOVA', Scheiner & Gurevitch, 2001). Independent data were calculated with ANOVA (e.g. root branching (Experiment 1) and GUS intensity (Experiment 2)) for subsequent comparison of means at the 5 % probability level, Tukey's honestly significant difference was used. All statistical analyses were implemented in SAS 9.1 (SAS Institute, Cary, Florida, USA).

2.4 Results

Experiment 1

Treatments (Sterile, Bacteria, Amoebae) significantly affected the concentrations of conjugated IAA (MANOVA; Roy's Greatest Root, $F_{2,6}=32.7$, $p=0.0006$) and free IAA (MANOVA; Roy's Greatest Root, $F_{2,6}=31.4$, $p=0.0007$) in *L. sativum.*

Strong treatment effects on IAA levels occurred in shoots, but IAA concentrations in roots were not affected. Presence of bacteria increased the internal concentrations of conjugated IAA in *L. sativum* shoots compared to sterile grown plants by a factor of 23 ($F_{2,6}$=19.66, p=0.002), whereas no increase occurred when the plants were grown in presence of bacteria plus amoebae (Fig. 2.1). In contrast, concentrations of bioactive free IAA in *L. sativum* shoots were similar in Sterile and Bacteria treatments, but increased almost fourfold in presence of amoebae ($F_{2,6}$=29.55, p=0.0008; Fig. 2.1). Correspondingly, the number of root tips of *L. sativum* increased twofold in the Amoebae treatment ($F_{2,24}$=6.3, p= 0.006; Fig. 2.1).

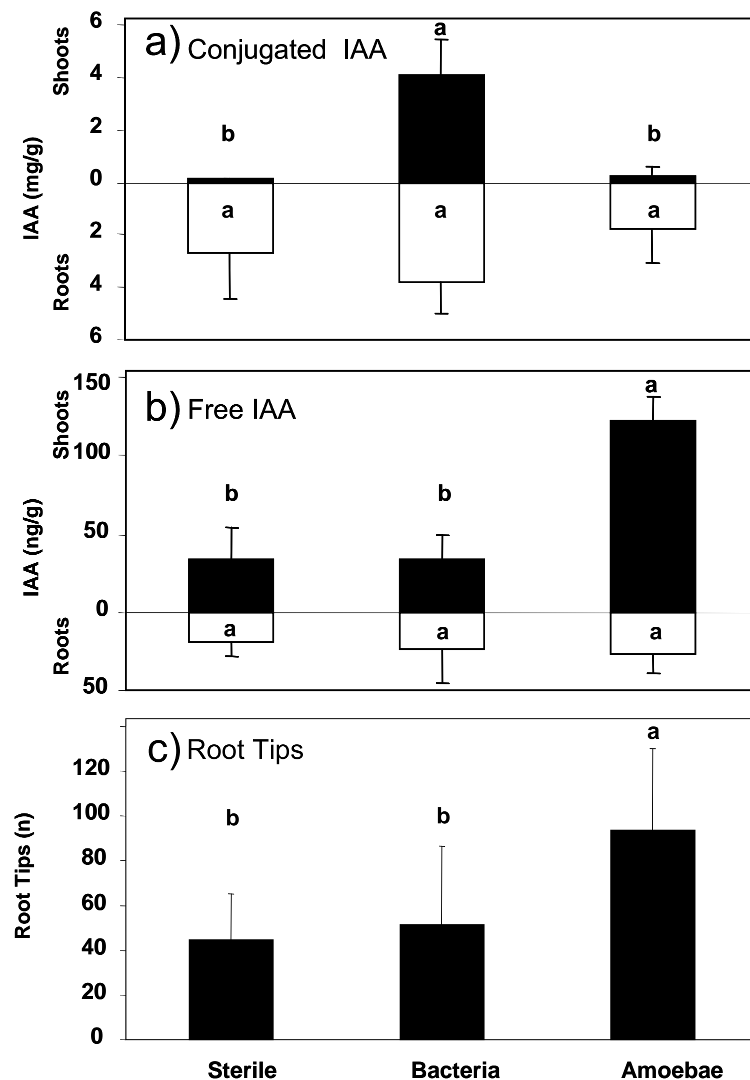

Fig. 2.1: Concentrations of (a) conjugated and (b) free IAA in roots and shoots; and c) number of root tips of *Lepidum sativum* grown under sterile conditions (Sterile), in presence of bacteria (Bacteria) or bacteria and amoeba (Amoebae). Significant differences are indicated by different letters (Tukey honestly significant difference test at p≤ 0.05).

Experiment 2

Amoebae had a general significant effect on the performance of *Arabidopsis* after 6 weeks (MANOVA; Roy's Greatest Root, $F_{6,25}$=2.58, p=0.044) and we could not detect any statistical difference between both *Arabidopsis* transformants in their sensitivity to effects of amoebae on plant growth (MANOVA, Roy's Greatest Root $F_{6,25}$=0.63, p=0.7 for the amoebae x plant interaction).

Table 2.1: Two-factorial analysis of variance (ANOVA) table of *F*- and *p*-values on the effect of amoebae (AMO) and plant transformants (PLA) on the number of root tips (n), root branching (root tips cm^{-1} root) and root volume (cm³) of six-week old *Arabidopsis thaliana*. The x-fold increase in Amoebae treatments compared to Bacteria treatments of the auxin-responsive (DR5) and the cytokinin-responsive (ARR5) *Arabidopsis* transformants is given in the lowermost two lines in italics. Stars indicate significant positive effects of amoebae on the different transformants at * p≤0.05 and ** Pp≤ 0.01.

		Shoot biomass		Root length		Number of root tips		Root branching		Root volume	
	df	F	p	F	p	F	p	F	p	F	P
AMO	1,31	0.82	0.373	9.52	**0.004**	15.83	**<.0001**	9.83	**0.004**	4.46	**0.043**
PLA	1,31	0.02	0.885	0.6	0.445	2.71	0.110	2.02	0.166	0.73	0.401
AMO x PLA	1,31	0.36	0.552	0.76	0.389	0.22	0.643	2.07	0.160	0.5	0.486
DR5		*1.04*		*1.28*		*1.88**		*1.48**		*1.25*	
ARR5		*1.24*		*1.63***		*1.90***		*1.18*		*1.71**	

After six weeks, amoebae did not affect shoot biomass of *A. thaliana* (Table 2.1), but root growth was strongly increased in Amoebae treatments of both, plants transformed with the auxin-reporter DR5 and the cytokinin-reporter ARR5 (Fig. 2.2). Amoebae in general caused an increase in total root length (Table 2.1, Fig. 2.2). On average, the number of root tips almost doubled in treatments with amoebae from 176 ± 81 to 332 ±129 in the Bacteria and Amoebae treatment, respectively. Also, root branching was denser in treatments with amoebae with 3.3 ± 0.9 and 4.5 ± 1.1 root

tips cm^{-1} in the Bacteria and Amoebae treatment, respectively (Table2.1). The increase in root volume in presence of amoebae mainly reflected the effects on root length.

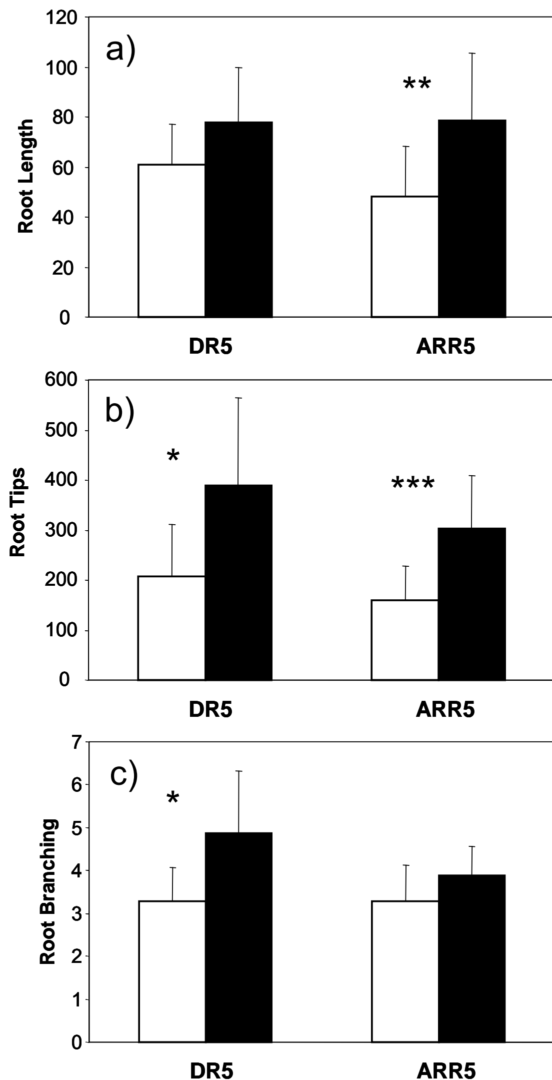

Fig. 2.2: Changes in root architecture of auxin-responsive (DR5) and cytokinin-responsive (ARR5) *Arabidopsis* transformants in absence (white bars) and presence (black bars) of the protozoan grazer *Acanthamoeba castellanii*: (a) total root length (cm), (b) number of root tips, and (c) root branching (root tips cm^{-1} root) of *A. thaliana*. Stars indicate significant positive effects of amoeba on the different Arabidopsis transformants at * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Despite increased root branching, auxin-responsive DR5::GUS coloration intensity was not affected by Amoebae ($F_{1,12}=0.31$, $p=0.59$ and $F_{1,12}=2.07$, $p=0.18$ for the two and six week old plants, respectively). Instead, cytokinin-responsive ARR5::GUS coloration intensity had increased in the Amoebae treatment by factors of 1.8 and 1.4

in two ($F_{1,12}$=18.7, P=0.001) and six week old plants ($F_{1,12}$=5.6, p=0.035), respectively (Fig. 2.3).

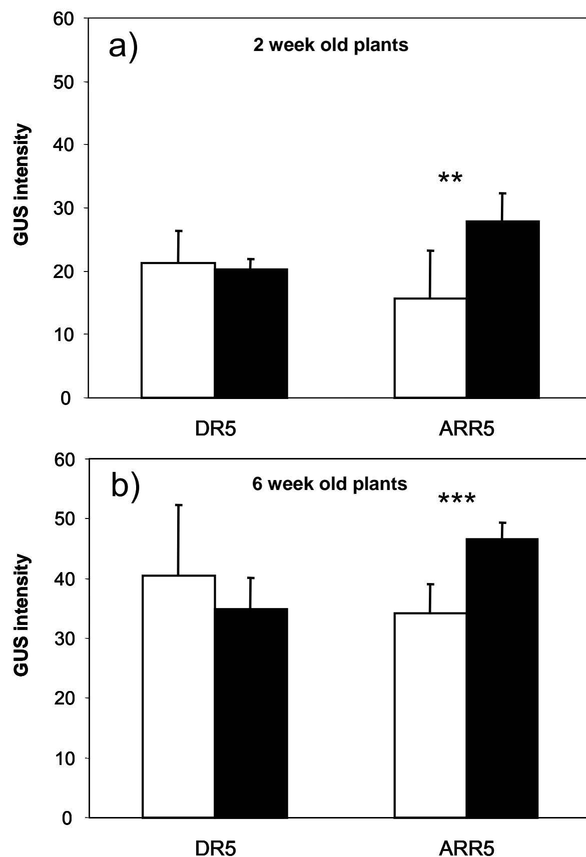

Fig. 2.3: Differences in GUS intensity of auxin-responsive (DR5) and cytokinin-responsive (ARR5) *Arabidopsis* transformants in absence (white bars) and presence (black bars) of the protozoan grazer *Acanthamoeba castellanii*: (a) two week old plants and (b) six week old *A. thaliana* plants. Stars indicate significant positive effects of amoebae at ** p≤0.01, and *** p≤0.001.

At harvest of *A. thaliana* after two weeks, presence of amoebae had increased the concentrations of NO_3^- in soil solution from 1.5 ± 0.5 to 2.2 ± 1.1 µg NO_3^--N mg^{-1} soil dry wt ($F_{1,30}$=4.7, p=0.039) and decreased the concentrations of NH_4^+ from 9.1 ± 2.0 to 7.6 ± 1.7 µg NH_4^+-N mg^{-1} soil dry wt ($F_{1,30}$=5.7, p=0.023), respectively. Accordingly, the NH_4^+/NO_3^- ratio decreased from 6.6 to 4.5 in Amoebae treatments ($F_{1,30}$=6.6, p=0.015), but the total amount of plant-available nitrogen in soil was not affected ($F_{1,30}$=1.5, p=0.24).

2.5 Discussion

Experiment 1

Root growth of *L. sativum* in our first experiment corresponded well to results from a previous study, where lateral roots of *L. sativum* increased in number and length when grown for 10 days on agar with a diverse soil bacterial population in presence of *A. castellanii* (Bonkowski & Brandt, 2002). Since the morphological changes in root growth resembled physiological effects caused by the plant hormone auxin (Himanen et al., 2002; Casimiro et al., 2003; Laskowski et al., 2006), Bonkowski & Brandt (2002) investigated auxin production by bacteria and indeed found that the proportion of IAA-producing bacteria in plate washes of the Bacteria treatment increased in the Amoebae treatment from 71 to 97 %, respectively.

In the present study, concentrations of conjugated auxin in plants strongly increased in presence of bacteria, suggesting that either directly IAA of bacterial origin accumulated in *L. sativum*, or that the presence of bacteria indirectly induced a strong increase in the plant-synthesis of auxins (Patten & Glick, 1996). IAA conjugates e.g. are involved in IAA transport and storage. Since high concentrations of free IAA can be toxic to plants and inhibit root expansion (Xie et al., 1996), the conjugation of IAA also functions as detoxification process (Seidel et al., 2006). The very high amounts of conjugated IAA due to the presence of bacteria suggests that soil bacteria strongly increased free IAA concentrations, which were further conjugated possibly to protect the plant from toxic IAA levels. In either case, the fact that root growth in bacteria treatments did not differ in comparison to sterile grown plants, although auxin concentrations increased, suggests that the hormone was inactivated by the plant through conversion into the non-active conjugated form in presence of bacteria (Seidel et al., 2006). It has long been suggested that particular bacterial isolates affect plant internal IAA concentrations (Barbieri et al.,

1986; Barbieri & Galli, 1993; Frankenberger & Arshad, 1995; Lambrecht et al., 2000; Dobbelaere et al., 2001; Patten & Glick, 2002; Vessey, 2003). Our data complement these studies, showing that plant-interactions with a natural mixed bacterial population may significantly affect the auxin balance of plants.

However, the physiological and morphological response of *L. sativum* to bacteria shifted fundamentally in treatments with amoebae. In presence of amoebae concentrations of conjugated auxin did not differ from those in sterile grown plants. However, concentrations of the bioactive free auxins which play a crucial role in root development (Malamy & Benfey, 1997; Casimiro et al., 2001; Himanen et al., 2002; Casimiro et al., 2003; Laskowski et al., 2006) strongly increased in *L. sativum* shoots, and exclusively in the treatment with amoebae *L. sativum* plants responded with increased root branching. Surprisingly, free IAA concentrations were only increased in shoots, but not in roots of *L. sativum* indicating that the presence of amoebae indirectly induced the synthesis of IAA in the plant shoot. Shoot derived IAA and its polar transport into the root is known to be crucial for the development of lateral roots (Reed et al., 1998; Casimiro et al., 2001; Bhalerao et al., 2002) and these results demonstrate that the protozoa induced increase in free IAA in shoots is linked to an increase in root branching. This experiment gives evidence that the regulation of increased root branching does probably not directly rely on the synthesis of IAA in the rhizosphere, but on changes in the finely tuned regulation mechanisms of auxin concentration and distribution within the plant.

In earlier experiments, protozoa have been shown to strongly change the composition and function of rhizosphere bacterial communities (Griffiths et al., 1999; Rønn et al., 2002; Bonkowski & Brandt, 2002; Kreuzer et al., 2006, Rosenberg, 2008) due to selective feeding on specific bacteria. Our data show that the response of plants to bacteria strikingly differed if a similar bacterial community was grazed by

protozoa. This demonstrates that bacterivores not simply enhanced overall bacterial effects on plant performance, but the interactions in the microbial food web induced altogether a completely different quality of plant responses, finally responsible for the observed changes in root branching. Overall, soil amoebae changed microbial community composition by selective grazing, thereby changing rhizosphere signalling and subsequently inducing plant internal modifications of auxin levels.

Experiment 2

Consistent with the results from *L. sativum*, both *Arabidopsis* reporter plants responded with increased root length and numbers of root tips in treatments with amoebae as compared to treatments with bacteria only. Complicating a simple explanation, however, was the observation that reporter plants did not show the expected auxin response in roots, but responded with increased levels of the auxin-antagonist cytokinin (Coenen & Lomax, 1997), which is considered to repress lateral root formation (Werner et al., 2001, 2003; Li et al., 2006; Laplaze et al., 2007). Despite increased root branching, GUS colour intensity of Arabidopsis DR5 reporter plants did not change, suggesting that total concentration of auxin in roots of *Arabidopsis* remained unaffected. The concentration window for auxins to promote root growth is narrow. In fact, the concentration of free IAA in amoebae treatments reached 5.4×10^{-7} M IAA in *L. sativum*, and was well within the range stimulating root growth. However, slightly higher (10^{-6}) free auxin levels already hamper plant growth (Scott, 1972; Mulkey et al., 1982; Xie et al., 1996; Seidel et al., 2006). The data from our first experiment confirm that plant IAA exists to 99 % in a conjugated state, and only about 1 % is bioactive but sufficient to provoke marked changes in root architecture. Presumably, DR5::GUS transformants were not sensitive enough to monitor these slight changes in the auxin balance (Ulmasov et al., 1997).

In contrast to auxin reporters, GUS coloration of *Arabidopsis* ARR5 transformants strongly increased, indicating that cytokinin concentrations increased almost twofold in presence of amoebae. It is well known that nitrogen availability tightly regulates cytokinin biosynthesis (Takei et al., 2002; Sakakibara, 2003). The rate-limiting step of cytokinin biosynthesis in *Arabidopsis* is catalyzed by ATP/ADP isopentenyltransferases (Miyawaki et al., 2004) and NO_3^- but not NH_4^+ stimulates the expression of ATP/ADP isopentenyltransferase genes (Miyawaki et al., 2004; Takei et al., 2004). In addition, it has been shown that in barley, maize and *Arabidopsis* roots cytokinin accumulates in response to increased nitrate supply (Samuleson & Larsson, 1993; Takei et al., 2002; Miyawaki et al., 2004). Protozoan grazing is described to liberate NH_4^+ (Clarholm, 1985, 2005) from consumed bacterial biomass and to stimulate nitrifying bacteria, presumably through predation on their faster growing bacterial competitors, resulting in high concentrations of NO_3^- in culture liquid and leachate of rhizosphere soil (Griffiths, 1989; Verhagen et al., 1994; Alphei et al., 1996; Bonkowski et al., 2000b). In fact, already in the two week old plants amoebae had significantly shifted the NH_4^+-to-NO_3^- ratio in soil in favour of nitrate and this may have led to the increased endogenous concentrations of cytokinin in *Arabidopsis*. Furthermore, cytokinin is assumed to increase free auxin levels (Coenen & Lomax, 1997), which again may stimulate root growth. Although these conclusions remain speculative, they provide a mechanistic framework of potential interactions that can be tested in future experiments.

Overall, results from both experiments indicate that mutual control mechanisms exist between plant hormone metabolism and microbial signalling, and that the fine tuning of the hormonal interactions of plants with free-living bacteria is strongly influenced by the presence of bacterial grazers, such as amoebae.

CHAPTER 3

GRAZING OF PROTOZOA ON RHIZOSPHERE BACTERIA ALTERS GROWTH AND REPRODUCTION OF *ARABIDOPSIS THALIANA*

3.1 Abstract

Plant roots are densely colonised with soil bacteria, which rely on carbon rich root exudates and form the basis of the rhizosphere bacterial food web with protozoa as most effective predators. We hypothesized that soil bacteria as well as soil protozoa promote plant growth since both benefit from an increased carbon allocation and exudation belowground. Therefore we established a well defined laboratory system with *Arabidopsis thaliana* as model plant allowing to investigate in detail the effect of rhizosphere interactions between soil bacteria and the bacterivorous soil protozoa *Acanthamoeba castellanii* on plant performance. We analysed growth and reproduction as well as tissue carbon and nitrogen concentrations of *A. thaliana.* Further, we investigated nitrogen availability and by using ^{15}N labelling we traced the incorporation of nitrogen from organic matter into the plants as affected by bacteria and amoebae. Protozoa and soil bacteria increased plant growth with the effects of protozoa exceeding those of bacteria only. *A. thaliana* immediately responded to the presence of protozoa by increasing carbon but not nitrogen uptake. Later protozoa enhanced plant uptake of nitrogen from organic material and prolonged vegetative growth of *A. thaliana* resulting in strongly increased seed production. It is concluded that the immediate plant response was based on changes in rhizosphere signalling inducing increased plant carbon fixation. The following increased plant nitrogen uptake presumably originated from nitrogen fixed in bacterial biomass made available by protozoan grazing. The results suggest that *A. thaliana* anticipated the upcoming

mobilization of nitrogen and initiated carbon fixation and root carbon allocation which payed off later by increased nutrient capture and strongly increased plant reproduction.

3.2 Introduction

Soil bacteria profoundly affect plant development in a beneficial or detrimental way. Plant growth promoting rhizobacteria improve plant growth by increasing nutrient supply, releasing phytohormones or analogues, triggering induced systemic resistance or operating as biocontrol agents against pathogens, whereas detrimental bacteria attack root tissue as pathogens (Persello-Cartieaux et al., 2003; Ping & Boland, 2004; Barea et al., 2005; Bais et al., 2006). In the field the diverse community of bacterial species colonizing plant roots are consumed by predators. Soil bacteria are generally limited by carbon and thus depend on the release of carbon rich exudates by plant roots (Wardle, 1992; Paterson et al., 1997; Aldén et al., 2001), which forms the basis of a complex microbial food web with protozoa as the most important predators (Bonkowski, 2004). Since bigger plants may allocate more carbon belowground and support a greater root biomass, resulting in increased bacterial biomass and therefore prey for bacterivores (Bonkowski & Brandt, 2002, Phillips et al., 2003), we hypothesize that soil bacteria and bacterivorous protozoa benefit from inducing an increased plant performance.

To investigate effects on plant growth caused by bacteria – protozoa interactions we developed a model laboratory system allowing to investigate effects of bacterial communities and bacterivorous protozoa on plant performance. We used *Arabidopsis thaliana* as a model plant since it allows time efficient analysis of plant growth and reproduction due to its small size and rapid life cycle. Further, the molecular toolbox

available for *A. thaliana* allows in depth analysis of plant responses to rhizosphere interactions in later experiments. As bacterivorous model organism we chose the naked amoeba *Acanthamoeba castellanii* which is one of the most common protozoan species in soil (Page, 1976).

This study presents the set up of the model system and uses it to investigate effects of a natural soil bacterial community and bacterivorous amoebae on the growth and reproduction of *A. thaliana*. In particular, we analysed rosette diameter, biomass and reproduction, as well as tissue carbon and nitrogen concentrations of *A. thaliana.* Further, by using ^{15}N labelling we traced the incorporation of nitrogen from organic matter into the plants as affected by bacteria and amoebae.

3.3 Materials and Methods

Compared to field conditions the model system was simplified in various respects, to allow e.g. harvesting of plants with undamaged roots and tracing plant uptake of nitrogen from organic vs. inorganic pools. Therefore, natural soil was replaced by sand mixed with organic matter as substrate for bacteria. Since we aimed to analyse plants grown with and without soil bacteria the development of a system which can be kept without contaminations throughout the experiment was essential.

Plants

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia were surface-sterilized by subsequent soaking in 5 % vol:vol filtered $Ca(ClO)_2$ 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. After sterilisation the seeds were washed twice in autoclaved deionised water and

dried on sterile filter disks. Square Petri dishes (VWR) containing 0.9 % plant agar (Duchefa, Haarlem, The Netherlands) with Gamborg medium plus vitamins (Duchefa) and 0.5 % sucrose were used for plant germination. In the square Petri dishes, a strip of 3 cm agar was removed and ten sterilized seeds were equally spaced on the small cutting edge of the agar. Subsequently, the Petri dishes were positioned upright and incubated at 4 °C for vernalisation and to synchronize germination for three days. For germination the seedlings were placed in a plant growth chamber with a day / night regime of 10 / 14 h at 22 °C / 19 °C and a photon flux density of 150 µmol m^{-2} s^{-1} for three weeks. Day light hours were chosen to support vegetative growth and to suppress the activation of the generative phase.

Bacteria

Soil bacteria used for inoculation were obtained by suspending 20 g fresh collected rhizosphere soil from a meadow (campus of the Faculty of Biology, Darmstadt University of Technology) in 200 ml distilled water and filtering the soil slurry through paper filters (Schleicher & Schuell, Dassel, Germany). To exclude soil protozoa, the filtrate was subsequently filtered through 5 µm and then through 1.2 µm isopore filters (Millipore, Schwalbach, Germany). To check for protozoan contaminations, the bacterial filtrate was cultured for three days in sterile NB-NMAS consisting of nutrient broth (NB; VWR) and Neff`s Modified Amobae Saline (NMAS; Page, 1976) at 1:9 v/v (NB-NMAS) prior to use.

Protozoa

The naked amoeba *A. castellanii*, isolated from woodland soil (Göttinger Wald, Lower Saxony, Germany; cf. Bonkowski & Brandt, 2002), was cultivated axenically in sterile PGY medium (2 % peptone, 1 % glucose and 0.5 % yeast extract; Sigma-Aldrich,

Steinheim, Germany; Rosenberg, 2008). Prior to inoculation, axenic amoebae were washed twice by centrifugation with half strength Hoagland (Sigma-Aldrich) at 1000 rpm for 10 min to remove excess nutrients.

Experimental design

The experiment was set up in plastic Magenta jars (height 10 cm, width 6.5 cm, thickness 6.5 cm; Sigma-Aldrich, St. Louis, USA), which were filled with 220 g quartz sand (1.0-1.2 mm Ø) thoroughly mixed with 0.5 g grass powder from dried and milled leaves of *Lolium perenne* (43.7 % C, 3.8 % N; termed organic matter (OM) in the following) to support bacterial growth and to quantify nutrient mobilization and uptake by the plants. The Magenta jars filled with sand/OM substrate were autoclaved three times; in between they were incubated for 48 h at room temperature to germinate and subsequently kill sporulating microorganisms. The Magenta systems were watered with 6 ml sterile deionised water to adjust moisture levels to about 60 % water holding capacity and checked for sterility by plating sand substrate on NB agar (NB plus 1.5 % agarose; VWR).

Three treatments were set up: (1) sterile Magenta systems, (2) Magenta systems inoculated with bacteria and (3) Magenta systems inoculated with bacteria plus *A. castellanii*. For bacterial inoculation 1.5 ml of the protozoa-free bacterial inoculum containing ca. 10^8 cfu was thoroughly mixed with the sand. Three days later 0.5 ml of washed axenic amoebae (ca. 5000 ind.) in half strength Hoagland were added to the bacteria plus amoebae treatment. Corresponding amounts of sterile NB-NMAS and half strength Hoagland were added to the sterile and bacteria treatments, respectively. Two days after the inoculation of amoebae one sterile *A. thaliana* seedling was planted into each Magenta jar and the plants were watered with 1 ml Gamborg B5-N nutrient solution (Zhang & Forde, 1998) plus

350 mg l^{-1} $^{15}NH_4{}^{15}NO_3$ (2.86 atom % ^{15}N l^{-1}; Campro Scientific, Berlin, Germany). Watering with Gamborg B5-N nutrient solution was repeated three days past plant inoculation (dpi), i.e. three days past plant transformation into the inoculated jars. During the experiment the jars were incubated in a plant growth chamber with a day / night regime of 10 / 14 h at 22 °C / 19 °C and a photon flux density of 150 µmol m^{-2} s^{-1}. To allow investigating plant reproduction the light regime was gradually changed to a day / night regime of 14 / 10 h at 14 dpi initiating flowering and seed production.

For each treatment (sterile, with bacteria and with bacteria plus amoebae) 60 replicates were set up resulting in 180 Magenta jars in total. From 10 replicates of each treatment, rosette diameter was monitored at 3 and 6 dpi; then the plants were grown until maturity to investigate number and biomass of seeds as measure of plant reproduction (five replicates only). Ten replicates of each treatment were destructively sampled at each 3 and 6 dpi, for shoot and root biomass measurements and subsequent analyses of shoot carbon and nitrogen concentration; also, concentrations of nitrate and ammonium in the sand substrate were analysed from these replicates. The remaining 30 replicates were kept without plants to measure concentrations of soil nitrate and ammonium in absence of plant roots at 0, 3 and 6 dpi (10 replicates each).

Plant performance and plant uptake of carbon and nitrogen

Plant rosette diameters of each leaf pair (except cotyledons) were measured manually with a pair of compasses immediately after transplanting the plants into Magenta jars (0 dpi), and at 3 and 6 dpi. The mean diameter of the leaf rosette was calculated after subtracting the rosette diameter 0 dpi from that 3 and 6 dpi. In the plants kept further the time to the onset of inflorescense production was monitored. At maturity seeds were collected in Arasystems plastic tubes (Beta Tech, Gent,

Belgium), seed biomass was determined and a subset of seeds were counted to calculate average seed weight.

Biomass of harvested plants was measured after drying of shoots and roots at 60 °C for 24 h. Due to the small size of the sterile grown plants shoots and roots of each replicate plant were pooled, chopped with fine scissors and dried again at 60 °C for one day before processing for total plant carbon and nitrogen analysis as well as isotope ratio analysis ($^{15}N/^{14}N$). An elemental analyser (NA 1500, Carlo Erba, Milan, Italy) was used for total plant carbon and nitrogen analysis. The system was coupled with a trapping box (type CN, Finnigan, Bremen, Germany) and an isotope ratio mass spectrometer (MAT 251, Finnigan, Bremen, Germany) for isotope ratio analysis. Acetanilide (C_8H_9NO; VWR) was used for internal calibration. The percentage of nitrogen taken up by the plants from the labelled nutrient solution was calculated using a two source mixing model (Peterson & Fry, 1987) as $\%N_{sol} = (R_{sample} – R_{unlabelled\ OM}) / (R_{labelled\ solution} - R_{unlabelled\ OM}) \times 100$ with R the ratio between the heavy and the light isotope ($^{15}N/^{14}N$) in the respective materials.

Analysis of plant available nitrogen

Concentrations of plant available ammonium and nitrate were analysed in the sand substrate of unplanted and planted systems immediately before transplanting *A. thaliana* into Magenta jars (0 dpi), and at 3 and 6 dpi. From each replicate 5 g of the sand substrate was suspended in 25 ml 0.1 M $CaCl_2$ x 2 H_2O and shaken for 1 h. The solution was filtered through paper filters (Schleicher & Schuell, Dassel, Germany) and subsequently used for ion chromatography. The ion chromatography system DX500 consisted of a gradient pump GP40, an electrochemical detector ED40, an UV-VIS-Detector AD20 and a PeakNet Chromatograpy Workstation

(Dionex, Idstein). IonPac AS14 separating columns and IonPac CS16 separating columns were used for the analysis of nitrate and ammonium, respectively.

Statistical analysis

Data on rosette diameter were analysed by two-factor repeated measures analysis of variance (rm-ANOVA) with Treatment (sterile control, with bacteria, with bacteria plus amoebae) as fixed factor and Time (measurements at 3 and 6 dpi) as repeated factor. Data on concentrations of carbon and nitrogen in plant tissue and percentage of nitrogen taken up by the plants from the nutrient solution were analysed by two-factor ANOVA with Treatment (see above) and Time (destructive samplings 3 and 6 dpi) as fixed factors. Prior to statistical analyses data on percentage of nitrogen taken up from the nutrient solution were arcus sinus square root transformed. Data on the availability of ammonium and nitrate in the sand substrate at 0, 3 and 6 dpi were analysed by three-factor ANOVA with Treatment (see above), *A. thaliana* (with and without) and Time (destructive samplings at 0, 3 and 6 dpi) as fixed factors. Differences between means were inspected at the 5 % probability level using Tukey's minimum significant difference test. Statistical analyses were executed using SAS 9.1 (Cary, Florida, USA).

3. 4. Results

Plant growth

Compared to sterile rhizosphere plants, rosette diameter of *A. thaliana* increased by factors of 2.6 and 2.4 in presence of bacteria at 3 and 6 dpi, respectively (Table 3.1, Fig. 3.1).

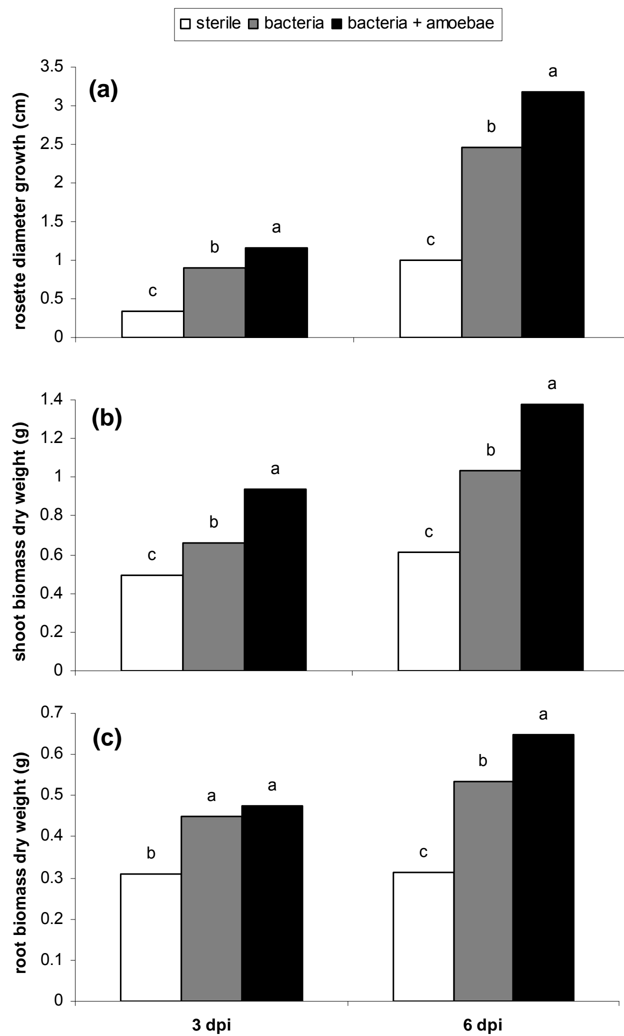

Fig. 3.1: Effect of bacteria and bacterivorous amoebae *(Acanthamoeba castellanii)* on (a) rosette diameter growth, (b) shoot biomass and (c) root biomass of *Arabidopsis thaliana* 3 and 6 days past plant inoculation (dpi); means of ten replicates per treatment are presented. Data marked with the same letter do not differ significantly (Tukey's minimum significant difference test, $P<0.05$).

Compared to plants grown with bacteria only, the additional presence of amoebae further enhanced rosette diameter of *A. thaliana* by factors of 1.3 and 1.3 at 3 and 6 dpi, respectively (Table 3.1, Fig. 3.1). Similarly, compared to sterile plants shoot biomass increased by factors of 1.3 and 1.7 in presence of bacteria, and further in presence of bacteria plus amoebae by factors of 1.4 and 1.3 at 3 and 6 dpi, respectively (Table 3.1, Fig. 3.1).

Respective factors for root biomass in presence of bacteria were 1.5 and 1.7; in presence of bacteria plus amoebae root biomass did not increase further at 3 dpi, but was further increased by a factor of 1.2 at 6 dpi (Table 3.1, Fig. 3.1).

Table 3.1: ANOVA table of F-values on the effect of treatment (sterile, with bacteria, with bacteria plus amoebae) and time (sampling day 3 and day 6 past plant inoculation) on rosette diameter, shoot biomass and root biomass of *Arabidopsis thaliana*.

	between subject effects			within subject effects					
	treat			time			treat x time		
factor	df	F	p	df	F	p	df	F	p
rosette diameter	2,27	54.72	<0.0001	1,27	136.18	<0.0001	2,27	10.89	0.0003
shoot biomass	2,27	116.65	<0.0001	1,27	108.53	<0.0001	2,27	11.23	0.0003
root biomass	2,27	88.81	<0.0001	1,27	12.50	0.0015	2,27	3.84	0.0341

Plant uptake of carbon and nitrogen

The amount of carbon in *A. thaliana* followed the same pattern as plant biomass; compared to sterile grown plants carbon increased by factors of 1.3 and 1.6 in the presence of bacteria.

Compared to plants grown with bacteria only, the additional presence of amoebae further enhanced the amount of carbon by factors of 1.2 and 1.2 at 3 and 6 dpi, respectively (Table 3.2, Fig. 3.2). In contrast to plant carbon, the amount of plant nitrogen was not increased in the presence of bacteria and amoebae compared to sterile rhizosphere plants at 3 dpi. However, at 6 dpi the amount of plant nitrogen in the presence of bacteria plus amoebae exceeded that of sterile rhizosphere plants and plants grown with bacteria only by factors of 2.0 and 1.7, respectively (Table 3.2, Fig. 3.2). Consequently, at 3 dpi the C-to-N ratio of *A. thaliana* plants in the presence of bacteria and bacteria plus amoebae were increased by factors of 1.3 and 1.4 compared to sterile rhizosphere plants (Table 3.2, Fig. 3.2).

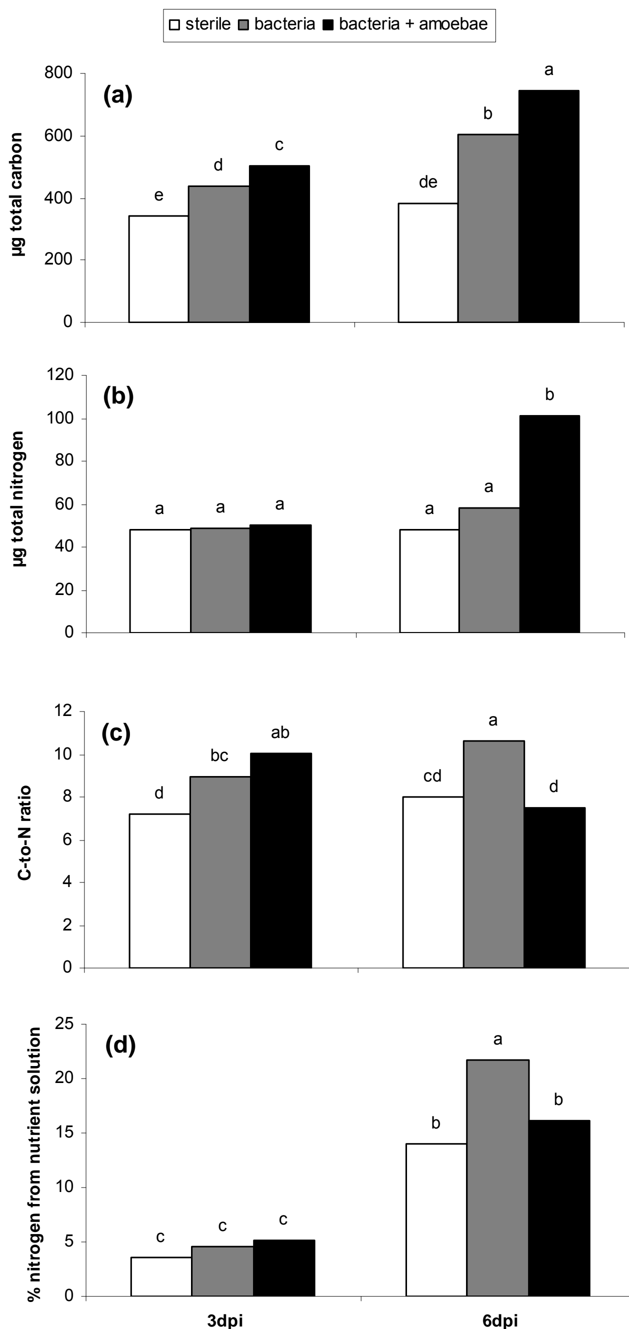

Fig. 3.2: Effect of bacteria and amoebae on the amount of (a) carbon, (b) nitrogen, (c) C – to - N ratio and (d) nitrogen uptake from the nutrient solution of *Arabidopsis thaliana* 3 and 6 days past plant inoculation (dpi); means of ten replicates per treatment. Data marked with the same letter do not differ significantly (Tukey's minimum significant difference test, $P<0.05$).

At 6 dpi the plant C-to-N ratio in the presence of bacteria exceeded that of sterile rhizosphere plants by a factor of 1.3; remarkably, the plant C-to-N ratio in the presence of bacteria only also exceeded that in the presence of bacteria plus amoebae by a factor of 1.4 (Table 3.2, Fig. 3.2).

Table 3.2: ANOVA table of F-values on the effect of treatment (sterile, with bacteria, with bacteria plus amoebae) and time (sampling day 3 and day 6 past plant inoculation) on carbon and nitrogen concentration as well as on carbon-to-nitrogen ratio and on the percentage of nitrogen taken up from the nutrient solution of *Arabidopsis thaliana*.

	treat			time			treat x time		
factor	df	F	p	df	F	p	df	F	P
carbon	2,54	127.80	<0.0001	1,54	122.81	<0.0001	2,54	18.74	<0.0001
nitrogen	2,54	62.91	<0.0001	1,54	87.52	<0.0001	2,54	53.06	<0.0001
C-to- N ratio	2,54	23.77	<0.0001	1,54	0.03	0.8747	2,54	24.93	<0.0001
N uptake from solution	2,54	15.61	<0.0001	1,54	416.32	<0.0001	2,54	11.94	<0.0001

At 3 dpi the percentage of nitrogen in plant tissue originating from the added mineral fertilizer (^{15}N labelled NH_4NO_3) did not differ significantly between experimental treatments (Table 3.2, Fig. 3.2). In contrast, at 6 dpi the percentage of plant nitrogen originating from mineral fertilizer in presence of bacteria exceeded that of sterile rhizosphere plants and plants grown in presence of bacteria plus amoebae by factors of 1.5 and 1.4, respectively (Table 3.2, Fig. 3.2), indicating that plants grown in presence of bacteria took up less nitrogen from the added OM, i.e. the organic component of the sand/OM substrate.

Plant reproduction

The duration of the vegetative growth phase of *A. thaliana* was prolonged by nine days in presence of bacteria plus amoebae compared to both, sterile rhizosphere plants and plants grown with bacteria only (Fig. 3.3).

Total seed biomass in presence of bacteria plus amoebae exceeded that of sterile rhizosphere plants and plants grown with bacteria only by factors of 7.5 and 3.1, respectively (Fig. 3.3; $F_{2,12}$=51.61, p<0.0001). This increase was due to increased

seed numbers since the biomass of individual seeds did not change significantly ($F_{2,12}$=0.34, p>0.7183).

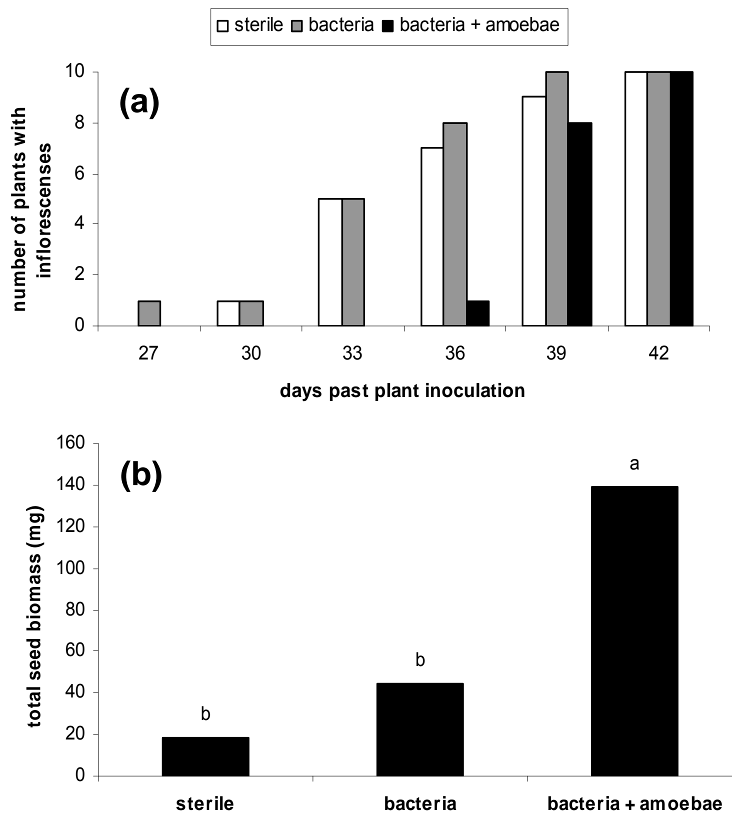

Fig. 3.3: Effect of soil bacteria and soil amoebae on (a) initiation point of inflorescence production presented as number of plants with inflorescences (means of ten replicates per treatment are presented) and (b) seed biomass production of *Arabidopsis thaliana* (means of five replicates per treatment are presented). Data marked with the same letter do not differ significantly (Tukey's minimum significant difference test, P<0.05).

Mineral nitrogen in the sand/OM substrate

In contrast to the sand/OM substrate with bacteria and with bacteria plus amoebae, high concentrations of ammonium and nitrate were present throughout the experiment in the sand/OM substrate of sterile rhizosphere plants (Table 3.3, 3.4).

In unplanted systems, the concentrations of ammonium in the sand/OM substrate decreased to 8, 32 and 13 % in presence of bacteria and to 8, 31 and 13 % in presence of bacteria plus amoebae of the concentrations in the sterile substrate at 0, 3 and 6 dpi, respectively. Also, in planted systems concentrations of ammonium decreased in presence of bacteria and bacteria plus amoebae (to 26 and 17 %, and

to 42 and 34 % of the concentrations in the sterile substrate at 3 and 6 dpi, respectively).

Table 3.3: Effect of bacteria (bact) and amoebae (amo) on the concentration of (a) ammonium and (b) nitrate (µg g^{-1} sand) in the sand substrate at 0, 3 and 6 days past plant inoculation (dpi) with and without *Arabidopsis thaliana* plants. Mean values ± standard deviations (SD) from 10 replicates per treatment are presented. Data marked with the same letter do not differ significantly (Tukey's minimum significant difference test, P<0.05).

(a)	0dpi	3dpi		6dpi	
		+ *A. thaliana*	- *A. thaliana*	+ *A. thaliana*	- *A. thaliana*
sterile	3.79±0.15 **b**	4.43±0.53 **b**	4.38±0.44 **b**	5.78±0.58 **a**	5.70±0.41 **a**
bact	0.32±0.22 **f**	1.14±0.32 **de**	1.39±0.58 **cde**	1.00±0.51 **ef**	0.73±0.29 **ef**
bact+amo	0.32±0.37 **f**	1.82±0.65 **cd**	1.34±0.35 **cde**	1.94±0.84 **c**	1.16±0.29 **de**
(b)	0dpi	3dpi		6dpi	
		+ *A. thaliana*	- *A. thaliana*	+ *A. thaliana*	- *A. thaliana*
sterile	48.01±6.13 **ab**	48.83±2.21 **ab**	46.98±4.72 **ab**	45.05±2.83 **b**	49.58±4.17 **a**
bact	1.78±0.98 **c**	2.09±0.86 **c**	2.03±0.22 **c**	1.72±0.08 **c**	1.15±0.12 **c**
bact+amo	0.70±0.12 **c**	1.50±0.37 **c**	1.85±0.58 **c**	1.44±0.28 **c**	1.19±0.20 **c**

Compared to the sand/OM substrate in presence of bacteria only, the additional presence of amoebae increased the availability of ammonium by 94 % in the planted system, but did not affect ammonium concentrations in the unplanted system at 6 dpi (Table 3.3, 3.4). In unplanted systems, concentrations of nitrate almost completely vanished in presence of bacteria to 4, 4 and 2 % and in presence of bacteria plus amoebae to 1, 4 and 2 % of the initial concentrations in the sterile substrate at 0, 3 and 6 dpi, respectively.

Table 3.4: ANOVA table of F-values on the effects of treatment (sterile, with bacteria, with bacteria plus amoebae), plant addition (with, without *Arabidopsis thaliana*) and time (sampling day 0, 3 and 6 past plant inoculation) on concentrations of ammonium and nitrate in the sand substrate.

	treat			plant			time		
factor	df	F	p	df	F	p	df	F	p
ammonium	2,135	1035.59	**<0.0001**	1,135	50.78	**<0.0001**	2,135	70.19	**<0.0001**
nitrate	2,135	5759.62	**<0.0001**	1,135	0.39	0.5322	2,135	0.70	0.4962
	treat x time			treat x plant			time x plant		
factor	df	F	p	df	F	p	df	F	p
ammonium	4,135	20.47	**<0.0001**	2,135	6.39	**0.0022**	1,135	0.00	1.0000
nitrate	4,135	0.26	0.9017	2,135	1.43	0.2428	1,135	4.00	**0.0475**
				treat x time x plant					
factor				df	F	p			
ammonium				2,135	0.00	1.0000			
nitrate				2,135	6.30	**0.0024**			

Similarly, in planted systems concentrations of nitrate in the presence of bacteria and in the presence of bacteria plus amoebae was strongly reduced reaching 4 and 4 %, and 3 and 3 % of the initial concentration in the sterile substrate at 3 and 6 dpi, respectively (Table 3.3, 3.4).

3.5 Discussion

We successfully established an experimental system, in which it is easy to investigate effects of soil bacteria and the bacterivorous amoebae *A. castellanii* on the growth and reproduction of *A. thaliana*. As expected, the plants strongly responded to the presence of microorganisms and protozoan grazers in the rhizosphere.

Effects of bacteria on plant growth

Arabidopsis thaliana grew poorly in the sterile sand/OM substrate despite high concentrations of mineral nitrogen, which are in the range of natural occurring nitrogen concentrations in the field (Britto & Kronzucker, 2002). The presence of soil bacteria markedly improved plant performance as measured by plant rosette diameter and plant biomass. Plant shoot and root biomass was already increased at 3 dpi indicating an immediate response of *A. thaliana* to the presence of soil bacteria. The immediate and strong growth response suggests that the presence of bacteria is essential for successful development of *A. thaliana*. Remarkably, the increase in plant growth by bacteria was independent of plant nitrogen availability and uptake. In fact, soil bacteria strongly decreased the concentrations of ammonium and nitrate in the sand/OM substrate indicating that soil bacteria strongly immobilized nitrogen compounds (Hodge et al., 2000). Additionally, the use of ^{15}N labelling demonstrated that plants grown in presence of bacteria took up more nitrogen from the nutrient solution and less from organic material at 6 dpi indicating that bacteria sequestered the nitrogen from organic matter. Despite plant nitrogen uptake remained unaffected, *A. thaliana* seedlings fixed more carbon in presence of bacteria resulting in an increased C-to-N ratio in plant tissue, and this already occurred at 3 dpi.

Soil microorganisms have been shown to increase net photosynthesis in a number of plant species (Meharg & Killham, 1991; Merbach & Ruppel, 1992). For example, Phillips et al. (1999) demonstrated that *Medicago sativa* responds to lumichrome, a bacterial signal compound, with increased net carbon assimilation. Further, plant growth is known to be stimulated by Plant Growth Promoting Rhizobacteria due to the release of plant hormones or analogues (Bloemberg & Lugtenberg, 2001; Bonkowski & Brandt, 2002; Persello-Cartieux, 2003; Ping & Boland, 2004; Tsavkelova et al., 2006). Similarly, the bacteria-mediated increase in plant growth in

our model system presumably also was due to signalling or hormonal effects since plant nitrogen nutrition remained unaffected. To our knowledge this is the first study comparing the performance of plants grown under sterile conditions with that of plants grown with a complex bacterial community in the rhizosphere resembling that in the field. Previous studies focussed on interactions between plants and certain soil bacteria strains advancing the understanding of interactions between specific bacteria and plants (Ping & Boland, 2004; Mulder et al., 2005; Bakker et al., 2007). However, in nature the rhizosphere of plants is colonised by a wide variety of different microbial species/strains exerting beneficial, neutral and detrimental effects on plants. Since most soil bacteria depend on plants as source of carbon, positive rather than antagonistic interactions are likely to dominate (Wall & Moore, 1999; Phillips, 2003).

Effects of protozoa on plant growth

Parallel to previous studies with other plant species (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Jentschke et al., 1995; Alphei et al., 1996; Bonkowski et al. 2001a; Bonkowski, 2004), results of this study indicate that *A. thaliana* benefits from the presence of amoebae in the rhizosphere. At 3 dpi shoot biomass as well as the amount of fixed carbon but not the amount of nitrogen taken up by *A. thaliana* was increased by the additional presence of amoebae. Remarkably, the increase in shoot growth predated the amoebae-mediated increase in root growth which occurred at 6 dpi but not at 3 dpi, suggesting systemic changes in plant growth initiated via increasing shoot carbon uptake early during plant development. Interestingly, increased root growth at 6 dpi was accompanied by a strong increase in plant nitrogen uptake, and in parallel with a strong reduction in tissue C-to-N ratio of *A. thaliana*.

Plant growth promotion by bacterivorous protozoa has traditionally been ascribed to the microbial loop in soil, i.e. the mobilization of nutrients locked up in bacterial biomass by protozoan grazing (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Clarholm, 2005). Results of the present investigation suggest that the immediate response of plants to amoebae at 3 dpi were unlikely caused by an increased plant nitrogen uptake. Rather, presence of amoebae appeared to first stimulate carbon fixation by shoots resulting in enhanced shoot biomass. We assume that root growth was subsequently promoted by increasing carbon allocation to roots. This subsequent increase in root growth presumably was responsible for the increased incorporation of nitrogen at 6 dpi. Further, increased nitrogen uptake by *A. thaliana* at 6 dpi was facilitated by increased ammonium concentrations in the sand/OM substrate in presence of bacteria plus amoebae compared to the systems where only bacteria were present. Interestingly, this difference only occurred in the planted but not in the unplanted systems, suggesting that *A. thaliana* actively participated in bacteria – amoebae interactions, presumably by increasing the availability of bacterial prey via rhizosphere priming effects (Cheng, 2008) due to enhanced root exudation, and subsequent stimulation of protozoan grazers.

Overall, the use of ^{15}N labelling demonstrated that plants grown in presence of bacteria plus amoebae incorporated more nitrogen from organic matter at 6 dpi suggesting that the protozoan-mediated changes were based on increased nitrogen transfer from OM by feeding on bacteria, i.e. the microbial loop in soil. However, the immediate systemic effect of amoebae on plant growth at 3 dpi was independent of nitrogen, suggesting that the early plant response was due to protozoan-mediated changes in rhizosphere signalling resulting in increased shoot carbon fixation and triggering increased allocation of carbon to the roots, thereby allowing more efficient exploitation of the nutrients made available by protozoan grazing on rhizosphere

bacteria. The timing of the response suggests that the plants sensed the presence of amoebae and anticipated the upcoming mobilization of nitrogen by amoebae. The fact that concentrations of ammonium in presence of amoebae were only increased in planted systems indicates that plants actively participated in the mobilization of nutrients by amoebae.

Effects of bacteria and protozoa on plant reproduction

Later in plant development, *A. thaliana* relocates its aboveground resources to the production of the inflorescence and ultimately into seeds, i.e. into reproduction (Hensel et al., 1993). Increased plant growth at early stages of plant development, e.g. as indicated by larger rosette diameter, is assumed to result in increased seed biomass (Dietrich et al., 2005). Indeed, early shoot growth and carbon fixation by *A. thaliana* in presence of soil bacteria resulted in increased seed biomass; remarkably, this increase was strongly further increased in presence of amoebae, demonstrating that in particular protozoa increased plant reproduction.

Shortage of nitrogen is known to initiate early flowering and seed set (Heil et al., 2000; Dietrich et al., 2005). The enhanced number of seeds in presence of amoebae was associated with both an increased uptake of nitrogen by the plants already at 6 dpi and a prolonged vegetative growth phase. Presumably, reduced nutrient limitation in presence of amoebae triggered the prolongation of the vegetative growth phase and combined with the amoebae-mediated increase in nutrient availability ultimately resulted in the very marked increase in seed production of *A. thaliana*. However, also the attenuation of plant defence, low water availability or other factors may have contributed to the prolonged vegetative growth and thus increased seed production in presence of bacteria plus amoebae (Becker & Apel, 1993; Nam, 1997; Weaver et al., 1998; Tian et al., 2003; Dietrich et al., 2005).

In conclusion, we successfully established a model system which allowed investigating the effects of complex bacterial communities and bacterivorous amoebae on growth and reproduction of *A. thaliana*. The results supported our hypothesis that soil bacteria as well as bacterivorous protozoa promote plant growth, with the effects of protozoa markedly exceeding those of bacteria only. Both caused an immediate growth response in *A. thaliana* which was not related to increased nitrogen availability and plant nitrogen uptake. Rather, the uptake of nitrogen remained unaffected while *A. thaliana* fixed more carbon in presence of bacteria and bacteria plus amoebae. Later, amoebae increased the availability and plant uptake of nitrogen resulting in the prolongation of the vegetative growth period, and consequently to increased seed production. The results suggest that rather than by increased nitrogen availability, the immediate effects of soil bacteria and amoebae on plant growth were due to changes in rhizosphere signalling, resulting in increased carbon fixation and carbon allocation to roots. The established model system is ideally suited to test these hypotheses and uncover the mechanisms responsible for protozoa-mediated changes in plant growth in future experiments using the molecular toolbox available for *A. thaliana*.

CHAPTER 4

TRANSCRIPTIONAL RESPONSE OF *ARABIDOPSIS THALIANA* TO BACTERIVOROUS SOIL PROTOZOA

4.1 Abstract

Plant growth promotion by bacterivorous soil protozoa is traditionally assigned to the mobilisation of nitrogen from consumed bacterial biomass, which improves the nitrogen supply and thus growth of plants. However, previous experiments suggested that the growth initiation of *Arabidopsis thaliana* due to the presence of protozoa is not only mediated by nitrogen. The early plant growth response likely is triggered by changes in rhizosphere signalling resulting from selective grazing on soil bacteria. To investigate the immediate plant growth response to protozoa we employed quantitative real time PCR with nitrogen responsive genes as well as a custom-made DNA array covering genes involved in plant signalling and stress response. As expected shoot growth of *A. thaliana* immediately increased due to soil protozoa. However, the initiation of the growth promotion was not associated with changes in the expression of nitrogen responsive genes, whereas later soil protozoa induced the up-regulation of ammonium assimilatory genes. DNA array expression analysis further demonstrated that soil protozoa rapidly down-regulate defence responses in roots, whereas in shoots defence responses were up-regulated. The results support the suggestion that the initiation of growth promotion of *A. thaliana* by soil protozoa is not mediated by nitrogen, whereas later protozoa increase plant ammonium availability. Presumably, soil protozoa trigger a down-regulation of defence responses in roots via changes in bacterial signalling, resulting in a redirection of

ressources into root growth allowing the plant to take benefit from protozoa-mediated increase in ammonium availability.

4.2 Introduction

Plant growth is strongly affected by microbial communities in the rhizosphere (Paterson, 2003; Barea et al., 2005). Many bacteria isolated from the vicinity of roots produce plant hormones and other signal molecules which have been shown to interfere with the hormonal balance in plants (Patten & Glick, 1996), manipulate root exudation (Phillips et al., 2004), root branching (Barbieri & Galli, 1993; De Leij et al., 2002; Vessey, 2003), root respiration (Phillips et al., 1999; Joseph & Phillips, 2003) or induce systemic resistance (Van Loon et al., 1998; Pieterse et al., 2002). However, it is largely unknown how plants integrate the information from and respond to the diverse signals of rhizosphere microbial communities. Moreover, under natural conditions bacteria in the rhizosphere are part of a complex foodweb and exposed to strong predation pressure of bacterivores, in particular protozoa. Plant growth promotion in presence of bacterivorous protozoa is well documented (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Jentschke et al., 1995; Alphei et al., 1996; Bonkowski et al. 2001a; 2004; Kreuzer et al., 2006) and need to be considered if we want to achieve a thorough understanding of rhizosphere processes (Griffiths et al., 2007). However, basic questions on how the interactions between bacteria and bacterivores affect plants remain as yet unresolved. It has been hypothesized that protozoa mobilise nitrogen locked-up in bacterial biomass thereby enhancing plant nutrient availability and plant growth (‘microbial loop in soil’ *sensu* Clarholm, 1985, 1994).

However, recent experiments indicate that protozoa also affect plant growth via non-

nitrogen mediated processes by structuring rhizobacterial populations thereby governing bacterial effects on plant performance (Bonkowski & Brandt, 2002; Kreuzer et al., 2006; Rosenberg, 2008). To gain insight into interactions between soil bacteria, protozoa and plants we established a well defined laboratory system allowing to investigate growth of *Arabidopsis thaliana* in response to a natural soil bacterial community in absence and presence of protozoan grazers such as the naked amoeba *Acanthamoeba castellanii* (Chapter 3). The results indicated an immediate (< 3 days past inoculation) plant growth response in presence of both bacteria and amoebae - with the effects of amoebae exceeding effects of bacteria only - despite plant nitrogen concentrations were not increased (Chapter 3). The results, therefore, support the hypothesis that bacterivorous amoebae not only enhance plant growth by microbial loop processes but by changes in rhizosphere signalling. This may be caused by grazing-induced shifts in the composition of the bacterial rhizosphere community or just by the presence of amoebae near the plant root. We hypothesize that *A. thaliana* responds to the presence of amoebae with immediate changes in expression patterns of genes involved in secondary and signalling metabolism. Furthermore, we hypothesize that the immediate growth response of *A. thaliana* is not correlated with a transcriptional regulation of nitrogen responsive genes.

To elucidate the immediate response of *A. thaliana* to the presence of amoebae we employed a custom-made DNA array covering about 1000 gene-specific target sequences involved in plant signalling and stress response (Glombitza et al., 2004). Since the applied DNA array did not harbour genes involved in nitrogen metabolism we analysed in addition five genes involved in nitrate transport (*NRT1.1*), nitrate reduction (*NIA2*) and ammonium assimilation (*GS-GLN2*, *GOGAT*, *ASN2*) using

quantitative real time PCR (qRT-PCR), which have been shown to be responsive to enhanced nitrogen supply (Wang et al., 2003).

4.3 Materials and Methods

Plant cultivation

Seeds of wild-type *Arabidopsis thaliana* accession Col-0 were surface sterilized by subsequent soaking in 5 % filtered $Ca(ClO)_2$ 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, respectively. After sterilisation, seeds were washed twice in autoclaved deionised water and dried on sterile filter disks. Sterilized seeds were incubated in upright positioned square Petri dishes (VWR) containing 0.9 % plant agar (Duchefa, Haarlem, The Netherlands) with Gamborg medium plus vitamins (Duchefa) and 0.5 % sucrose 3 days at 4 °C to synchronize germination. Plants were grown in a growth chamber with a day / night regime of 10 / 14 h at 22 °C / 19 °C and a photon flux density of 150 µmol m^{-2} s^{-1} for three weeks prior to transplantation. Day light hours were chosen to support vegetative growth and to suppress the activation of the generative phase.

Bacterial inoculum

A bacterial inoculum was obtained by suspending 20 g freshly collected rhizosphere soil from a meadow (campus of the Faculty of Biology, Darmstadt University of Technology) in 200 ml distilled water and filtering the soil slurry through paper filters (Schleicher & Schuell, Dassel, Germany). To exclude soil protozoa, the filtrate was subsequently filtered through sterile 5 and 1.2 µm isopore filters (Millipore, Schwalbach, Germany). To check for protozoan contaminations, the bacterial filtrate

was cultured for three days in sterile NB-NMAS consisting of nutrient broth (NB; VWR) and Neff`s Modified Amobae Saline (NMAS; Page, 1976) at 1:9 v/v (NB-NMAS).

Protozoan inoculum

The naked amoeba *Acanthamoeba castellanii,* isolated from woodland soil (Göttinger Wald, Lower Saxony, Germany; cf. Bonkowski & Brandt, 2002), was axenically cultivated in sterile PGY medium (2 % peptone, 1 % glucose and 0.5 % yeast extract; Sigma-Aldrich, Steinheim, Germany; Rosenberg, 2008). Prior to inoculation, axenic amoebae were washed twice by centrifugation with half strength Hoagland (Sigma-Aldrich) at 1000 rpm for 10 min to remove excess nutrients.

Experimental system and set up

The experiment was set up in Magenta jars (Sigma-Aldrich, St. Louis, USA), which were filled with 220 g quartz sand (1-1.2 mm Ø) thoroughly mixed with 0.5 g grass powder from dried and milled leaves of *Lolium perenne* (43.7 % C, 3.8 % N) to support bacterial growth. The Magenta jars were autoclaved three times with subsequent incubation periods of 48 h in between at room temperature to kill sporulating microorganisms. The Magenta systems were watered with 6 ml sterile deionised water to adjust moisture levels to 4 % water content and checked for sterility by plating some sand substrate on NB agar (NB plus 1.5 % agarose; VWR).

Three treatments were investigated: (1) sterile plants in sterile sand (Sterile), (2) plants in sand inoculated with bacteria (Bacteria) and (3) plants in sand inoculated with bacteria plus axenic *A. castellanii* (Protozoa). For bacterial inoculation 1.5 ml of the protozoa-free bacterial inoculum containing ca. 10^8 cfu was thoroughly mixed with the sand. Three days later 0.5 ml of washed axenic amoebae (ca. 5000 ind.) in half

strength Hoagland were added to the Protozoa treatment. Corresponding amounts of sterile NB-NMAS and half strength Hoagland were added to Sterile and Bacteria treatments, respectively. Two days after the inoculation of amoebae one sterile *A. thaliana* seedling was planted into each Magenta jar and the plants were watered with 1 ml Gamborg B5-N nutrient solution (Zhang & Forde, 1998) plus 350 mg l^{-1} NH_4NO_3. Watering with Gamborg B5-N nutrient solution was repeated 3 days past plant inoculation (dpi). The planted systems were incubated in a growth chamber with a day / night regime of 10 / 14 h at 22 °C / 19 °C and a photon flux density of 150 µmol m^{-2} s^{-1}.

The experiment was set up twice at exactly identical conditions, the second eight weeks after the first. Plant material from the first experiment was used for the microarray analysis, and plant material from the second experiment was used for qRT-PCR. The effect of soil bacteria was determined by comparing gene expression between plants grown in Sterile treatments and plants grown in Bacteria treatments (Bacteria versus Sterile: BvsS), the effect of protozoa was determined by comparing gene expression between plants in Bacteria and Protozoa treatments (Bacteria versus Protozoa: BvsP). In addition, differences in gene expression between plants grown in Sterile treatments and in Protozoa treatments were analysed (Sterile versus Protozoa: SvsP).

Microarray experiment

For each of the three treatments 66 replicates were set up giving 198 Magenta jars in total. From 10 replicates of each treatment, rosette diameter was monitored 3, 4 and 5 dpi. Plant rosette diameters of each leaf pair (except cotyledons) were measured manually with a pair of compasses immediately after transplanting the plants into Magenta jars (0 dpi) and 3, 4 and 5 dpi, respectively. The increase in mean diameter

of the leaf rosette was calculated after subtracting the rosette diameter at 0 dpi from that at 3, 4 and 5 dpi. Three and 5 dpi, 36 and 30 plants per treatment were harvested, respectively. Twelve plants (3 dpi) and 10 plants (5 dpi) per treatment were pooled to obtain three independent replicates from each harvest for RNA extraction. Plant roots and shoots were separated and stored at -80 °C until analysis.

RNA extraction and generation of labelled cDNA

From each replicate 100 mg were ground to powder in liquid nitrogen and placed in lysis buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM Na_2EDTA, 1 % LiDS, 5 mM DTT, pH 8). Samples were centrifuged and the supernatant transferred to a new tube. Sixty µl Dynabeads Oligo$(dT)_{25}$ (Invitrogen, Dynal AS, Oslo, Norway) were added to the supernatant. The sample was incubated for 5 min at room temperature to allow binding of mRNA to Oligo $(dT)_{25}$ Dynabeads. Dynabeads bind mRNA and allow magnetic separation of RNA from the solution. Beads with mRNA were washed twice in buffer with lithium-dodecylsulfate (10 mM Tris-HCl, 150 mM LiCl, 1 mM Na_2EDTA, 0.1 % LiDS, 0.05 % Tween, pH 8), before being transferred to a new tube and washed twice in buffer without LiDS (10 mM Tris, 150 mM LiCl, 1 mM Na_2EDTA, 0.05 % Tween, pH 8).

For cDNA synthesis beads were washed twice in 1x reverse transcriptase (RT) buffer (diluted from 5x RT: 250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$, pH 8) and transferred to a new tube with 1x RT buffer. Synthesis of cDNA was performed in 100 µl 1x RT buffer, 1 mM DTT, 0.5 mM dNTPs, 104 U RNase Inhibitor and 600 U of SuperScript II (Invitrogen, Karlsruhe, Germany) at 42 °C for 1 h. Then, samples were washed twice in RT buffer plus 0.05 % Tween and transferred to a new tube. To detach RNA from beads, TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8.5) was added and the sample was incubated at 95 °C for 2 min. The supernatant containing

the mRNA was removed and cDNA bound to the beads was washed twice in TE buffer.

Beads carrying first-strand cDNA were washed twice with ddH_2O and denatured in ddH_2O and 10x decamer solution (DECAprime II Kit; Applied Biosystems, Foster City, USA) at 95 °C for 2 min. Samples were kept on ice until second-strand synthesis was performed by addition of [α-^{33}P] dATP and exonuclease-free Klenow DNA polymerase (Strip-EZ® DNA Probe Synthesis Kit; Ambion, Huntington, UK). Second-strand synthesis was performed at 37 °C for 2 h with repeated gentle shaking of the reaction vial. After incubation 1x SSC (150mM NaCl, 15mM Na-citrate, pH 7.0) was added and the supernatant removed. TE buffer was added and the samples incubated at 95 °C for 3 min for denaturing the double-stranded cDNA. The supernatant containing [α-^{33}P]-labelled cDNA was transferred to a new tube and the process repeated once. The samples were filtered through an Anapore filter to remove any particles (Whatman, Maidstone, England).

DNA array hybridisation

Gene expression patterns were assessed with a custom-made cDNA array harbouring gene-specific fragments of about 1000 genes. The selected genes focused on primary metabolism, transport, signalling, stress response and secondary metabolism**.** Gene-specific PCR-amplified DNA fragments had been spotted onto Nylon Hybond N+ membranes (GE Healthcare, Freiburg, Germany) in duplicate as described by Glombitza et al. (2004). Before hybridization to labelled cDNA, each membrane was hybridized with a reference oligonucleotide targeting a common sequence derived from flanking vector sequences used for PCR amplification (Thimm et al., 2001; Glombitza et al., 2004).

Filters were prepared for hybridisation by addition of hybridisation buffer (5x SSC; 5x Denhardt's solution; 0.5 % SDS; 100µg ml $^{-1}$ denaturated, sheared herring sperm DNA) at 65 °C. Labelled probes were added to hybridisation buffer and filters were hybridised at 65 °C for 20 h. After hybridisation filters were washed twice in 2x SSC, 0.1 % SDS and once in 0.2x SDS, 1 % SDS.

Array analysis

Filters were exposed to imaging plates which were scanned with a FLA-3000 image reader (Fuji, Düsseldorf, Germany) and analysed using ArrayVision 8.0 software (Imaging Research Inc., Haverhill, UK). The local background signal within each subgrid of the filters was subtracted from corresponding expression values; values were normalised with respect to the DNA amount spotted (reference oligo nucleotide expression) and the total signal intensity of a particular filter according to the Haruspex protocol (http://haruspex.mpimp-golm.mpg.de/gxdb; Thimm et al., 2001). The expression ratios were obtained by comparing treatments (mean of duplicated spots) with the corresponding controls. Expression ratios in the range between 0.7 and 1.4 were excluded from further analysis. We considered genes with ratios 1.4 to 1.8 and p-value <0.05 as well as genes with ratios >1.8 and p-value <0.1 as up-regulated; those with ratios 0.7 to 0.5 and p-value <0.05 and those with ratios <0.5 and p-values <0.1 as down-regulated.

Quantitative real time PCR experiment

Quantitative real time PCR (qRT-PCR) was employed to determine the expression of nitrogen responsive, but also to confirm the microarray data were confirmed by using plant tissue from the second independent experiment.

For each treatment 44 replicates were set up giving 132 Magenta jars in total. As in the microarray experiment, rosette diameter was monitored 3, 4 and 5 dpi from 10 replicates of each treatment. Twentyfour and 20 plants per treatment were harvested 3 and 5 dpi, respectively. Of those 12 (3 dpi) and 10 plants (5 dpi) per treatment were pooled to obtain two independent replicates. Only one replicate was used for the array confirmation, but both replicates were analyzed for their expression of nitrogen responsive genes. Plant roots and shoots were separated and frozen in Eppendorf tubes in liquid nitrogen. Samples were stored at -80 °C until analysis.

RNA extraction

RNA was isolated from 100 mg grounded plant material per replicate using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer´s protocol. The obtained RNA was digested with RNase – free recombinant DNase (Promega GmbH, Mannheim, Germany) to eliminate contaminating DNA. RNA concentration was determined with a Nanodrop spectrophotometer (Wilmington, USA). Complete degradation of DNA in the RNA samples was confirmed by the absence of amplified UBQ5 PCR products.

cDNA synthesis

RNA (1µg) was reverse transcribed in a 20 µl reaction containing 1 x First-Strand Buffer (Invitrogen), 10mM DTT (Invitrogen), 1mM dNTP (Fermentas, St. Leon-Roth, Germany), 20 U RNase Inhibitor (Fermentas), 0.17µg Oligo dT (stock solution: 500 µg/ml; Promega) and 5 U SuperScript II Reverse Transcriptase (Invitrogen) at 42 °C for 30 min, at 50 °C for 40 min and at 95 °C for 5 min, respectively. Quality of the transcription and contaminations were checked by the presence or absence of amplified *UBQ5* (At3g62250) PCR products in samples incubated with and without

reverse transcriptase samples, respectively. Template cDNA for qRT-PCR analyses was diluted 1:10 for the array verification and 1:20 for the analysis of nitrogen responsive genes with HPLC-pure water (Roth, Karlsruhe, Germany).

Quantitative real time PCR

Forward and reverse primers were designed from the respective gene sequences using PrimerExpress 3.0 software (Applied Biosystems). For all primers annealing temperature was 55 °C, primer length ranged between 16 and 21 and PCR amplicon length comprised 120 to 150 base pairs. Oligonucleotides were tested for specificity by BLAST analyses at http://signal.salk.edu/cgi-bin/tdnaexpress. For the verification of the array data *PAL*, *CSD1*, *CAT3*, *UGT72B1*, *GSTF10*, *PDF2.2*, *PIP1;5*, *TIP2;3* and *NDPK2* were chosen as target genes for expression analysis using qRT-PCR; the corresponding primers are illustrated in Table 4.1. To analyse the nitrogen response of *A. thaliana* to Bacteria and Protozoa treatments, the nitrate transporter *NRT1.1*, nitrate reductase NIA2, glutamine synthetase *GS-GLN2*, glutamate synthase *GOGAT* and asparagine synthetase *ASN2* were chosen as target genes for qRT-PCR (Wang et al., 2003); the corresponding primers are illustrated in Table 4.1. Prior to the qRT-PCR analysis, amplicons were tested for their specificity by gel electrophoresis and qRT-PCR melting curves indicating a single amplification product as well as for their linear amplification being dependent on cDNA concentrations.

Power SYBR Green PCR Master Mix containing a Hot Start Taq Polymerase (Applied Biosystems) was used for all qRT-PCR reactions containing 4 µl diluted template cDNA (1:10 and 1:20, respectively) in 25 µl; each reaction was duplicated. Analysis was performed using StepOne Real time PCR System (Applied Biosystems). A melting curve was added to check product specificity of the primer pairs in each reaction. Data were processed using the comparative C_t method ($2^{-\Delta\Delta Ct}$)

using *UBQ5* (At3g62250) and S16 encoding ribosomal protein *S16* (At3g04230) separately (Table 4.1) as endogenous control genes to normalise the expression levels of the target genes.

Table 4.1: Primer sequences of target and control genes used in qRT-PCR analysis

Target gene	AGI code	Forward primer	Reverse primer
PAL	At2g37040	5´-TCCCGAACAGGATCAAGG-3´	5´-ACTCGTTGAGACATTCCATCAT-3´
CSD1	At1g08830	5´-AGGCATCATTGGTCTCCAG-3´	5´-TGCTTTGAGCCACACTAAGC-3´
CAT3	At1g20620	5´-AGGCAAGACAGGTTTGTTAAGA-3´	5´-CTGAAACTTGGTAAAAAGGACG-3´
UGT72B1	At4g01070	5´-GAAGAGAAGAGGTGGCTAGAGTG-3´	5´-AAGGCCACAAGACTAAGTGCT-3´
GSTF10	At2g30870	5´-GTCTAGCTGATTTGGCTCACC-3´	5´-GAGTACTTAGCGGAAACCTCCT-3´
PDF2.2	At2g02100	5´-TGTGAGTCGCAGAGCCATA-3´	5´-AGATCAGCAATGTCTGGTGC-3´
PIP1;5	At4g23400	5´-GGAGCTGCCATCATCTACAA-3´	5´-TTATGTCTTGGACTTGAAAGGAAT-3´
TIP2;3	At5g47450	5´-CCATGAATCCAGCAAGGTC-3´	5´-GGTTTCTACCGCTTCATAAGAAC-3´
NDPK2	At5g63310	5´-TGGCTAAGGGAGTGATCCTT-3´	5´-TCAAGCCATAGATTGGCAGT-3´
NRT1.1	At1g12110	5´-GGGCCGTCTTTACAATTTCTA-3´	5´-ATCCCCACCTCAGCTAGTCT-3´
NIA2	At1g37130	5´-GGCATACAGTACCGGGTTTAT-3´	5´-TGAACCGCAAACTGAATCA-3´
GS-GLN2	At5g35630	5´-GGCGAAAGGAAAAGGTTACT-3´	5´-GGGCTTCAGCCTCAAGAG-3´
GOGAT	At5g53460	5´-TCACCAAAACGGATGATGA-3´	5´-TCTCGACTTTTGCTTCAGATG-3´
ASN2	At5g65010	5´-AGCTGTAGAATGGGATGCAA-3´	5´-ACTAAATCCGATCCAGCCTTA-3´
Control gene	**AGI code**	**Forward primer**	**Reverse primer**
UBQ5	At3g62250	5´-GATGGATCTGGAAAGGTTCAG-3´	5´-ATCTACCGCTACAACAGATCAAG-3´
S16	At3g04230	5´-CCGGCGAAAGAGTCTGTTCA-3´	5´-GGCGAACCGTTGAGCTTAATC-3´

Statistical analysis

Effects of Treatment (Sterile, Bacteria, Protozoa) and Time (measurements 3 and 6 dpi) on rosette diameter were analysed by two-factor repeated measures analysis of variance (rm-ANOVA). Differences between means were calculated at the 5 % probability level using Tukey's minimum significant difference test. Statistical analyses were performed using SAS 9.1 (Cary, Florida, USA). Data on induction ratios were analysed by t-tests performed in Excel (Microsoft Office).

4.4 Results

Plant growth

Plant growth as measured by changes in rosette diameter was significantly increased in Bacteria and Protozoa treatments (Fig. 4.1), but the effects varied with time (array experiment: $F_{2,27}$=194.68, p<0.0001, qRT-PCR experiment: $F_{2,27}$=100.18, p<0.0001). Compared to Sterile treatments the rosette diameter of *A. thaliana* in Bacteria treatments increased by factors of 2.9 ($F_{2,27}$=78.29, p<0.0001), 2.4 ($F_{2,27}$=140.81, p<0.0001) and 2.2 ($F_{2,27}$=134.32, p<0.0001) in the array experiment and by factors of 3.5 ($F_{2,27}$=79.01, p<0.0001), 2.8 ($F_{2,27}$=53.46, p<0.0001) and 2.0 ($F_{2,27}$=117.47, p<0.0001) in the qRT-PCR experiment 3, 4 and 5 dpi, respectively (Fig. 4.1). The additional presence of protozoa did not further enhance rosette diameter 3 dpi. However, compared to plants grown with bacteria only, protozoa further increased rosette diameter of *A. thaliana* by factors of 1.3 and 1.5 in the array experiment and by factors of 1.4 and 1.4 in the qRT-PCR experiment 4 and 5 dpi, respectively (Fig.4.1).

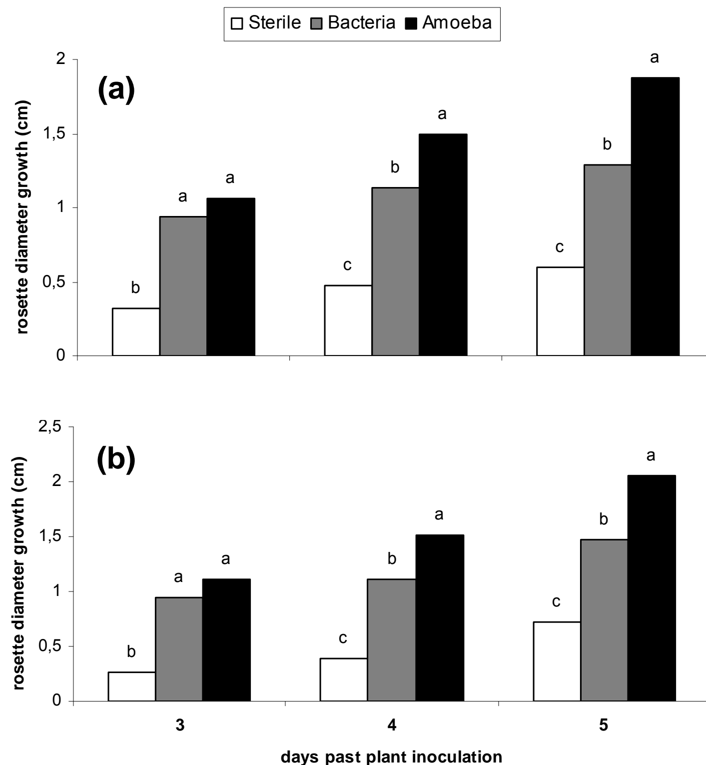

Fig. 4.1: Effect of bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*) on rosette diameter growth of *Arabidopsis thaliana* in (a) the array experiment and (b) the qRT - PCR experiment 3, 4 and 5 days past plant inoculation; means of ten replicates per treatment are presented. Data marked with the same letter do not differ significantly (Tukey's minimum significant difference test, $P<0.05$).

Differential gene expression induced by soil bacteria and protozoa

In roots the presence of soil bacteria induced the up-regulation of 58 and 61 genes 3 and 5 dpi, respectively, with 30 genes up-regulated at both dates (see Appendix). In contrast, 43 and 46 genes were down-regulated in Bacteria treatments compared to Sterile treatments 3 and 5 dpi, respectively, with 32 genes down-regulated at both dates (see Appendix). In shoots bacteria induced the up-regulation of 26 and 8 genes 3 and 5 dpi, respectively, with two genes regulated at both dates. Down-regulated were 39 and 17 genes 3 and 5 dpi, respectively, with 12 genes down-regulated at both dates (see Appendix).

Only two genes encoding aquaporins (*TIP2;2*, *TIP2;3*) up-regulated in Bacteria treatments in roots were further up-regulated in Protozoa treatments 3 dpi (Fig. 4.2, Table 4.2). In shoots, the expression of four genes (*PDF1.1*, *PDF1.2*, *TOC1*, *NIT1*; Fig. 4.2, Table 4.3) altered by bacteria was changed further in protozoa treatments.

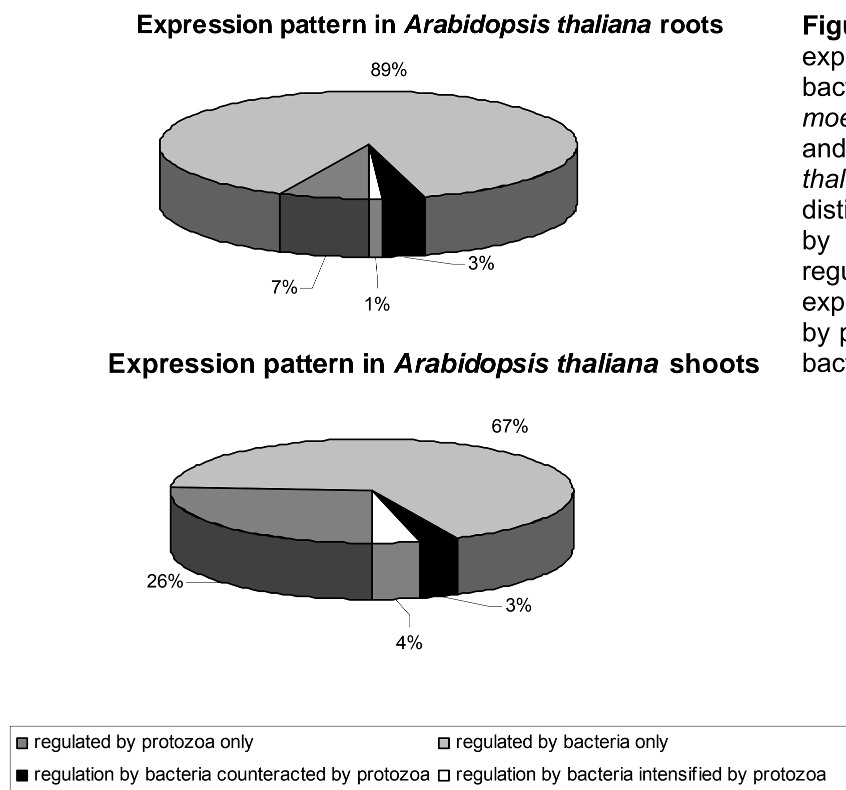

Figure 4.2: Changes in gene expression by soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*) in roots (n=161) and shoots (n=100) of *Arabidopsis thaliana*. Four effects are distinguished: (1) genes regulated by bacteria only, (2) genes regulated by protozoa only, (3) expression by bacteria intensified by protozoa, and (4) expression by bacteria counteracted by protozoa.

Expression patterns induced in Protozoa treatments compared to Bacteria treatments were rather inconsistent with differences between sampling days (3 and 5 dpi) and tissues (shoot and root; Table 4.2, 4.3). In roots 3 dpi, four genes (*UGT76E10*, *UGT76E12*, *NCED5*, *LTP*) were up-regulated and two genes (*GSTF10* and *CCS1/LYS7*) were down-regulated in presence of protozoa (Table 4.2). Five dpi, three genes were up-regulated (*TIP2;2*, *TIP2;3*, *TOC1*) and nine genes (*UGT72B1*, *UGT72C1*, *UGT72E2*, a PR thaumatin protein, *CSD1*, *LTP*, a chitinase, *PAL*, *DFR*) were down-regulated (Table 4.2). In shoots 3 dpi four genes (*PDF1.1*, *PDF1.2*, *NDPK1*, *TOC1*) were up-regulated and eight genes (*PIP1;1*, *PIP1;5*, *PIP2;1*; *PIP2;*2, *NIP6;1*, *WRKY26*, *CAT*2, *CAT3*) were down-regulated (Table 4.3).

Five dpi, 18 genes (*UGT83A1*, *CYP89A9*, *PIP2;6*, *WRKY68*, β-1,3-glucanase, *PDF2.2*, *PDF2.3*, *NDPK2*, *NDPK3*, *CHI*, *ETR1*, *PAP*, *ASB1*, *50S*, *SYP125*, *PLDγ2*,

MAM1, *CYCLIN A1;2*) were up-regulated and three genes (*CYP79C1*, *NIT1*, *NIT4*) were down-regulated (Table 4.3).

Table 4.2: Changes in the induction / repression of selected genes in *Arabidopsis thaliana* roots as indicated by microarray analyses 3 and 5 days past plant inoculation (dpi) with soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*); means of three replicates and significant differences (p-values; Student's t-test) are demonstrated. Genes up-regulated by a factor 1.4 to 1.8 with p-value <0.05 are marked light blue, genes up-regulated by a factor >1.8 and p-value <0.1 are marked dark blue. Genes down-regulated by a factor 0.7 to 0.5 with p-value <0.05 are marked light red, genes down-regulated by a factor <0.5 and p-value <0.1 are marked dark red. S = Sterile treatment, B = Bacteria treatment, P = Protozoa treatment.

		root 3 dpi						**root 5 dpi**					
		SvsB		**SvsP**		**BvsP**		**SvsB**		**SvsP**		**BvsP**	
AGI-Code	**Gene**	**mean±SD**	**p**	**mean±SD**	**p**	**mean±SD**	**p**	**mean±SD**	**p**	**mean±SD**	**p**	**mean±SD**	**p**
aquaporins													
At4g17340	*TIP2;2*	2.85±1.31	0,08	2.00±0.29	0,01	0.77±0.21	0,24	2.24±0.29	0,01	3.47±0.34	0,00	1.57±0.23	0,03
At5g47450	*TIP2;3*	3.01±1.13	0,05	1.74±0.14	0,00	0.62±0.16	0,11	1.87±0.24	0,03	3.52±0.55	0,01	1.91±0.49	0,04
stress related													
At1g12520	*CCS1/LYS7*	2.29±0.97	0,04	1.34±0.49	0,39	0.60±0.14	0,04	1.68±0.03	0,00	1.74±0.21	0,02	1.04±0.11	0,64
At2g30870	*GSTF10*	1.96±0.61	0,11	1.21±0.32	0,41	0.63±0.12	0,04	1.00±0.12	0,89	0.85±0.03	0,02	0.86±0.08	0,10
At4g01070	*UGT72B1*	1.64±0.60	0,16	1.28±0.16	0,08	0.83±0.21	0,30	1.42±0.16	0,04	0.92±0.11	0,33	0.65±0.05	0,02
At4g36770	*UGT72C1*	2.24±0.58	0,02	1.75±0.14	0,01	0.81±0.17	0,18	1.87±0.12	0,00	1.14±0.25	0,44	0.60±0.09	0,03
At5g66690	*UGT72E2*	2.68±0.41	0,01	2.23±0.28	0,01	0.84±0.07	0,06	2.78±0.37	0,01	1.67±0.08	0,01	0.61±0.10	0,04
At1g77700	*THAUMATIN*	1.21±0.23	0,26	0.97±0.21	0,81	0.80±0.09	0,08	1.21±0.14	0,11	0.74±0.02	0,00	0.61±0.06	0,02
At2g43580	*CHITINASE*	1.41±0.51	0,31	0.86±0.34	0,60	0.61±0.14	0,07	1.20±0.18	0,16	0.64±0.15	0,09	0.53±0.04	0,00
At1g08830	*CSD1*	2.18±0.47	0,00	1.38±0.47	0,38	0.63±0.18	0,09	1.83±0.37	0,03	1.25±0.42	0,50	0.67±0.13	0,05
At2g37040	*PAL1*	1.89±0.22	0,01	1.53±0.11	0,00	0.81±0.07	0,06	1.66±0.22	0,01	1.18±0.33	0,46	0.70±0.10	0,04
At2g45400	DFR	1.37±0.16	0,04	1.27±0.43	0,40	0.91±0.21	0,54	1.15±0.12	0,14	0.76±0.14	0,10	0.66±0.10	0,03
growth related													
At5g61380	*TOC1*	1.46±0.41	0,18	1.67±0.46	0,12	1.18±0.35	0,57	1.17±0.10	0,07	1.63±0.15	0,01	1.40±0.09	0,00
unknown function													
At2g15050	*LTP*	1.28±0.31	0,28	1.11±0.38	0,76	0.86±0.16	0,26	1.08±0.16	0,63	0.73±0.15	0,16	0.67±0.04	0,02
At1g36150	*LTP*	0.82±0.21	0,24	1.21±0.36	0,44	1.47±0.15	0,01	0.98±0.14	0,77	0.97±0.17	0,71	0.98±0.10	0,78
At3g46650	*UGT76E10P*	0.82±0.11	0,13	1.19±0.14	0,06	1.46±0.02	0,04	1.13±0.12	0,19	1.37±0.31	0,17	1.21±0.23	0,25
At3g46660	*UGT76E12*	0.72±0.10	0,14	1.16±0.19	0,23	1.62±0.26	0,04	1.03±0.11	0,70	1.06±0.05	0,18	1.03±0.12	0,65
At1g30100	*NCED5*	0.78±0.13	0,19	1.25±0.34	0,44	1.58±0.19	0,02	1.12±0.16	0,32	1.24±0.15	0,09	1.12±0.18	0,38

Table 4.3: Changes in the induction / repression of selected genes in *Arabidopsis thaliana* shoots as indicated by microarray analyses 3 and 5 days past plant inoculation (dpi) with soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*); means of three replicates and significant differences (P-values; Student's t-test) are demonstrated. Genes up-regulated by a factor 1.4 to 1.8 with p-value <0.05 are marked light blue, genes up-regulated by a factor >1.8 and p-value <0.1 are marked dark blue. Genes down-regulated by a factor 0.7 to 0.5 with p-value <0.05 are marked light red, genes down-regulated by a factor <0.5 and p-value <0.1 are marked dark red. S = Sterile treatment, B = Bacteria treatment, P = Protozoa treatment.

		shoot 3 dpi						shoot 5 dpi					
		SvsB		SvsP		BvsP		SvsB		SvsP		BvsP	
AGI-Code	Gene	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p
aquaporins													
At3g61430	*PIP1;1*	1.20±0.22	0,22	0.76±0.06	0,04	0.64±0.07	0,02	0.86±0.08	0,11	0.78±0.07	0,04	0.91±0.09	0,22
At4g23400	*PIP1;5*	2.19±0.66	0,04	1.41±0.63	0,44	0.63±0.10	0,02	1.11±0.22	0,51	1.02±0.22	0,99	0.92±0.17	0,50
At3g53420	*PIP2;1*	1.48±0.44	0,21	1.01±0.30	0,84	0.68±0.03	0,01	0.85±0.16	0,24	0.80±0.05	0,03	0.95±0.13	0,49
At2g37170	*PIP2;2*	1.58±0.49	0,15	0.99±0.30	0,79	0.62±0.03	0,01	0.86±0.12	0,17	0.76±0.05	0,01	0.89±0.13	0,28
At1g80760	*NIP6;1*	1.89±0.41	0,01	1.32±0.46	0,34	0.69±0.09	0,05	1.22±0.73	0,78	1.52±0.27	0,11	1.56±0.95	0,49
At2g39010	*PIP2;6*	1.32±0.13	0,05	1.21±0.19	0,17	0.92±0.12	0,36	1.02±0.38	0,80	1.72±0.53	0,08	1.72±0.11	0,01
stress related													
At4g35090	*CAT2*	0.57±0.10	0,06	0.33±0.10	0,05	0.57±0.08	0,04	0.61±0.08	0,01	0.45±0.07	0,02	0.75±0.18	0,15
At1g20620	*CAT3*	4.00±1.21	0,01	2.05±1.39	0,27	0.49±0.21	0,07	1.77±0.90	0,25	1.86±0.40	0,03	1.16±0.33	0,65
At5g07100	*WRKY26*	1.13±0.21	0,42	0.76±0.14	0,10	0.68±0.07	0,03	0.97±0.32	0,73	0.96±0.14	0,60	1.04±0.22	0,89
At1g75830	*PDF1.1*	26±11.90	0,06	27.64±16.58	0,09	5.10±1.82	0,00	1.13±0.08	0,09	0.96±0.14	0,69	0.85±0.11	0,13
At5g44420	*PDF1.2*	14.66±3.12	0,03	34.37±20.05	0,09	4.36±0.36	0,09	1.66±0.28	0,03	1.30±0.29	0,23	0.78±0.12	0,08
At2g02100	*PDF2.2*	1.69±0.66	0,16	2.32±0.27	0,00	1.46±0.36	0,15	1.44±0.48	0,23	3.14±0.99	0,03	2.21±0.26	0,01
At2g02130	*PDF2.3*	1.36±0.28	0,10	1.61±0.11	0,00	1.20±0.15	0,15	0.95±0.27	0,67	1.68±0.16	0,01	1.84±0.33	0,03
At1g66340	*ETR1*	1.67±0.17	0,03	2.29±0.58	0,05	1.40±0.47	0,28	1.36±0.30	0,15	3.32±0.99	0,03	2.47±0.61	0,04
At1g74550	*CYP98A9*	1.23±0.07	0,02	1.72±0.22	0,02	1.39±0.18	0,05	0.86±0.18	0,32	1.79±0.40	0,06	2.12±0.55	0,05
At1g53520	*CHI*	1.03±0.07	0,49	1.12±0.07	0,11	1.08±0.04	0,10	0.89±0.09	0,18	1.29±0.17	0,10	1.45±0.04	0,02
At5g57890	*ASB1*	1.00±0.13	0,94	1.11±0.15	0,35	1.11±0.07	0,11	1.19±0.58	0,99	1.66±0.68	0,27	1.43±0.13	0,02
At4g11830	*PLDgamma 2*	1.23±0.23	0,21	1.21±0.09	0,02	1.00±0.16	0,96	0.89±0.18	0,38	1.33±0.17	0,06	1.52±0.25	0,05
At5g23020	*MAM1*	1.65±0.52	0,20	1.48±0.66	0,30	0.95±0.45	0,65	0.94±0.08	0,33	1.61±0.36	0,07	1.71±0.27	0,04
At1g51110	*PAP*	1.14±0.16	0,29	1.08±0.06	0,10	0.96±0.13	0,61	1.02±0.50	0,74	1.50±0.54	0,31	1.54±0.25	0,04
At5g63310	*NDPK2*	0.98±0.18	0,87	1.53±0.26	0,06	1.62±0.50	0,16	0.66±0.13	0,08	2.32±0.70	0,05	3.46±0.43	0,01
At3g44310	*NIT1*	0.49±0.07	0,01	0.46±0.02	0,00	0.96±0.19	0,66	0.69±0.05	0,02	0.48±0.00	0,01	0.70±0.05	0,01
At5g22300	*NIT4*	0.84±0.24	0,32	1.12±0.44	0,74	1.33±0.27	0,17	1.18±0.12	0,11	0.76±0.15	0,12	0.63±0.07	0,01
growth related													
At4g09320	*NDPK1*	1.08±0.20	0,65	1.78±0.23	0,00	1.66±0.22	0,01	0.89±0.25	0,48	1.37±0.38	0,26	1.71±0.99	0,32
At5g63310	*NDPK2*	0.98±0.18	0,87	1.53±0.26	0,06	1.62±0.50	0,16	0.66±0.13	0,08	2.32±0.70	0,05	3.46±0.43	0,01
At4g11010	*NDPK3*	1.16±0.21	0,28	1.36±0.24	0,14	1.19±0.31	0,40	0.76±0.15	0,13	1.13±0.11	0,17	1.50±0.14	0,02
At5g61380	*TOC1*	1.53±0.15	0,01	2.40±0.16	0,01	1.58±0.22	0,05	1.04±0.26	0,84	1.74±0.20	0,02	1.77±0.59	0,08
At2g33450	*50S-CL28*	1.19±0.12	0,13	1.28±0.32	0,25	1.09±0.35	0,80	0.84±0.05	0,04	1.74±0.09	0,00	2.08±0.05	0,00
At1g77390	*CYCLIN A1;2*	1.22±0.44	0,56	1.69±0.85	0,21	1.35±0.19	0,07	0.95±0.25	0,68	1.85±0.49	0,07	1.94±0.07	0,01
other function													
At1g11250	*SYP125*	0.94±0.21	0,55	1.17±0.41	0,62	1.26±0.43	0,45	0.87±0.19	0,36	1.93±0.31	0,03	2.24±0.17	0,00
unknown function													
At1g79370	*CYP79C1*	0.96±0.11	0,55	1.03±0.28	0,96	1.09±0.37	0,83	1.19±0.03	0,00	0.82±0.15	0,16	0.69±0.14	0,05
At3g62340	*WRKY68*	1.13±0.32	0,65	1.11±0.08	0,17	1.07±0.43	0,94	0.98±0.09	0,72	1.40±0.23	0,09	1.43±0.14	0,05
At3g02100	*UGT83A1*	1.10±0.27	0,68	1.00±0.26	0,80	0.91±0.08	0,18	0.86±0.14	0,25	1.29±0.29	0,21	1.50±0.10	0,02

Of the number of genes regulated by bacteria 30 % were affected in shoots of *A. thaliana* only, whereas of those regulated by protozoa 63 % were regulated in shoots only (Fig. 4.3). Furthermore, 55 % of the genes regulated by bacteria were regulated in roots only, whereas 35 % of the genes regulated by protozoa were regulated in roots only.

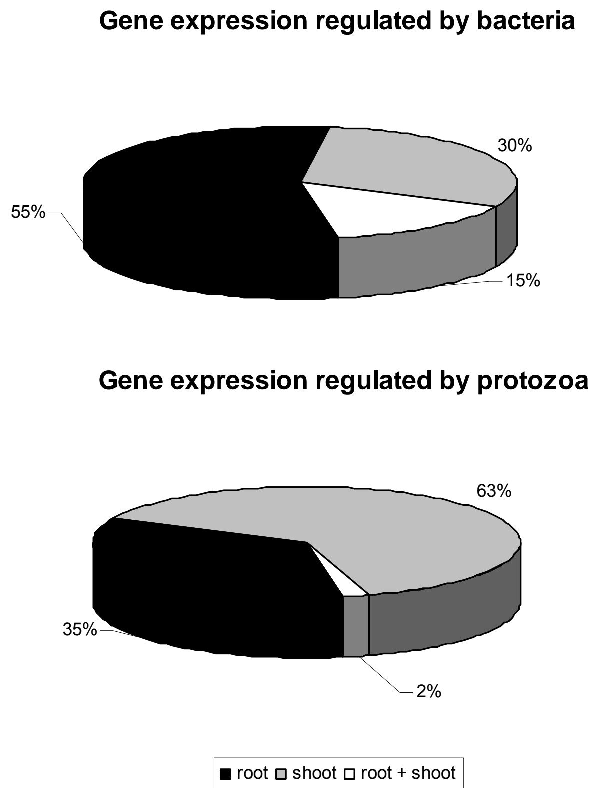

Figure 4.3: Gene expression regulated by soil bacteria (n=254) and bacterivorous protozoa (*Acanthamoeba castellanii*) (n=52) in shoots and roots only, and in both compartments (root + shoot) of *Arabidopsis thaliana*.

Verification of gene expression data by qRT-PCR

The results obtained from qRT-PCR analysis supported the gene expression patterns in roots and shoots obtained from the array (Table 4.4, Table 4.5). Quantitative RT-PCR confirmed the regulation of *CSD1* and *PAL1* in roots 3 dpi as well as the regulation of all analysed genes in shoots 3 dpi and *CAT3* and *PIP1;5* in shoots 5 dpi with almost identical induction and repression values.

In most cases qRT – PCR results verified the expression patterns but exceeded the strength of the expression ratios of the array analysis (root 3 dpi: *GSTF10*, *CAT3*, *UGT72B1*, *PIP1;5*, *TIP2;3*; root 5 dpi: *CAT3*, *CSD1*, *PAL1*, *TIP2;3*, *PIP1;5*; shoot 5 dpi: *PDF2.2*, *NDPK2*). However, the results of three qRT-PCR analyses differed from the array results (root 5 dpi: *GSTF10*; shoot 5 dpi: *GSTF10*, *PAL1*).

Table 4.4: Induction / repression of selected genes by soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*) as indicated by microarray and quantitative real time PCR analyses in roots of *Arabidopsis thaliana* 3 and 5 days past plant inoculation (dpi).
S = Sterile; B = Bacteria; P = Protozoa.

			CSD1	*PAL1*	*GSTF10*	*CAT3*	*UGT72B1*	*TIP2;3*	*PIP1;5*
			At1g08830	At2g37040	At2g30870	At1g20620	At4g01070	At5g47450	At4g23400
root 3dpi	**SvsB**	**array**	2.18	1.89	1.96	1.95	1.64	3.01	1.99
		Ubi	1.59	2.00	4.38	4.42	2.61	6.22	4.28
		S16	1.55	1.94	4.85	4.89	2.89	5.40	3.27
	SvsP	**array**	1.38	1.53	1.21	1.20	1.28	1.74	1.68
		Ubi	0.91	1.60	0.92	0.98	0.93	2.22	2.13
		S16	0.93	1.63	1.08	1.15	1.09	2.29	2.20
	BvsP	**array**	0.63	0.81	0.63	0.66	0.83	0.62	0.84
		Ubi	0.57	0.80	0.21	0.22	0.36	0.36	0.50
		S16	0.60	0.84	0.22	0.24	0.38	0.42	0.59
root 5dpi	**SvsB**	**array**	1.83	1.66	1.00	1.46	1.42	1.87	1.66
		Ubi	2.35	2.98	2.20	3.88	1.95	6.05	6.82
		S16	2.08	2.63	2.29	5.26	1.73	4.74	5.34
	SvsP	**array**	1.25	1.18	0.85	1.53	0.92	3.52	1.61
		Ubi	1.32	1.21	0.97	1.17	0.97	14.19	7.80
		S16	1.17	1.07	1.10	1.34	0.80	9.70	5.33
	BvsP	**array**	0.67	0.70	0.86	1.05	0.65	1.91	0.98
		Ubi	0.56	0.41	0.44	0.30	0.50	2.35	1.14
		S16	0.56	0.41	0.37	0.25	0.46	2.05	1.00

Table 4.5: Induction / repression of selected genes by soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*) as indicated by microarray and quantitative real time PCR analyses in shoots of *Arabidopsis thaliana* 3 and 5 days past plant inoculation (dpi).
S = Sterile; B = Bacteria; P = Protozoa.

			GSTF10	*CAT3*	*PDF2.2*	*PAL1*	*PIP1;5*	*NDPK2*
			At2g30870	At1g20620	At2g02100	At2g37040	At4g23400	At5g63310
shoot 3dpi	**SvsB**	**array**	2.04	4.00	1.69	1.06	2.19	0.98
		Ubi	2.96	4.76	2.27	0.78	2.98	1.21
		S16	3.14	5.05	2.24	0.88	3.16	1.28
	SvsP	**array**	2.14	2.05	2.32	0.97	1.41	1.53
		Ubi	2.20	2.00	3.34	1.03	1.37	2.12
		S16	2.51	2.28	4.34	1.18	1.56	2.42
	BvsP	**array**	1.05	0.49	1.46	1.08	0.63	1.62
		Ubi	0.75	0.42	1.47	1.37	0.46	1.76
		S16	0.80	0.45	1.93	1.37	0.50	1.89
shoot 5dpi	**SvsB**	**array**	2.46	1.77	1.44	1.35	1.11	0.66
		Ubi	2.02	1.77	3.02	0.82	1.03	0.90
		S16	2.09	1.83	2.83	0.85	0.97	0.84
	SvsP	**array**	2.32	1.86	3.14	1.12	1.02	2.32
		Ubi	3.59	2.11	7.20	1.59	0.90	4.71
		S16	3.65	2.15	7.42	1.62	0.93	4.85
	BvsP	**array**	0.97	1.16	2.21	0.85	0.92	3.46
		Ubi	1.78	1.19	2.38	1.94	0.87	5.23
		S16	1.75	1.17	2.62	1.91	0.96	5.76

The array experiment did not reveal changes of GSTF10 gene expression in shoots or roots 5 dpi by amoebae, but results from qRT – PCR analyses showed a down – regulation of *GSTF10* gene expression in roots and an up – regulation of *GSTF10* gene expression in shoots 5 dpi. Similarly, results obtained from array analyses did not show a regulation of *PAL* gene expression in shoots by amoebae, whereas qRT-PCR analyses revealed an up – regulation in the shoot 5 dpi.

Nitrogen responsive genes

The expression of the nitrate transporter *NRT1.1* and the nitrate reductase *NIA2* was not affected in Bacteria and Protozoa treatments, neither in roots nor in shoots of *A. thaliana* at any sampling date (Table 4.6). Ammonium assimilatory genes *GS-GLN2*, *GOGAT* and *ASN2* were up-regulated in the Protozoa treatment 5 dpi but not 3 dpi (Table 4.6).

Table 4.6: Induction / repression of selected nitrogen responsive genes by soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*) indicated by quantitative real time PCR analyses; data are means of two replicates.

			NRT1.1	***NIA2***	***GS-GLN2***	***GOGAT***	***ASN2***
			At1g12110	At1g37130	At5g35630	At5g53460	At5g65010
root 3dpi	**SvsB**	**Ubi**	1.01±0.16	1.13±0.18	1.06±0.09	1.27±0.15	1.05±0.31
		S16	1.01±0.04	1.14±0.06	1.07±0.04	1.19±0.01	1.11±0.27
	SvsP	**Ubi**	1.10±0.06	1.13±0.18	0.98±0.01	1.28±0.05	1.11±0.15
		S16	1.09±0.10	1.12±0.03	0.97±0.14	1.30±0.08	1.15±0.08
	BvsP	**Ubi**	1.05±0.11	1.01±0.01	0.93±0.08	1.02±0.08	1.08±0.18
		S16	1.03±0.13	0.98±0.02	0.91±0.10	1.09±0.07	1.06±0.18
root 5dpi	**SvsB**	**Ubi**	1.05±0.04	1.04±0.06	1.03±0.02	0.84±0.11	1.03±0.03
		S16	1.02±0.08	1.01±0.11	0.99±0.01	0.95±0.12	0.97±0.04
	SvsP	**Ubi**	1.26±0.45	1.10±0.40	**3.36±1.49**	**3.61±0.54**	**3.65±1.56**
		S16	1.25±0.16	1.09±0.15	**3.28±0.74**	**4.05±0.71**	**3.45±0.91**
	BvsP	**Ubi**	1.22±0.47	1.08±0.45	**3.27±1.39**	**4.40±1.25**	**3.53±1.44**
		S16	1.24±0.25	1.09±0.25	**3.31±0.79**	**4.29±0.21**	**3.56±0.83**
shoot 3dpi	**SvsB**	**Ubi**	not analysed	0.85±0.01	0.86±0.09	0.89±0.02	0.85±0.04
		S16		0.98±0.04	1.00±0.13	1.03±0.01	0.99±0.08
	SvsP	**Ubi**		0.92±0.09	0.93±0.03	0.9±0.01	0.91±0.04
		S16		0.91±0.04	0.94±0.17	0.91±0.12	0.92±0.09
	BvsP	**Ubi**		1.09±0.12	1.08±0.07	1.02±0.01	1.08±0.11
		S16		0.93±0.00	0.94±0.05	0.88±0.11	0.92±0.01
shoot 5dpi	**SvsB**	**Ubi**	not analysed	1.20±0.17	1.06±0.01	1.26±0.11	1.25±0.00
		S16		1.15±0.04	1.03±0.16	1.21±0.11	1.21±0.21
	SvsP	**Ubi**		1.17±0.06	**1.68±0.16**	**2.89±0.02**	**2.9±0.42**
		S16		1.19±0.14	**1.73±0.26**	**2.96±0.15**	**2.98±0.60**
	BvsP	**Ubi**		0.99±0.19	**1.59±0.18**	**2.32±0.21**	**2.32±0.34**
		S16		1.04±0.08	**1.69±0.01**	**2.46±0.07**	**2.46±0.08**

4.5 Discussion

Plant growth

The present results support recent findings on the rapid nitrogen-independent growth response of *A. thaliana* in presence of both, soil bacteria and protozoa in the rhizosphere. Using the same experimental set up we observed that the presence of bacteria and protozoa results in an immediate (< 3 dpi) increase in rosette diameter of *A. thaliana*, with effects of protozoa exceeding those of bacteria only (Chapter 3). Results of this study confirmed the immediate plant growth response for both bacteria and protozoa. However, in the present experiments the increase in plant growth induced by protozoa was delayed by one day as compared to the experiment in Chapter 3.

Expression of nitrogen responsive genes

Plant roots are known to compete with soil bacteria for nitrogen (Hodge et al., 2000). Expression analysis of nitrogen responsive genes (*NRT1.1*, *NIA2*, *GS-GLN2*, *GOGAT*, *ASN2*) conducted in the presented study showed that none of the chosen genes was regulated by the presence of bacteria indicating that early plant promotion by soil bacteria is not mediated by nitrogen. Mineralisation of nitrogen in soil has been assumed to be driven by microbial feeding fauna in particular by protozoa (Griffiths, 1994) Indeed, protozoa have been shown to increase plant nitrogen acquisition and growth supporting the microbial loop concept (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Clarholm, 2005). However, other studies indicate that indirect nutrient-independent effects also contribute to plant growth promoting effects by protozoa (Jentschke et al., 1995; Bonkowski & Brandt, 2002; Bonkowski, 2004). The expression analyses of nitrogen responsive genes

support this view. The observed early initiation (3 dpi) in plant growth promotion (rosette diameter) by amoebae unlikely was caused by increased plant nitrogen acquisition since neither the expression of *NRT1.1* and *NIA2*, nor that of *GS-GLN2*, *GOGAT* and *ASN2* was affected by amoebae. Still 5 dpi nitrate responsive genes *NRT1.1* and *NIA2* did not respond, but the ammonium assimilatory genes *GS-GLN2*, *GOGAT* and *ASN2* were up-regulated in the Protozoa treatment, suggesting that after a lag of about 5 days amoebae increased the availability of ammonium but not of nitrate. Also, up-regulation of *TIP2;3* in the DNA array analysis suggests that amoebae increased ammonium uptake of the plants 5 dpi but not 3 dpi (Table 4.2). *TIP2;3* is known to transport ammonium and methylammonium across membranes of vacuoles (Jahn et al., 2004; Kaldenhoff & Fischer, 2006). Overall, the results enforce the view that the initiation in plant growth promotion induced by protozoa is independent of increased nitrogen availability and plant nitrogen uptake. However, later, i.e. after about 5 days, plant nitrogen acquisition and growth start to be caused by the microbial loop mechanism. Supporting this scenario, we found the protozoa-mediated increase in root growth of *A. thaliana* to predate the increase in plant N uptake (Chapter 3).

DNA array gene expression analysis

Results from DNA array analysis covering stress and signalling response genes demonstrate that soil bacteria particularly affected gene expression in roots, whereas amoebae in particular systemically influenced gene expression in shoots of *A. thaliana* (Fig. 4.3). The regulation of only six genes affected by bacteria was intensified by amoebae (Fig. 4.2) indicating that effects of amoebae on plant growth are not only caused by enhancing bacterial effects on plants. Rather, the presence of

soil bacteria plus amoebae induces a different response of plants than the presence of bacteria alone.

We were particularly interested in genes affected in presence of soil protozoa compared to bacteria only. Therefore, we examined the function of genes differentially expressed in these two treatments. Expression patterns in *A. thaliana* roots 3 and 5 dpi suggest that amoebae down-regulated a number of genes involved in plant defence responses which in part were up-regulated by bacteria. In roots *LYS7* and *GSTF10* involved in detoxification processes (Marrs, 1996; Culotta et al., 1997; Dixon et al., 2002; Chu et al., 2005) were down-regulated by amoebae 3 dpi. Similarly, amoebae down-regulated a number of defence genes in roots 5 dpi, such as *CSD1* which detoxify superoxide radicals (Bowler et al., 1992; Chu et al., 2005), genes involved in lignin biosynthesis (*UGT72E2*, *UGT72C1*; Lanot et al., 2006; Lim et al., 2005), genes of to the pathogenesis related (PR) family proteins (*thaumatin*, *chitinase*; Van Loon & Van Strien, 1999; Kasprzewska, 2003), genes responding to benzoates (*UGT72B1*; Lim et al., 2002) and genes involved in phenylpropanoid (PAL; Hahlbrook & Scheel, 1989) and flavonoid biosynthesis (*DFR*; Winkel-Shirley, 2001, 2002).

In *A. thaliana* shoots the gene expression response partly matched that in roots. As in roots genes involved in detoxification processes (*CAT2*, *CAT3*; Blokhina et al., 2003) and stress in general (*WRKY* 26; Tosti et al., 2006; Zhang et al., 2007) were down-regulated in shoots 3 dpi. However, both at 3 and 5 dpi a group of plant defensins (*PDF1.1*; *PDF1.2*; *PDF2.2*; *PDF2.3*, Thomma et al., 2002; Turner et al., 2002; Devoto & Turner, 2003), genes involved in glucosinolate biosynthesis (*MAM1*, Textor et al., 2004; Field et al., 2006; *ASB1*, Niyogi & Fink, 1992; Niyogi et al., 1993), in phenylpropanoid biosynthesis (*CYP89A9*; Gachon et al., 2005), flavonoid biosynthesis (*CHI*, Winkel-Shirley, 2001, 2002) and several other stress related

genes (*ETR1*, Li & Guo, 2007; *PAP*, Peltier et al., 2004; *PLDγ2*, Wang, 2005; *NDPK2*, Moon et al., 2003) were up-regulated by amoebae in shoots. In addition, a gene involved in glucosinolate degradation was down-regulated (*NIT1*, Vorwerk et al., 2001). These patterns indicate that contrary to root responses amoebae systemically up-regulated defence responses in shoots of *A. thaliana*. The increased shoot defence response, however, did not come at the expense of plant growth. Rather, plant rosette diameter was increased in presence of amoebae and this increase was reflected by the up-regulation of genes related to primary metabolism and thus growth and development (*NDPK*'s, Choi et al., 2005; *TOC1*, Matsushika et al., 2000; Murakami et al., 2004; *CYCLIN A1;2*, Burssens et al., 1998; Huntley & Murray, 1999; *50S-Cl28*, Yokoi & Sugiura, 1992).

The results suggest that the presence of amoebae in the rhizosphere of plants triggers an immediate down-regulation of bacteria-induced defence responses in roots of *A. thaliana,* most likely via reducing bacterial density and changing bacterial community composition (Rosenberg, 2008). The reduced costs for secondary metabolism (Van Hulten et al., 2006) presumably enabled the plants to redirect resources into root growth (Chapter 3) thereby increasing nutrient exploitation. The up-regulation of nitrogen–responsive genes 5 dpi in roots and shoots of *A. thaliana* indicates a subsequent enhanced capture of nitrogen released by protozoa from consumed microbial biomass. Amoebae thus alter the performance of *A. thaliana* by (1) decreasing defence responses in roots, (2) increasing defence response in shoots and (3) improving the availability and uptake of nitrogen.

The up-regulation of defence response genes in shoots of *A. thaliana* resembles the induced systemic resistance (ISR) by Plant Growth Promoting Rhizobacteria (PGPR; Pieterse et al., 2002). Similar to ISR induced by PGPRs, amoebae affected genes related to jasmonate and ethylene pathways such as *PDF*s and *ETR1*

(Pieterse et al., 2002). However, ISR is not associated with a direct activation of defence-related genes, rather, plants exhibit a faster and stronger activation of defence responses after being attacked by pathogens (Van Wees et al., 1999; Conrath et al., 2002; Verhagen et al., 2004). This “priming” combines the advantage of enhanced disease protection and low costs compared to the induction of direct defences (Van Hulten et al., 2006). Interestingly, the present study suggests that amoebae up-regulated distinct defence responses in *A. thaliana*, but in parallel increased plant growth and reproduction due to enhanced carbon and nitrogen capture (see also Chapter 3). Presumably, plants growing in presence of protozoa can afford additional costs for defence induction due to their improved nutrient and energy status allowing investment in both defence and growth.

CHAPTER 5

GENERAL DISCUSSION

Plant growth promotion by soil protozoa has been repeatedly described and is traditionally assigned to the ‚microbial loop' (Clarholm, 1985; Kuikman & Van Veen, 1989; Kuikman et al., 1991; Jentschke et al., 1995; Alphei et al., 1996; Bonkowski et al. 2001a; Bonkowski, 2004). The 'microbial loop' suggests that bacterivorous soil protozoa release excess nitrogen from consumed bacterial biomass into the soil, which improves the nitrogen supply of plants and thus plant growth (Clarholm, 1985, Clarholm et al., 2006). However, several studies argue that protozoa induced plant growth promotion is not solely caused by increased nitrogen supply (Robinson et al., 1989; Griffith & Robinson, 1992; Bonkowski & Brandt, 2002; Bonkowski, 2004). For example, Bonkowski & Brandt (2002) demonstrated that soil amoebae enhance the proportion of IAA synthesizing soil bacteria presumably by selective grazing. They concluded that this enhanced the release of IAA synthesized by bacteria into the rhizosphere stimulating the initiation of lateral root production and thus soil and nutrient capture and plant growth. The present PhD thesis was performed to further elucidate and evaluate the involvement of nitrogen and auxin in plant – bacteria – soil amoebae interactions. First, we continued the experiment described in Bonkowski & Brandt (2002) and investigated the effects of soil bacteria and the bacterivorous soil amoeba *Acanthamoeba castellanii* on root branching and plant internal auxin concentrations of L*epidium. sativum*. Subsequently, since *L. sativum* is inappropriate as model plant, we started to design an experimental set up which was appropriate for investigating morphological, physiological and gene transcriptional responses of *Arabidopsis thaliana* to a natural occurring soil bacterial community and to *A. castellanii*. The same system was concurrently used to obtain information on

changes in the soil bacterial community composition due to selective feeding of *A. castellanii* on the diverse soil bacterial community (Rosenberg, 2008).

5.1. Effects of bacterivorous soil protozoa on root architecture and hormonal balance in *Lepidium sativum*

The plant hormone auxin, indole-3-acetic acid (IAA), which is particularly synthesized in plant shoots, is known to be the key signal in the initiation of lateral root production. (Celenza et al., 1995; Malamy & Benfey, 1997; Casimiro et al., 2001; Himanen et al., 2002; Casimiro et al., 2003; Laskowski et al., 2006). Bacterivorous soil protozoa have been found to induce root branching by enhancing the number of lateral roots which suggests that IAA is involved in interactions between soil protozoa and plants (Jentschke et al., 1995; Alphei et al., 1996; Bonkowski & Brandt, 2002; Kreuzer et al., 2006). In addition, protozoa induced changes in root architecture resemble effects of particular strains of soil bacteria, which are assumed to release bacterial synthesized IAA into the rhizosphere (Barbieri et al., 1986; Barbieri & Galli, 1993; Frankenberger & Arshad, 1995; Lambrecht et al., 2000; Dobbelaere et al., 2001; Patten & Glick, 2002; Vessey, 2003). Our results support previous results on effects of soil protozoa on root architecture (Jentschke et al., 1995; Alphei et al., 1996; Bonkowski & Brandt, 2002; Kreuzer et al., 2006) since root branching was increased due to the presence of soil bacteria plus the soil amoeba *A. castellanii*, whereas soil bacteria alone did not promote root branching in *L. sativum* compared to sterile grown plants. Analysis of free and conjugated IAA levels indicated that soil bacteria as well as soil amoebae modify the internal IAA metabolism of *L. sativum*. Soil bacteria strongly enhanced the concentration of conjugated IAA, whereas soil amoebae increased free IAA levels in shoots. Surprisingly, both soil bacteria and soil amoebae only influenced IAA concentrations systemically in shoots but not in roots

indicating that rather the plant internal auxin synthesis in shoots than auxin uptake via the roots was influenced. In bacteria treatments root branching was not induced although conjugated IAA concentration was enhanced in *L. sativum* shoots. Compared to free IAA, which is the bioactive form of IAA, conjugated IAA is considered to be involved in IAA transport, storage and detoxification (Seidel et al., 2006). The high amounts of conjugated IAA in presence of bacteria suggests that soil bacteria strongly increased free IAA concentrations in plant shoots, which were further conjugated possibly to protect the plant from toxic IAA levels. In either case, the fact that root growth in bacteria treatments did not differ in comparison to sterile grown plants, although auxin concentrations strongly increased, suggests that the hormone was inactivated by the plant through conversion into the non-active conjugated form (Seidel et al., 2006). It has long been suggested that particular bacterial isolates affect plant internal IAA concentrations (Patten & Glick, 1996). Our data support these suggestions showing that plant-interactions with a natural mixed bacterial population affect the internal auxin balance of plants.

L. sativum responded to soil amoebae with an increase in lateral root production accompanied with an increase in free IAA concentrations in shoots. Shoot derived IAA and its polar transport into the root is known to be essential for the development of lateral roots (Reed et al., 1998; Casimiro et al., 2001; Bhalerao et al., 2002) and these results suggest that the amoebae-induced increase in free IAA in shoots is presumably linked to an increase in root branching. Our results further suggest that soil amoebae increase root branching via systemically increasing free IAA levels in plant shoots presumably by changes in rhizosphere signalling which might induce the synthesis of IAA in plant shoots. In previous experiments protozoa have been shown to strongly change the composition and function of rhizosphere bacterial communities due to selective feeding on certain bacteria (Griffiths et al., 1999; Rønn et al., 2002;

Bonkowski & Brandt, 2002; Kreuzer et al., 2006, Rosenberg, 2008). Thus, changes in rhizosphere signalling my either directly originate from soil amoebae or indirectly from changes in the soil bacteria community composition.

5.2. Effects of bacterivorous soil protozoa on Arabidopsis thaliana

5.2.1 The Arabidopsis System

We successfully established an experimental system with *A. thaliana* as model plant which allows investigating interactions between microorganisms in the rhizosphere. *Arabidopsis thaliana* is the most important model system for investigating plant-microbe interactions because of its small size, rapid life cycle and its well characterized genetic background (O'Callaghan et al., 2001; Mantelin & Touraine, 2004; De Vos, 2006). These advantages enable time efficient analyses of plant growth and reproduction as well as extensive gene expression analyses. The developed system allows to maintain sterility for several weeks which is crucial for experiments using microorganisms. Natural soil was substituted by a sand/organic matter (OM) substrate, which enables to harvest the tiny roots of *A. thaliana* without damage. Although using a sand substrate instead of soil, the system is suitable for imitating natural conditions since several uncultured bacterial strains were detected by sequencing of particular PCR products (Rosenberg, 2008). As shown for different other plant species, *A. thaliana* strongly responded to the presence of a diverse microbial community and the bacterivorous soil amoeba *A. castellanii* with an increase in plant growth, indicating that interactions between soil bacteria, protozoan grazers and *A. thaliana* resemble those in the field.

5.2.2 Initiation of Growth Promotion and Reproduction

As expected, soil bacteria as well as soil amoebae increased shoot growth with effects of amoebae exceeding effects of soil bacteria only. Carbon rich root exudates foster soil bacteria and thus bacterivores (Milchunas et al., 1985; Semenov et al., 1999; Cheng & Gershenson, 2007; Griffiths et al., 2007); consequently both benefit from increased plant growth which is associated with increased root exudation. The plant itself relied on the presence of microorganisms in the rhizosphere as *A. thaliana* grown under sterile conditions performed poorly despite of high concentrations of mineral nitrogen in the sand/OM substrate.

The promotion of shoot growth induced by soil bacteria and soil amoebae already increased 3 days past plant inoculation (dpi) indicating a rapid response of A. *thaliana* to microbial changes in the rhizosphere. Interestingly, the nitrogen content of the plants was neither increased by soil bacteria nor by amoebae 3 dpi. Further, in both treatments mineral nitrogen concentrations in the sand/OM substrate were decreased compared to sterile treatments suggesting that soil bacteria immobilized nitrogen compounds (Hodge et al., 2000). Thus, contrary to the ‘microbial loop’ concept (Clarholm, 1985, Clarholm et al., 2006), the addition of amoebae to soil bacteria did not result in an increase in plant available nitrogen and plant nitrogen uptake 3 dpi. This suggests that the immediate response of *A. thaliana* to bacteria as well as to amoebae were unlikely caused by improved nitrogen supply in the rhizosphere. Despite plant nitrogen uptake remained unaffected, carbon allocation in *A. thaliana* increased in presence of bacteria and was further increased in presence of bacteria plus amoebae, and this already occurred 3 dpi. Soil microorganisms have been shown to enhance net photosynthesis (Meharg & Killham, 1991; Merbach & Ruppel, 1992). One of presumably thousands of signals originating from microorganisms in the rhizosphere is the signal compound lumichrome, which

increased net carbon allocation in *Medicago sativa* (Phillips et al., 1999). Further, plant growth is known to be stimulated by the release of bacterial synthesized plant hormones or analogues (Bloemberg & Lugtenberg, 2001; Persello-Cartieux, 2003; Ping & Boland, 2004; Tsavkelova et al., 2006). Thus, the increase in carbon allocation and the associated shoot growth induced by soil amoebae suggests that signalling or hormonal effects originating from microorganisms in the rhizosphere were responsible for the increased plant growth.

While plant growth promotion induced by soil bacteria was independent of nitrogen 3 dpi, presence of amoebae increased the concentration of plant available nitrogen in the rhizosphere as well as plant nitrogen uptake 6 dpi. In addition, the use of ^{15}N labelling demonstrated that plants grown in presence of bacteria plus amoebae incorporated more nitrogen from organic matter and thus less from the nutrient solution 6 dpi, suggesting that the amoebae-mediated changes were based on an increased nitrogen transfer from OM by grazing on bacteria. These results support the concept of the 'microbial loop', which describes that soil bacteria sequester nitrogen from OM in bacterial biomass, which is partly re-mineralized and released in the rhizosphere due to protozoan grazing (Clarholm, 1985, Clarholm et al., 2006).

Our results suggest that the early response of *A. thaliana* to soil amoebae relies on changes in rhizosphere signalling which triggers the systemic allocation of carbon in plant shoots 3 dpi. The enhanced carbon allocation above the ground further enables increased carbon allocation into the roots and thus root growth allowing to take advantage of ammonium made available by protozoan grazing. The results imply that the plants sensed the presence of amoebae by changes in rhizosphere signalling and anticipated the upcoming mobilization of nitrogen. Using the same experimental system Rosenberg (2008) demonstrated that, *A. castellanii* immediately (0 and 3 dpi) changed the bacterial community composition in the rhizosphere of *A. thaliana,* which

might be responsible for the suggested changes in rhizosphere signalling. However, one must take in account the possibility that also the amoebae itself may release signals inducing immediate systemic plant responses.

The immediate plant growth promotion of *A. thaliana* by soil amoebae was reflected in plant reproduction. Nitrogen deficiency is known to reduce the vegetative growth phase and initiate an early seed set of *A. thaliana* (Heil et al. 2000; Dietrich et al., 2005). Plants grown in presence of soil bacteria plus amoebae extended the vegetative growth phase accompanied with an increased seed biomass. Presumably, reduced nitrogen limitation in presence of amoebae, which already occurred 6 dpi, triggered the prolongation of the vegetative growth phase and combined with the amoebae-mediated increase in nitrogen availability resulted in the strong increase in seed production of *A. thaliana*.

5.2.3 Transcriptional Nitrogen and Stress Response

Results described above (Chapter 5.2.2) indicate that the immediate plant growth promotion by soil bacteria as well as by soil amoebae does not rely on an improved nitrogen supply and uptake, and thus not on the ‘microbial loop’ mechanism (Clarholm, 1985; Clarholm et al., 2006). This conclusion was further supported by transcription analysis of five genes involved in nitrate transport (*NRT1.1*), nitrate reduction (*NIA2*) and ammonium assimilation (*GS-GLN2, GOGAT, ASN2*; Wang et al., 2003). None of these genes responded to the presence of soil bacteria or soil amoebae 3 dpi indicating that the observed early promotion of *A. thaliana* is not mediated by nitrogen. However, two days later (5 dpi) the ammonium assimilatory genes *GS-GLN2*, *GOGAT* and *ASN2* were up-regulated by soil amoebae, whereas nitrate responsive genes *NRT1.1* and *NIA2* did not respond. This supports the

scenario described above (Chapter 5.2.2), i.e. that soil amoebae increase ammonium but not nitrate availability in the rhizosphere and also plant nitrogen allocation 6 dpi. In conclusion, the results of nitrogen responsive gene expression enforce the view that the immediate (3 dpi) plant growth promotion induced by amoebae is independent of increased nitrogen availability and plant nitrogen uptake. However, later, i.e. after about 5 days, plant nitrogen acquisition and growth start to be caused by the 'microbial loop' mechanism (Clarholm, 1985; Clarholm et al., 2006). Previously, we suggested that *A. thaliana* may be able to recognize the presence of amoebae by changes in rhizosphere signalling and anticipate the upcoming mobilization of nitrogen. Nitrate is known not only to function as a metabolic substrate for assimilation, but also as a signal molecule modulating the pattern of plant growth and development by regulating the expression of a number of genes (Takei et al., 2002; Sakakibara, 2003). However, the concentration of available nitrate in the rhizosphere (see Chapter 5.2.2) and the expression of nitrate responsive genes *NRT1.1* and *NIA2* argues against nitrate as signalling substance in the immediate plant growth promotion induced by soil amoebae. Previous investigations suggest that selective grazing of protozoa on soil bacteria favours nitrifying bacteria resulting in hotspots of nitrate concentrations around plant roots (Griffiths, 1989; Alphei et al., 1996). Since our results show that ammonium but not nitrate is involved in the immediate response of plants to soil amoebae and since nitrifying bacteria generally occur in low densities and grow slowly (Griffiths, 1989; Alphei et al., 1996) we assume that an increased nitrification due to protozoan grazing is rather a long-term effect in plant – protozoa interactions and is not responsible for the early plant growth promotion. However, we observed also in our system an increase in the relative abundance of *Nitrospira* due to protozoan grazing using fluorescence *in situ*

hybridization (Rosenberg, 2008), but this was not accompanied by increased nitrate concentrations and plant nitrogen uptake.

Expression analysis of about 1000 genes related to stress and signalling responses in *A. thaliana* (Glombitza et al., 2004) revealed that soil amoebae induced a down-regulation of defence genes in roots 3 and 5 dpi. The response comprised genes involved in detoxification mechanisms, genes belonging to the pathogenesis related (PR) family proteins and genes involved in lignin, phenylpropanoid and flavonoid biosynthesis indicating that soil amoebae reduce diverse defence pathways. The local down-regulation of defence responses suggests that selective grazing attenuates effects of detrimental soil bacteria on plants. Using the same experimental system, Rosenberg (2008) demonstrated that bacterial community composition changes rapidly in presence of soil amoebae. Results of the gene expression analysis suggest that soil amoebae either reduce the proportion of detrimental bacteria via direct grazing or indirectly by improving the competitiveness and therefore density of non-detrimental soil bacteria. Presumably, costs saved due to the reduced investment in detoxification and defence enabled the plants to redirect resources into root growth and thus soil and nutrient exploitation (see Chapter 5.2.2). The up-regulation of nitrogen–responsive genes 5 dpi in roots and shoots of *A. thaliana* indicates a subsequent enhanced capture of nitrogen released by amoebae originating from consumed microbial biomass according to the microbial loop mechanism (Clarholm, 1985, 1994, Clarholm et al., 2006).

Contrary to gene expression patterns in roots, shoots of *A. thaliana* responded to soil amoebae with an up-regulation of stress related genes such as plant defensins, ethylene responsive genes and genes involved in glucosinolate, phenylpropanoid and flavonoid biosynthesis indicating opposed responses locally and systemically. Similarly, the development of an induced systemic resistance (ISR) triggered by

particular Plant Growth Promoting Rhizobacteria (PGPR) is dependant on intact jasmonate and ethylene signalling pathways (Pieterse et al., 2002). However, contrary to the observed effects of soil amoebae, ISR is not associated with a direct activation of defence-related genes, rather; plants exhibit a faster and stronger activation of defence responses after being challenged by pathogens (Van Wees et al. 1999; Conrath et al. 2002, Verhagen et al. 2004). This “priming” combines the advantage of enhanced disease protection and low costs compared to the induction of direct defences (Van Hulten et al., 2006). Surprisingly, soil amoebae systemically up-regulated defence responses but also increased plant growth. Presumably, the improved nutrient and energy status of *A. thaliana* (see Chapter 5.2.2) induced by soil amoebae allows affording additional investment in defence.

Although defence mechanisms related to jasmonate and ethylene pathways were up-regulated by soil amoebae in plant shoots it was previously demonstrated that aphid performance on barley plants is promoted in presence of soil protozoa (Bonkowski et al., 2001b). Our results from transcription analysis rather presume a reduction of herbivores due to protozoa. However, aphids are particularly limited in nitrogen and rely on specific amino acids in the phloem (Douglas, 1988; Febvay et al., 1995; Docherty et al., 1997) and thus benefit from increased plant nitrogen uptake in presence of protozoa. Moreover, plant defence against aphids relies on jasmonate as well as salicylic acid related pathways (Moran & Thompson 2001, de Vos et al., 2005) which were not affected according to our microarray analysis. Thus, we assume that the protozoa-induced defence is rather effective against pathogens which are suppressed by a jasmonate related plant defence and that the spectrum of plant pathogens which may be affected by soil protozoa thus is limited. Additionally, although aphid performance on barley was increased due to soil protozoa plant reproduction still was enhanced (Bonkowski et al., 2001b) indicating that despite

enhanced herbivore pressure the plant performance was promoted in presence of protozoa.

5.3 Conclusions

The present study demonstrated that bacterivorous soil amoebae promote shoot as well as root growth of plants, with the increased root growth presumably being caused by the initiation of lateral roots and thus increased root branching resulting in increased exploitation of soil nutrients. The increased plant growth and the prolongation of the vegetative growth phase by soil amoebae strongly enhanced plant reproduction. Concurrently the abundance of soil amoebae in the rhizosphere increased (Rosenberg, 2008) suggesting mutualistic interactions between plants and soil protozoa (Bonkowski 2004). By enhancing plant growth soil amoebae benefit from increased root exudation of carbon rich substances allocated from shoots into roots, which promote the growth of soil bacteria and subsequently growth of bacterial predators such as amoebae (Milchunas et al., 1985; Semenov et al., 1999; Cheng & Gershenson, 2007; Griffiths et al., 2007).

Growth promotion of *A. thaliana* by the abundant and widespread soil amoeba *A. castellanii* already occurred 3 dpi indicating immediate effects of soil amoebae on plant growth. This early plant promotion was not associated with nitrogen-mediated effects, since neither the availability of plant available nitrogen nor plant nitrogen uptake nor the transcriptional regulation of nitrogen responsive genes was affected by soil amoebae. However, 5 dpi the availability of ammonium, plant nitrogen uptake and also the regulation of ammonium assimilatory genes was induced by soil amoebae. Consistent with the ‘microbial loop’ theory this suggests that soil protozoa release sequestered ammonium from grazed bacterial biomass into the rhizosphere

and thus improve plant nitrogen supply and plant growth (Clarholm, 1985, Clarholm et al., 2006). Based on the differential responses in time we suggest that the plant anticipated the upcoming mobilization of nitrogen and increased carbon fixation, and thus shoot and root growth, thereby facilitating the capture of nitrogen made available by soil amoebae later. Anticipation of upcoming improved nitrogen supply requires recognition processes in the plant, i.e. in rhizosphere signalling. Nitrate, which also functions as a signal compound (Takei et al., 2002; Sakakibara, 2003), is unlikely to be involved in this early recognition since soil amoebae neither increased the concentration of nitrate in the rhizosphere nor regulated nitrate responsive genes during the immediate plant response. Expression analysis of genes involved in plant signalling and stress response did not reflect how plants may recognize changes in rhizosphere signalling or the presence of soil amoebae. However, gene expression analysis showed that the presence of soil amoebae immediately reduced defence responses in plant roots, whereas in shoots defence responses were activated. Presumably, via selective grazing soil amoebae reduce the impact of detrimental rhizosphere bacteria on the plant resulting in decreased investment in defence in roots. The reduced costs for secondary metabolism (Van Hulten et al., 2006) presumably enabled the plants to redirect resources into root growth thereby increasing nitrogen exploitation. Concurrently, soil amoebae induced defence responses in shoots, which however, did not come at the expense of plant growth. Rather, soil amoebae increased plant growth and reproduction, presumably due to enhanced carbon and nitrogen capture.

Previous investigations suggest that auxin might be a signal compound in plant – protozoa interactions (Bonkowski & Brandt, 2002). Soil amoebae were assumed to enhance the bacterial production of IAA in the rhizosphere by selective grazing, which subsequently induces the formation of lateral roots resulting in an improved

soil exploitation and further plant growth. Our results from experiments with *L. sativum* support that soil amoebae induce root branching by modifying auxin metabolism in plants, which presumably is responsible for the initiation of lateral roots. However, soil amoebae only influenced IAA concentrations systemically in shoots but not in roots indicating that protozoa altered the internal auxin synthesis in shoots rather than auxin uptake via the roots. The observation that *A. thaliana* reporter plants did not show the expected auxin response despite of increased numbers of lateral roots, but rather increased levels of cytokinin (Coenen & Lomax, 1997) which is considered to repress lateral root formation (Werner et al., 2001, 2003; Li et al., 2006; Laplaze et al., 2007), further challenged the 'auxin theory'. Cytokinin biosynthesis is stimulated by nitrate (Samuleson & Larsson, 1993; Takei et al., 2002; Sakakibara, 2003; Miyawaki et al., 2004; Takei et al., 2004) suggesting that the increased concentrations of cytokinin were due to the amoebae-mediated increase in the supply of nitrate. In fact, cytokinin is assumed to increase free auxin levels (Coenen & Lomax, 1997) and therefore may have been responsible for the observed increase in root growth.

This is the first study which focussed on the initiation of plant growth promotion by soil protozoa. The results obtained provided novel information on chronological processes of the response of plants to soil protozoa. The immediate increase in plant growth is reflected later in increased reproduction. Therefore, uncovering the mechanisms responsible for the early plant response is necessary to understand the later plant responses including plant reproduction and life cycle. Although we obtained hints on the mechanisms involved in the immediate plant – soil bacteria – protozoa interaction the responsible elicitor for the plant growth promotion still has to be uncovered. Progress is expected by using specific bacterial strains, whole genome arrays and *A. thaliana* mutants other than the ones used in this study.

Methods and experimental systems developed in this study allow to implement the respective experiments in near future.

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EIDESSTATTLICHE ERKLÄRUNG

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Darmstadt, den 22. April 2008

APPENDIX

Changes in the induction / repression of selected genes in *Arabidopsis thaliana* roots as indicated by microarray analyses 3 and 5 days past plant inoculation (dpi) with soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*); means of three replicates and significant differences (P-values; Student's t-test) are demonstrated. Genes up-regulated by a factor 1.4 to 1.8 with p-value <0.05 are marked light blue, genes up-regulated by a factor >1.8 and p-value <0.1 are marked dark blue. Genes down-regulated by a factor 0.7 to 0.5 with p-value <0.05 are marked light red, genes down-regulated by a factor <0.5 and p-value <0.1 are marked dark red. S = Sterile treatment, B = Bacteria treatment, P = Protozoa treatment.

	Rd3						Rd5					
	SvsB		SvsP		BvsP		SvsB		SvsP		BvsP	
AGI-Code	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p	mean±SD	p
At2g29750	2.78±0.90	0.03	2.41±0.43	0.01	0.9±0.13	0.32	3.02±0.29	0.01	2.47±0.55	0.03	0.82±0.20	0.24
At1g07260	1.32±0.29	0.20	1.15±0.16	0.23	0.9±0.20	0.42	1.65±0.20	0.02	1.64±0.06	0.01	1.01±0.16	1.00
At1g07250	1.31±0.44	0.36	1.26±0.09	0.03	1.04±0.32	0.97	1.57±0.16	0.02	1.55±0.25	0.05	0.98±0.06	0.75
At4g36770	2.24±0.58	0.02	1.75±0.14	0.01	0.81±0.17	0.18	1.87±0.12	0.00	1.14±0.25	0.44	0.60±0.09	0.03
At2g18570	2.10±0.86	0.09	2.00±0.53	0.04	1.01±0.30	0.87	1.61±0.11	0.01	1.23±0.14	0.09	0.76±0.08	0.04
At2g18560	0.89±0.22	0.34	0.84±0.14	0.18	0.96±0.08	0.43	0.38±0.03	0.01	0.53±0.08	0.01	1.40±0.21	0.09
At3g50740	3.18±1.11	0.06	2.16±0.75	0.10	0.69±0.23	0.16	2.14±0.11	0.00	2.01±0.46	0.04	0.93±0.19	0.57
At5g66690	2.68±0.41	0.01	2.23±0.28	0.01	0.84±0.07	0.06	2.78±0.37	0.01	1.67±0.08	0.01	0.61±0.10	0.04
At4g34131	0.56±0.28	0.14	0.42±0.07	0.06	0.85±0.34	0.39	0.53±0.05	0.01	0.55±0.07	0.01	1.04±0.15	0.69
At2g15490	0.30±0.09	0.05	0.25±0.07	0.05	0.85±0.13	0.21	0.29±0.04	0.01	0.30±0.05	0.01	1.04±0.06	0.39
At2g15480	0.52±0.17	0.07	0.44±0.11	0.06	0.92±0.39	0.57	0.59±0.03	0.01	0.58±0.03	0.00	0.98±0.08	0.65
At2g36800	0.78±0.09	0.07	0.77±0.07	0.04	0.99±0.11	0.81	0.63±0.10	0.04	0.65±0.14	0.08	1.03±0.10	0.61
At3g53160	0.62±0.14	0.03	0.77±0.13	0.09	1.27±0.27	0.25	0.90±0.17	0.40	0.84±0.14	0.18	0.94±0.13	0.47
At3g53150	1.05±0.21	0.79	1.10±0.37	0.77	1.07±0.34	0.92	1.48±0.19	0.02	1.35±0.34	0.19	0.91±0.16	0.41
At1g05670	0.14±0.06	0.03	0.14±0.04	0.03	1.06±0.17	0.73	0.18±0.02	0.00	0.19±0.03	0.00	1.06±0.10	0.44
At2g43840	0.42±0.08	0.01	0.55±0.22	0.19	1.19±0.30	0.43	0.51±0.05	0.02	0.58±0.10	0.04	1.14±0.08	0.08
At1g05680	0.10±0.03	0.02	0.09±0.02	0.02	0.90±0.08	0.21	0.12±0.01	0.00	0.12±0.02	0.01	1.07±0.09	0.32
At1g05560	0.33±0.12	0.05	0.33±0.10	0.05	1.04±0.22	0.92	0.27±0.04	0.01	0.28±0.04	0.01	1.01±0.03	0.76
At4g15550	0.57±0.20	0.09	0.55±0.25	0.11	0.94±0.09	0.44	0.57±0.11	0.03	0.53±0.07	0.02	0.94±0.09	0.36
At5g38040	0.30±0.12	0.08	0.30±0.09	0.06	1.05±0.32	0.94	0.32±0.04	0.01	0.29±0.04	0.01	0.93±0.15	0.45
At5g17040	0.23±0.13	0.09	0.24±0.11	0.08	1.11±0.28	0.61	0.18±0.02	0.01	0.20±0.04	0.01	1.14±0.15	0.27
At1g22360	0.84±0.08	0.08	0.86±0.14	0.24	1.04±0.27	0.93	0.68±0.06	0.01	0.71±0.04	0.01	1.04±0.02	0.08
At1g22340	1.76±0.60	0.12	1.43±0.27	0.07	0.85±0.21	0.30	1.57±0.15	0.01	1.50±0.14	0.02	0.96±0.06	0.37
At2g30140	0.46±0.06	0.01	0.48±0.08	0.03	1.06±0.19	0.75	0.47±0.02	0.00	0.47±0.05	0.00	1.00±0.07	0.92
At1g51210	0.75±0.25	0.22	0.62±0.30	0.17	0.80±0.13	0.08	0.91±0.04	0.10	1.06±0.06	0.21	1.17±0.01	0.00
At1g06000	0.70±0.12	0.05	0.91±0.11	0.30	1.32±0.28	0.19	0.87±0.18	0.33	0.88±0.06	0.08	1.04±0.23	0.84
At1g11680	1.59±0.10	0.01	1.41±0.23	0.10	0.89±0.20	0.44	1.23±0.17	0.13	1.01±0.21	1.00	0.82±0.14	0.19
At5g42590	1.88±0.27	0.00	1.59±0.21	0.01	0.85±0.06	0.05	1.16±0.12	0.14	1.08±0.17	0.56	0.93±0.15	0.46
At1g13080	1.79±0.05	0.00	1.67±0.14	0.01	0.93±0.05	0.15	1.41±0.18	0.06	1.87±0.22	0.03	1.34±0.16	0.06
At2g24180	0.46±0.10	0.02	0.48±0.05	0.01	1.05±0.11	0.59	0.56±0.11	0.04	0.55±0.09	0.03	1.00±0.08	0.87
At1g13110	1.55±0.21	0.03	1.23±0.04	0.00	0.80±0.09	0.07	1.25±0.07	0.04	1.23±0.16	0.11	0.98±0.13	0.79
At3g26150	1.31±0.15	0.10	1.26±0.19	0.14	0.97±0.22	0.73	1.74±0.15	0.02	2.07±0.53	0.06	1.21±0.42	0.49
At3g26210	1.60±0.24	0.02	1.48±0.48	0.19	0.91±0.17	0.45	1.05±0.10	0.49	1.10±0.11	0.25	1.05±0.16	0.65
At1g13100	1.70±0.29	0.04	1.51±0.29	0.05	0.90±0.13	0.30	1.40±0.08	0.01	1.53±0.17	0.03	1.10±0.18	0.44
At2g30490	2.27±0.76	0.13	1.48±0.47	0.25	0.66±0.06	0.06	1.57±0.02	0.00	1.39±0.19	0.07	0.89±0.12	0.28
At1g01190	1.19±0.12	0.11	1.17±0.06	0.04	0.99±0.05	0.62	1.94±0.29	0.03	1.84±0.30	0.03	0.95±0.10	0.44
At4g39950	1.38±0.37	0.20	1.47±0.22	0.08	1.11±0.28	0.60	1.88±0.43	0.04	2.17±0.67	0.07	1.14±0.11	0.18
At2g22330	1.06±0.10	0.39	1.23±0.08	0.05	1.17±0.17	0.22	1.79±0.32	0.03	2.23±0.36	0.02	1.27±0.29	0.23
At3g28740	0.58±0.16	0.08	0.61±0.18	0.11	1.05±0.26	0.80	0.39±0.05	0.00	0.42±0.05	0.00	1.09±0.17	0.50
At4g31500	1.60±0.20	0.04	1.49±0.35	0.12	0.93±0.15	0.51	1.29±0.12	0.05	1.37±0.16	0.05	1.07±0.09	0.34
At5g23190	1.76±0.38	0.05	1.25±0.16	0.11	0.72±0.10	0.06	1.44±0.11	0.01	1.36±0.12	0.04	0.95±0.14	0.57
At1g64930	0.50±0.11	0.05	0.54±0.15	0.07	1.07±0.10	0.36	0.66±0.13	0.07	0.70±0.06	0.03	1.06±0.11	0.45
At3g50660	1.11±0.12	0.27	1.42±0.44	0.19	1.26±0.31	0.24	1.64±0.10	0.01	1.65±0.18	0.01	1.01±0.09	0.98
At1g74540	1.95±0.47	0.05	1.76±0.42	0.07	0.90±0.02	0.01	1.32±0.04	0.01	1.36±0.09	0.03	1.03±0.04	0.32
At1g74550	1.51±0.00	0.02	1.37±0.43	0.31	0.91±0.29	0.63	1.20±0.10	0.06	1.05±0.08	0.46	0.88±0.08	0.11
At5g47990	0.98±0.24	0.79	1.14±0.22	0.45	1.18±0.07	0.04	0.64±0.02	0.00	0.64±0.05	0.00	1.01±0.11	0.89

At3g20130	1.55±0.23	0.05	1.35±0.22	0.11	0.87±0.02	0.00	1.37±0.32	0.14	1.29±0.23	0.14	0.95±0.10	0.41
At3g20960	1.57±0.55	0.14	1.63±0.72	0.18	1.03±0.14	0.74	1.49±0.18	0.05	1.77±0.30	0.04	1.21±0.34	0.42
At4g22690	1.58±0.51	0.13	1.52±0.53	0.16	0.97±0.15	0.78	1.54±0.15	0.03	1.53±0.08	0.00	1.00±0.09	0.87
At5g45340	1.81±0.56	0.09	1.66±0.54	0.12	0.92±0.08	0.23	1.36±0.26	0.13	1.20±0.11	0.07	0.89±0.09	0.21
At5g36130	1.83±0.34	0.05	1.97±0.28	0.02	1.10±0.26	0.62	2.06±0.18	0.00	1.61±0.23	0.03	0.78±0.04	0.02
At2g29490	0.50±0.08	0.02	0.67±0.13	0.05	1.36±0.22	0.10	0.61±0.15	0.10	0.60±0.13	0.05	1.01±0.25	0.97
At2g29480	0.62±0.07	0.01	0.77±0.06	0.03	1.25±0.10	0.02	0.76±0.10	0.05	0.72±0.14	0.07	0.98±0.29	0.83
At2g29460	0.56±0.08	0.00	0.73±0.10	0.02	1.29±0.08	0.03	0.68±0.01	0.00	0.62±0.11	0.03	0.92±0.16	0.46
At2g29450	0.49±0.10	0.05	0.53±0.18	0.09	1.05±0.17	0.70	0.63±0.05	0.01	0.63±0.06	0.01	1.01±0.04	0.69
At2g29450	0.57±0.07	0.04	0.46±0.04	0.03	0.81±0.06	0.05	0.68±0.11	0.05	0.62±0.10	0.04	0.92±0.13	0.36
At2g29440	0.67±0.03	0.01	0.76±0.09	0.06	1.14±0.13	0.21	0.89±0.09	0.15	0.78±0.06	0.02	0.89±0.15	0.33
At2g29420	0.38±0.20	0.10	0.34±0.07	0.05	1.07±0.45	0.95	0.48±0.07	0.02	0.43±0.05	0.02	0.91±0.10	0.25
At3g09270	0.37±0.11	0.01	0.51±0.15	0.07	1.12±0.34	0.74	0.50±0.07	0.02	0.54±0.16	0.07	1.07±0.23	0.68
At5g62480	1.21±0.27	0.32	1.08±0.03	0.05	0.93±0.26	0.56	1.61±0.10	0.01	1.24±0.14	0.09	0.77±0.11	0.08
At1g74590	0.59±0.14	0.05	0.63±0.20	0.10	1.05±0.12	0.45	0.88±0.11	0.22	1.02±0.18	0.99	1.15±0.10	0.11
At1g78380	0.28±0.14	0.07	0.28±0.12	0.07	1.05±0.25	0.96	0.35±0.05	0.00	0.32±0.07	0.01	0.91±0.09	0.20
At1g78370	1.29±0.15	0.08	1.43±0.44	0.17	1.10±0.28	0.71	1.70±0.22	0.03	1.75±0.16	0.01	1.04±0.06	0.43
At1g17170	0.36±0.11	0.06	0.27±0.06	0.04	0.77±0.10	0.07	0.30±0.03	0.00	0.24±0.02	0.00	0.80±0.13	0.18
At1g17180	0.23±0.09	0.05	0.20±0.10	0.06	0.86±0.07	0.08	0.26±0.06	0.02	0.21±0.00	0.01	0.82±0.20	0.28
At1g17190	2.64±0.30	0.02	2.48±0.80	0.07	0.94±0.27	0.65	1.49±0.21	0.04	1.47±0.25	0.07	1.01±0.26	0.95
At4g02520	0.72±0.13	0.09	0.69±0.13	0.07	0.97±0.06	0.43	0.59±0.00	0.00	0.61±0.03	0.01	1.04±0.06	0.35
At1g02950	1.51±0.68	0.39	1.25±0.29	0.30	0.90±0.23	0.47	1.57±0.03	0.01	1.25±0.34	0.30	0.80±0.23	0.25
At3g61430	2.85±1.03	0.07	2.11±0.49	0.04	0.77±0.16	0.20	1.53±0.27	0.06	1.69±0.12	0.01	1.12±0.20	0.40
At1g01620	3.75±1.06	0.12	2.35±0.60	0.05	0.82±0.19	0.28	1.46±0.03	0.00	1.59±0.03	0.00	1.08±0.00	0.01
At4g23400	1.99±0.60	0.07	1.68±0.46	0.10	0.84±0.04	0.04	1.66±0.10	0.01	1.61±0.09	0.01	0.98±0.11	0.68
At3g53420	1.68±0.61	0.22	1.25±0.11	0.05	0.81±0.28	0.37	1.63±0.17	0.01	1.57±0.40	0.09	0.96±0.14	0.63
At2g37170	1.69±0.72	0.25	1.25±0.23	0.35	0.83±0.56	0.60	1.70±0.27	0.01	1.75±0.54	0.08	1.02±0.16	0.90
At5g60660	2.94±1.20	0.07	2.31±0.36	0.01	0.84±0.22	0.36	2.36±0.24	0.00	2.36±0.38	0.01	1.00±0.10	1.00
At4g35100	2.22±1.04	0.16	1.82±0.14	0.00	0.92±0.35	0.54	1.60±0.13	0.01	1.64±0.16	0.02	1.03±0.07	0.51
At4g17340	2.85±1.31	0.08	2.00±0.29	0.01	0.77±0.21	0.24	2.24±0.29	0.01	3.47±0.34	0.00	1.57±0.23	0.03
At5g47450	3.01±1.13	0.05	1.74±0.14	0.00	0.62±0.16	0.11	1.87±0.24	0.03	3.52±0.55	0.01	1.91±0.49	0.04
At2g36830	2.59±0.87	0.06	1.94±0.46	0.06	0.77±0.12	0.13	2.35±0.15	0.00	2.45±0.15	0.00	1.04±0.03	0.12
At3g26520	3.17±1.19	0.07	2.41±0.29	0.01	0.80±0.17	0.26	2.91±0.45	0.00	3.23±0.73	0.01	1.10±0.08	0.14
At3g16240	2.49±0.87	0.07	2.63±0.75	0.04	1.08±0.14	0.42	1.94±0.30	0.05	1.69±0.26	0.03	0.90±0.25	0.51
At4g19030	1.67±0.30	0.04	1.65±0.24	0.03	0.99±0.10	0.86	1.44±0.24	0.08	1.05±0.07	0.33	0.75±0.17	0.14
At4g10380	1.61±0.15	0.03	1.48±0.29	0.08	0.93±0.20	0.55	1.50±0.15	0.02	1.31±0.14	0.05	0.88±0.03	0.01
At1g80760	2.16±0.56	0.08	1.60±0.06	0.05	0.74±0.22	0.40	1.72±0.45	0.07	1.32±0.05	0.01	0.80±0.18	0.22
At3g06100	2.11±0.99	0.14	1.60±0.37	0.06	0.81±0.19	0.28	1.89±0.09	0.00	2.36±0.28	0.01	1.25±0.11	0.05
At4g31550	1.44±0.20	0.04	1.45±0.35	0.14	1.00±0.11	0.96	1.56±0.21	0.04	1.40±0.10	0.02	0.91±0.12	0.29
At5g64811	0.44±0.14	0.06	0.43±0.11	0.05	1.01±0.15	0.92	0.45±0.06	0.01	0.43±0.03	0.00	0.95±0.07	0.34
At2g40740	0.70±0.03	0.01	0.71±0.21	0.17	1.03±0.33	0.96	0.66±0.06	0.01	0.58±0.03	0.00	0.88±0.09	0.14
At1g29280	1.69±0.12	0.01	1.61±0.07	0.00	0.95±0.05	0.28	1.20±0.06	0.04	0.99±0.17	0.83	0.82±0.18	0.22
At3g58710	2.05±0.28	0.01	1.93±0.11	0.00	0.95±0.08	0.39	1.91±0.35	0.06	1.96±0.50	0.06	1.04±0.28	0.95
At3g56400	0.67±0.03	0.03	0.65±0.18	0.13	0.98±0.24	0.80	0.67±0.05	0.02	0.70±0.04	0.01	1.05±0.03	0.10
At5g13080	0.35±0.09	0.02	0.41±0.07	0.01	1.22±0.12	0.05	0.47±0.11	0.03	0.47±0.04	0.01	1.05±0.24	0.86
At3g10660	1.93±0.38	0.03	1.83±0.59	0.13	0.93±0.19	0.61	1.17±0.04	0.02	1.13±0.06	0.07	0.96±0.03	0.17
At4g23650	1.74±0.23	0.01	1.29±0.35	0.28	0.73±0.10	0.05	1.85±0.36	0.03	1.81±0.57	0.10	0.97±0.13	0.72
At4g35310	2.02±0.29	0.01	1.40±0.30	0.11	0.70±0.14	0.08	1.79±0.38	0.03	1.55±0.04	0.02	0.90±0.19	0.44
At4g04740	1.23±0.27	0.25	1.50±0.71	0.27	1.19±0.33	0.38	1.47±0.20	0.05	1.51±0.21	0.04	1.03±0.01	0.06
At4g38230	1.49±0.08	0.01	1.55±0.20	0.04	1.04±0.15	0.71	1.63±0.15	0.01	1.63±0.16	0.02	1.00±0.04	0.92
At4g04700	1.76±0.27	0.01	1.56±0.25	0.03	0.89±0.04	0.02	1.20±0.20	0.23	1.38±0.13	0.04	1.17±0.23	0.33
At4g04695	1.65±0.17	0.02	1.70±0.16	0.01	1.04±0.08	0.56	1.14±0.05	0.05	1.18±0.10	0.10	1.03±0.04	0.31
At3g57530	1.44±0.09	0.01	1.47±0.14	0.03	1.02±0.13	0.87	1.14±0.06	0.05	1.33±0.19	0.07	1.17±0.13	0.14
At3g49370	1.25±0.50	0.46	1.55±0.31	0.05	1.31±0.37	0.19	0.89±0.10	0.24	1.03±0.26	0.99	1.14±0.17	0.30
At2g43600	0.67±0.07	0.04	0.59±0.08	0.04	0.89±0.09	0.18	0.77±0.10	0.09	0.94±0.20	0.61	1.21±0.11	0.07

At2g27500	1.49±0.20	0.05	1.30±0.28	0.21	0.90±0.32	0.59	1.51±0.21	0.02	1.47±0.28	0.09	0.97±0.16	0.76
At2g19990	1.30±0.36	0.24	1.56±0.84	0.36	1.14±0.28	0.50	1.45±0.18	0.05	1.40±0.16	0.02	0.97±0.11	0.61
At4g33720	5.47±1.84	0.04	5.76±1.50	0.01	1.08±0.15	0.60	5.75±1.30	0.02	7.09±1.59	0.02	1.32±0.60	0.48
At4g25790	2.80±0.40	0.00	2.07±0.46	0.03	0.73±0.06	0.02	2.40±0.23	0.00	2.21±0.48	0.03	0.92±0.13	0.40
At2g02120	1.07±0.09	0.32	1.33±0.13	0.06	1.24±0.05	0.03	1.51±0.21	0.03	1.68±0.22	0.02	1.11±0.01	0.01
At2g02100	1.25±0.19	0.15	1.31±0.17	0.08	1.06±0.06	0.21	1.39±0.10	0.01	1.45±0.10	0.01	1.05±0.09	0.47
At2g02130	1.26±0.04	0.01	1.37±0.21	0.11	1.09±0.20	0.54	1.28±0.13	0.06	1.60±0.14	0.02	1.26±0.18	0.10
At2g43590	0.71±0.12	0.06	0.69±0.11	0.04	0.98±0.05	0.57	0.51±0.01	0.00	0.46±0.04	0.00	0.89±0.06	0.10
At2g43620	1.77±0.34	0.03	1.62±0.05	0.02	0.94±0.21	0.65	1.41±0.09	0.01	1.50±0.09	0.01	1.06±0.05	0.17
At3g16920	1.44±0.17	0.05	1.30±0.38	0.29	0.90±0.18	0.43	1.03±0.13	0.78	1.01±0.12	0.94	0.98±0.03	0.39
At3g44310	0.53±0.12	0.04	0.61±0.17	0.09	1.14±0.24	0.44	0.49±0.03	0.00	0.57±0.10	0.03	1.17±0.27	0.41
At3g44310	0.63±0.04	0.01	0.63±0.10	0.04	0.99±0.10	0.85	0.49±0.03	0.00	0.57±0.05	0.01	1.16±0.03	0.02
At3g44300	0.40±0.09	0.04	0.43±0.18	0.07	1.06±0.26	0.77	0.34±0.01	0.00	0.43±0.05	0.01	1.24±0.12	0.06
At3g44300	0.39±0.06	0.03	0.41±0.13	0.05	1.03±0.16	0.79	0.31±0.01	0.00	0.35±0.03	0.00	1.12±0.06	0.09
At1g20620	1.95±1.00	0.22	1.20±0.29	0.36	0.66±0.15	0.19	1.46±0.10	0.01	1.53±0.03	0.00	1.05±0.09	0.42
At1g08830	2.18±0.47	0.00	1.38±0.47	0.38	0.63±0.18	0.09	1.83	0.03	1.25±0.42	0.50	0.67±0.13	0.05
At1g12520	2.29±0.97	0.04	1.34±0.49	0.39	0.60±0.14	0.04	1.68±0.03	0.00	1.74±0.21	0.02	1.04±0.11	0.64
At5g01870	0.42±0.06	0.03	0.62±0.33	0.24	1.41±0.61	0.40	0.47±0.06	0.01	0.37±0.08	0.01	0.78±0.06	0.02
At3g18280	1.14±0.27	0.61	1.13±0.27	0.44	1.03±0.33	0.78	0.65±0.06	0.02	0.71±0.11	0.06	1.10±0.18	0.44
At2g38530	0.52±0.20	0.13	0.42±0.11	0.08	0.85±0.15	0.21	0.54±0.05	0.02	0.52±0.03	0.01	0.97±0.12	0.66
At2g38540	0.52±0.13	0.09	0.49±0.09	0.06	0.95±0.11	0.51	0.59±0.06	0.01	0.53±0.07	0.01	0.89±0.02	0.01
At5g59320	0.54±0.07	0.02	0.66±0.19	0.14	1.25±0.39	0.49	0.75±0.24	0.25	0.72±0.20	0.21	0.97±0.08	0.54
At4g23900	1.41±0.08	0.01	1.46±0.11	0.03	1.03±0.09	0.58	1.63±0.24	0.03	1.71±0.21	0.02	1.05±0.04	0.13
At2g37040	1.89±0.22	0.01	1.53±0.11	0.00	0.81±0.07	0.06	1.66±0.22	0.01	1.18±0.33	0.46	0.70±0.10	0.04
At3g10340	2.19±0.18	0.01	1.72±0.23	0.02	0.79±0.13	0.14	1.54±0.31	0.10	1.44±0.08	0.01	0.97±0.28	0.72
At3g53260	1.54±0.10	0.02	1.28±0.26	0.23	0.83±0.18	0.28	0.99±0.12	0.87	1.07±0.09	0.33	1.09±0.08	0.19
At3g50210	1.68±0.29	0.04	1.61±0.36	0.07	0.96±0.17	0.71	1.31±0.04	0.00	1.11±0.03	0.02	0.85±0.03	0.02
At1g51680	1.73±0.12	0.02	1.57±0.28	0.06	0.92±0.22	0.53	1.36±0.14	0.04	1.22±0.09	0.04	0.90±0.03	0.04
At3g21240	1.97±0.57	0.08	1.40±0.02	0.01	0.76±0.24	0.24	1.68±0.12	0.01	1.47±0.07	0.00	0.88±0.02	0.02
At1g66340	1.27±0.11	0.07	1.42±0.13	0.07	1.12±0.12	0.23	1.58±0.23	0.05	1.69±0.18	0.02	1.09±0.26	0.65
At3g04720	0.58±0.07	0.05	0.57±0.12	0.09	0.97±0.18	0.73	0.55±0.01	0.00	0.65±0.07	0.02	1.20±0.14	0.11
At3g50630	1.53±0.21	0.06	1.45±0.14	0.04	0.95±0.05	0.22	1.90±0.31	0.02	1.73±0.10	0.02	0.93+0.19	0.59
At2g27970	1.33±0.33	0.24	1.29±0.21	0.11	0.99±0.18	0.78	1.57±0.15	0.01	1.28±0.07	0.01	0.81±0.03	0.01
At1g30400	0.86±0.04	0.03	0.85±0.15	0.24	0.99±0.19	0.86	0.68±0.06	0.01	0.72±0.08	0.03	1.05±0.10	0.49
At4g02600	1.20±0.07	0.02	1.15±0.19	0.34	0.96±0.19	0.81	1.69±0.14	0.01	1.70±0.25	0.04	1.01±0.17	0.96
At5g19440	0.71±0.17	0.11	0.75±0.11	0.08	1.08±0.12	0.37	0.62±0.06	0.01	0.62±0.03	0.00	1.00±0.05	0.95
At2g21020	1.68±0.28	0.02	1.64±0.36	0.08	1.00±0.29	0.89	1.91±0.44	0.03	1.88±0.37	0.02	0.99±0.04	0.71
At3g63080	1.31±0.20	0.09	1.35±0.21	0.16	1.06±0.32	0.75	1.50±0.09	0.00	1.49±0.31	0.10	0.99±0.16	0.92
At5g05410	0.70±0.07	0.04	0.85±0.13	0.20	1.20±0.08	0.04	0.98±0.03	0.29	0.91±0.06	0.14	0.93±0.04	0.11
At5g66120	0.43±0.08	0.04	0.35±0.12	0.04	0.81±0.10	0.10	0.47±0.04	0.01	0.36±0.05	0.01	0.76±0.06	0.02
At1g66800	1.90±0.44	0.05	2.30±0.67	0.07	1.26±0.50	0.49	1.16±0.15	0.20	1.29±0.20	0.10	1.12±0.06	0.09
At2g38340	0.67±0.09	0.03	0.84±0.14	0.15	1.27±0.29	0.21	0.99±0.12	0.85	1.22±0.17	0.14	1.23±0.08	0.04
At4g35790	2.06±0.46	0.05	1.58±0.33	0.12	0.78±0.20	0.21	1.26±0.01	0.00	1.07±0.13	0.46	0.85±0.11	0.15
At2g43430	1.53±0.10	0.00	1.15±0.28	0.54	0.75±0.15	0.13	1.07±0.09	0.29	0.91±0.15	0.39	0.84±0.07	0.07
At5g39340	0.82±0.10	0.09	0.83±0.08	0.07	1.03±0.22	0.96	0.70±0.03	0.00	0.77±0.03	0.01	1.10±0.06	0.09
At5g55990	1.08±0.04	0.05	1.26±0.08	0.03	1.16±0.04	0.03	1.45±0.06	0.01	1.51±0.10	0.02	1.04±0.03	0.17
At1g80100	0.49±0.15	0.04	0.46±0.06	0.02	0.96±0.14	0.61	0.53±0.05	0.00	0.48±0.03	0.00	0.91±0.11	0.29
At2g27150	0.41±0.09	0.04	0.41±0.09	0.04	1.01±0.12	0.97	0.36±0.01	0.00	0.38±0.02	0.00	1.07±0.05	0.14
At4g11830	1.36±0.24	0.14	1.23±0.23	0.21	0.91±0.17	0.41	1.54±0.23	0.04	1.31±0.22	0.12	0.85±0.12	0.16

Changes in the induction / repression of selected genes in *Arabidopsis thaliana* shoots as indicated by microarray analyses 3 and 5 days past plant inoculation (dpi) with soil bacteria and bacterivorous protozoa (*Acanthamoeba castellanii*); means of three replicates and significant differences (P-values; Student's t-test) are demonstrated. Genes up-regulated by a factor 1.4 to 1.8 with p-value <0.05 are marked light blue, genes up-regulated by a factor >1.8 and p-value <0.1 are marked dark blue. Genes down-regulated by a factor 0.7 to 0.5 with p-value <0.05 are marked light red, genes down-regulated by a factor <0.5 and p-value <0.1 are marked dark red. S = Sterile treatment, B = Bacteria treatment, P = Protozoa treatment.

	Sd3						Sd5					
	SvsB		SvsP		BvsP		SvsB		SvsP		BvsP	
AGI-Code	mean	p	mean	p	mean	p	mean	p	mean	p	mean	p
At4g01070	0.67±0.05	0.00	0.73±0.26	0.22	1.11±0.48	0.85	0.83±0.19	0.25	0.51±0.10	0.01	0.66±0.26	0.16
At2g18560	0.74±0.22	0.20	0.72±0.09	0.07	1.01±0.18	0.93	0.64±0.03	0.01	0.54±0.18	0.07	0.83±0.24	0.36
At4g34131	0.16±0.03	0.02	0.24±0.13	0.09	0.98±0.32	0.83	0.48±0.05	0.04	0.46±0.05	0.04	0.96±0.04	0.19
At2g15490	0.28±0.17	0.10	0.29±0.16	0.09	1.05±0.22	0.71	0.88±0.10	0.16	0.95±0.14	0.61	1.10±0.28	0.62
At2g15480	0.22±0.10	0.05	0.22±0.08	0.04	1.02±0.33	0.91	0.69±0.17	0.11	0.54±0.13	0.05	0.79±0.02	0.01
At2g31750	0.48±0.08	0.01	0.50±0.04	0.00	1.08±0.22	0.72	0.76±0.13	0.09	0.81±0.10	0.10	1.07±0.07	0.17
At1g05670	0.22±0.01	0.01	0.29±0.07	0.02	1.31±0.27	0.18	0.59±0.02	0.00	0.57±0.09	0.01	0.97±0.13	0.79
At2g43840	0.54±0.06	0.02	0.58±0.06	0.03	1.06±0.06	0.22	0.84±0.20	0.30	0.72±0.15	0.09	0.88±0.23	0.40
At2g43820	0.73±0.24	0.20	0.49±0.13	0.06	0.71±0.26	0.20	0.66±0.09	0.04	0.68±0.10	0.04	1.04±0.10	0.56
At1g05680	0.16±0.02	0.02	0.19±0.04	0.02	1.21±0.11	0.07	0.39±0.06	0.01	0.33±0.04	0.00	0.84±0.13	0.20
At1g05560	0.30±0.06	0.03	0.36±0.13	0.05	1.19±0.21	0.27	0.51±0.04	0.00	0.48±0.07	0.01	0.94±0.10	0.35
At4g15550	0.51±0.20	0.10	0.47±0.21	0.11	0.90±0.18	0.40	0.55±0.19	0.08	0.49±0.04	0.02	0.95±0.23	0.65
At3g11340	0.52±0.02	0.01	0.59±0.09	0.02	1.13±0.14	0.28	0.76±0.05	0.02	0.62±0.15	0.03	0.81±0.22	0.27
At5g05870	0.64±0.05	0.01	0.64±0.11	0.04	1.00±0.15	0.96	1.02±0.11	0.74	0.93±0.12	0.38	0.91±0.12	0.34
At5g38040	0.43±0.06	0.04	0.46±0.06	0.04	1.06±0.07	0.26	0.61±0.06	0.02	0.56±0.05	0.01	0.93±0.05	0.10
At5g17040	0.29±0.05	0.03	0.31±0.05	0.03	1.07±0.10	0.36	0.51±0.03	0.00	0.46±0.05	0.00	0.92±0.13	0.36
At2g36970	0.67±0.12	0.05	0.67±0.15	0.08	1.01±0.15	0.96	0.98±0.12	0.75	0.94±0.15	0.53	0.96±0.06	0.36
At2g28080	0.70±0.09	0.05	0.74±0.05	0.03	1.05±0.06	0.28	0.97±0.03	0.18	1.15±0.20	0.34	1.18±0.19	0.27
At2g30140	0.43±0.12	0.05	0.47±0.09	0.05	1.12±0.11	0.15	0.73±0.12	0.09	0.79±0.05	0.01	1.11±0.24	0.55
At1g51210	0.71±0.24	0.18	0.62±0.24	0.14	0.89±0.33	0.57	0.67±0.07	0.02	0.62±0.03	0.00	0.94±0.13	0.51
At1g10400	0.88±0.18	0.37	1.03±0.03	0.24	1.21±0.27	0.33	0.56±0.06	0.02	0.71±0.19	0.13	1.28±0.35	0.30
At3g26220	0.63±0.08	0.03	0.54±0.12	0.04	0.88±0.24	0.42	0.99±0.25	0.88	0.82±0.12	0.13	0.84±0.12	0.17
At3g26290	0.57±0.04	0.01	0.60±0.06	0.05	1.07±0.17	0.77	1.08±0.16	0.46	0.84±0.20	0.33	0.79±0.17	0.16
At3g28740	0.08±0.01	0.01	0.07±0.01	0.01	0.94±0.05	0.20	0.11±0.02	0.01	0.09±0.03	0.01	0.85±0.16	0.26
At4g00360	1.25±0.23	0.20	1.28±0.28	0.18	1.03±0.15	0.89	1.84±0.32	0.03	1.39±0.42	0.28	0.75±0.13	0.10
At1g24540	0.67±0.10	0.05	0.67±0.08	0.05	1.01±0.13	0.94	0.90±0.09	0.17	0.67±0.11	0.03	0.76±0.17	0.15
At1g64930	0.38±0.04	0.00	0.36±0.02	0.00	0.94±0.06	0.21	1.00±0.09	0.94	0.80±0.19	0.19	0.81±0.23	0.27
At1g64940	0.52±0.10	0.05	0.69±0.14	0.03	1.38±0.49	0.35	0.97±0.20	0.77	0.90±0.09	0.21	0.96±0.23	0.67
At3g03470	0.64±0.05	0.01	0.57±0.03	0.00	0.89±0.05	0.06	1.08±0.10	0.35	0.96±0.35	0.76	0.88±0.27	0.50
At4g12320	1.57±0.20	0.03	1.25±0.11	0.07	0.81±0.16	0.21	1.19±0.26	0.33	1.11±0.08	0.14	0.96±0.19	0.67
At4g12330	1.67±0.17	0.01	1.33±0.33	0.23	0.79±0.17	0.18	1.31±0.13	0.06	1.30±0.23	0.15	1.00±0.26	0.92
At2g29420	0.24±0.09	0.08	0.28±0.16	0.10	1.12±0.27	0.58	0.49±0.09	0.03	0.34±0.09	0.02	0.69±0.11	0.06
At3g09270	0.37±0.12	0.07	0.33±0.06	0.04	0.92±0.16	0.44	0.62±0.06	0.01	0.58±0.03	0.00	0.94±0.12	0.50
At1g78380	0.22±0.06	0.04	0.25±0.07	0.04	1.15±0.07	0.06	0.31±0.08	0.02	0.30±0.06	0.00	1.02±0.44	0.96
At1g17170	0.37±0.07	0.03	0.37±0.08	0.04	0.99±0.06	0.81	0.76±0.07	0.04	0.77±0.12	0.09	1.01±0.05	0.69
At4g02520	1.67±0.17	0.01	1.45±0.38	0.19	0.86±0.15	0.26	1.54±0.56	0.27	1.32±0.07	0.07	0.96±0.45	0.74
At2g02930	1.57±0.23	0.01	1.32±0.26	0.12	0.83±0.08	0.06	1.08±0.23	0.64	1.03±0.11	0.72	0.97±0.11	0.62
At2g30870	2.04±0.12	0.01	2.14±0.24	0.01	1.05±0.10	0.55	2.46±0.54	0.01	2.32±0.25	0.02	0.97±0.23	0.80
At1g01620	1.86±0.30	0.03	1.27±0.44	0.46	0.68±0.20	0.13	1.12±0.17	0.33	0.97±0.13	0.70	0.87±0.07	0.08
At4g23400	2.19±0.66	0.04	1.41±0.63	0.44	0.63±0.10	0.02	1.11±0.22	0.51	1.02±0.22	0.99	0.92±0.17	0.50
At2g36830	1.56±0.19	0.02	1.54±0.36	0.09	0.99±0.19	0.82	1.10±0.16	0.39	1.21±0.08	0.03	1.11±0.09	0.16
At3g26520	1.83±0.61	0.04	1.59±0.65	0.19	0.86±0.16	0.27	1.12±0.46	0.85	1.13±0.21	0.39	1.16±0.63	0.81
At3g16240	1.81±0.13	0.01	1.56±0.19	0.01	0.86±0.05	0.07	1.09±0.62	0.96	1.48±0.02	0.06	1.89±1.44	0.43
At1g80760	1.89±0.41	0.01	1.32±0.46	0.34	0.69±0.09	0.05	1.22±0.73	0.78	1.52±0.27	0.11	1.56±0.95	0.49
At3g56400	1.90±0.87	0.19	3.39±1.48	0.09	1.87±0.71	0.16	1.58±0.17	0.04	1.42±0.61	0.40	0.90±0.41	0.62
At5g66210	0.67±0.10	0.02	0.71±0.24	0.18	1.11±0.56	0.97	1.11±0.16	0.37	1.22±0.27	0.27	1.09±0.17	0.42
At2g43600	2.11±0.19	0.01	2.13±0.31	0.02	1.00±0.06	0.96	1.62±0.45	0.12	1.99±0.38	0.04	1.27±0.30	0.25
At2g19990	1.12±0.12	0.19	1.01±0.18	0.92	0.90±0.10	0.25	2.21±1.02	0.09	1.66±0.35	0.01	0.82±0.22	0.28

At1g75830	26.00±11.90	0.06	27.64±16.58	0.09	5.10±1.82	0.00	1.13±0.08	0.09	0.96±0.14	0.69	0.85±0.11	0.13
At5g44420	14.66±3.12	0.03	34.37±20.05	0.09	4.36±0.36	0.09	1.66±0.28	0.03	1.30±0.29	0.23	0.78±0.12	0.08
At2g02120	1.47±0.09	0.02	1.85±0.09	0.00	1.26±0.11	0.03	0.91±0.01	0.01	1.17±0.15	0.20	1.28±0.15	0.09
At2g43620	1.62±0.21	0.02	1.45±0.11	0.02	0.90±0.08	0.18	1.17±0.08	0.06	1.38±0.21	0.08	1.18±0.09	0.09
At3g44310	0.49±0.07	0.01	0.46±0.02	0.00	0.96±0.19	0.66	0.69±0.05	0.02	0.48±0.00	0.01	0.70±0.05	0.01
At3g44310	0.45±0.03	0.00	0.42±0.06	0.01	0.94±0.18	0.55	0.81±0.19	0.21	0.61±0.15	0.09	0.75±0.12	0.08
At3g44300	0.41±0.04	0.02	0.39±0.04	0.02	0.94±0.02	0.05	0.81±0.15	0.16	0.71±0.04	0.01	0.90±0.17	0.42
At3g44300	0.37±0.01	0.01	0.41±0.03	0.02	1.11±0.09	0.18	0.71±0.07	0.05	0.59±0.14	0.07	0.83±0.11	0.13
At3g44320	0.64±0.04	0.01	0.71±0.07	0.01	1.11±0.16	0.33	1.04±0.27	1.00	1.02±0.30	0.90	0.97±0.05	0.51
At4g35090	0.57±0.10	0.06	0.33±0.10	0.05	0.57±0.08	0.04	0.61±0.08	0.01	0.45±0.07	0.02	0.75±0.18	0.15
At1g20620	4.00±1.21	0.01	2.05±1.39	0.27	0.49±0.21	0.07	1.77±0.90	0.25	1.86±0.40	0.03	1.16±0.33	0.65
At1g08830	1.31±0.35	0.22	1.16±0.14	0.15	0.90±0.12	0.30	2.74±0.78	0.06	2.37±0.87	0.10	0.95±0.55	0.64
At2g28190	1.14±0.10	0.15	1.06±0.17	0.65	0.94±0.21	0.61	1.50±0.25	0.04	1.12±0.45	0.77	0.74±0.25	0.23
At4g25100	2.46±0.51	0.01	1.83±0.47	0.04	0.74±0.05	0.03	0.98±0.19	0.80	1.17±0.44	0.57	1.17±0.20	0.29
At2g15050	2.17±0.79	0.10	1.82±1.04	0.28	0.85±0.38	0.52	1.08±0.20	0.56	1.17±0.26	0.35	1.08±0.08	0.24
At3g50210	1.52±0.16	0.02	1.57±0.17	0.02	1.03±0.02	0.14	1.24±0.09	0.04	1.13±0.05	0.05	0.92±0.07	0.18
At3g48990	0.65±0.09	0.04	0.52±0.07	0.02	0.81±0.14	0.15	0.67±0.15	0.08	0.71±0.09	0.04	1.10±0.27	0.64
At5g05270	0.62±0.01	0.01	0.69±0.09	0.04	1.10±0.12	0.27	0.78±0.06	0.02	0.73±0.11	0.05	0.95±0.22	0.67
At2g34660	0.62±0.02	0.01	0.61±0.06	0.02	0.98±0.06	0.60	1.05±0.06	0.28	0.87±0.05	0.04	0.83±0.06	0.04
At1g66340	1.67±0.17	0.03	2.29±0.58	0.05	1.40±0.47	0.28	1.36±0.30	0.15	3.32±0.99	0.03	2.47±0.61	0.04
At3g04720	2.14±0.31	0.04	2.42±0.26	0.00	1.15±0.27	0.49	1.40±0.17	0.08	1.72±0.36	0.07	1.24±0.31	0.28
At3g21250	0.66±0.04	0.00	0.68±0.22	0.12	1.02±0.27	0.85	0.85±0.13	0.17	0.70±0.23	0.16	0.83±0.25	0.36
At5g61380	1.53±0.15	0.01	2.40±0.16	0.01	1.58±0.22	0.05	1.04±0.26	0.84	1.74±0.20	0.02	1.77±0.59	0.08
At3g53480	1.32±0.24	0.14	1.36±0.23	0.11	1.04±0.10	0.56	1.50±0.13	0.02	1.52±0.19	0.04	1.02±0.21	0.92
At4g25490	1.48±0.20	0.02	1.15±0.48	0.74	0.77±0.25	0.25	1.17±0.12	0.10	0.93±0.23	0.63	0.79±0.15	0.13
At5g39340	1.95±0.53	0.08	2.40±0.70	0.10	1.34±0.62	0.56	1.02±0.04	0.51	1.16±0.13	0.16	1.14±0.16	0.24
At2g27150	0.41±0.07	0.02	0.45±0.07	0.03	1.11±0.20	0.49	0.70±0.15	0.11	0.59±0.18	0.10	0.83±0.11	0.13
At2g18790	2.05±0.35	0.07	4.00±1.20	0.01	1.62±0.99	0.49	0.95±0.15	0.58	1.02±0.16	0.91	1.10±0.28	0.69