Impact of the plant growth-promoting rhizobacterium *Raoultella terrigena* TFi08N on plant growth and root architecture

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Summary

The input of mineral fertilizers in agriculture increased drastically in the last 50 years. The use of mineral fertilizers, in particular nitrogen fertilizers requires a high input of energy and can cause nitrogen leakage out of agro-ecosystems. An opportunity to solve this problem is the use of biofertilizers, which contain plant growth promoting rhizobacteria (PGPR). PGPR are bacteria, which occur in the rhizosphere of plants and stimulate plant growth. The use of associative rhizobacteria is a possibility to make plants profiting from growth promotion and to substitute mineral fertilizers. However, this requires a predictable growth stimulation and in-vitro test system allowing to investigate the mechanisms underlying plant growth promotion.

It was therefore the aim of this thesis to establish an in-vitro plant culture system that allows investigating newly isolated rhizobacteria for their plant growth promotion effects and characterizing the mechanisms responsible for growth stimulation. Besides several rhizobacteria, isolated in frame of the EU-Rhibac project, *Raoultella terrigena* TFi08N, a soil bacterium isolated in the frame of the former EU-Micro-N-Fix Project (2001-2006) is until now not characterized as a PGPR.

To exclude an influence of other microorganisms and to study changes in root morphology, pre-germinated *Arabidopsis thaliana* plants were placed on vertically-oriented agar plates to which PGPR were added just before agar solidification. Growth promotion effects were then analysed after another two weeks.

In the first part of this thesis, different bacterial strains were investigated for their ability to promote plant growth. A considerable improvement of plant growth, in terms of enhanced dry matter production was found for *Raoultella terrigena* TFi08N, *Azospirillum brasilense* SP245, *Bacillus megaterium* M3 and *Bacillus subtilis* OSU142 but not for *Pseudomonas fluorescens* C139. As inoculation with *Raoultella terrigena* resulted in strong plant stimulation and as it represents a so far uncharacterized PGPR strain, it was decided to study the mechanisms responsible for plant growth promotion conferred by this bacterium in more detail.

Raoultella was found to stimulate Arabidopsis growth best when cells were harvested at an OD of approximately 1.0-2.5 and inoculated at a density of 10^7-10^8 cfu mL^{-1}. Furthermore, growth promotion depended on medium pH and the supplied N form. While non-inoculated Arabidopsis plants developed quite well on nitrate but not on ammonium as a sole N source, inoculation with Raoultella reversed the ammonium-dependent growth repression in particular when plants were grown on unbuffered medium or at low medium pH. A strong

stimulation of lateral and primary root length, shoot dry weight and shoot N concentration was not only observed on ammonium but also when plants were grown on urea, where a physiological acidification of the rhizosphere is absent. With the exception of glutamate, *Raoultella* stimulated Arabidopsis growth also when grown on histidine, arginine or glutamine.

In the second part of the thesis mechanisms were investigated by which *Raoultella* may stimulate plant growth. Evidence for compensatory pH changes in the rhizosphere, a nitrification of supplied ammonium or the release of growth-promoting volatile substances by *Raoultella* were not found. Regarding the genetic constitution of Arabidopsis, it was observed that the four investigated accession lines responded differently to *Raoultella* inoculation and that expression of the major root plasma membrane H^+-ATPase, AHA2, is required to confer plant growth stimulation.

Analysis of auxin and cytokinin reporter lines suggested that an altered phytohormone homeostasis may contribute to Raoultella-mediated growth stimulation. Further considering that Raoultella is able to produce auxin, its growth stimulatory effect may rely on an auxin-induced stimulation of lateral root growth and auxin-stimulated proton extrusion at the root plasma membrane that improves nutrient uptake and plant growth. This conclusion is supported by the fact that Raoultella improved Arabidopsis growth also under P deficiency.

Although this thesis could not yet fully elucidate the mechanism of plant growth stimulation by *Raoultella terrigena* TFi08N, it contributes to a better understanding of the possible modes of action of PGPR by defining growth conditions and plant factors required for growth stimulation by *Raoultella terrigena* TFi08N.

Zusammenfassung

Der Einsatz von mineralischen Düngemitteln in der landwirtschaftlichen Pflanzenproduktion ist sehr energieaufwendig und verursacht Stickstoffverluste aus Agrarökosysteme. Eine Möglichkeit dieses Problem zu mindern, ist die Verwendung von Biofertilizern, die pflanzenwachstumsfördernde Rhizosphärenbakterien (PGPR) enthalten, zu einer verbesserten Nährstoffaufnahme führen können und damit beitragen, mineralische Düngemittel einzusparen. Dies erfordert jedoch eine verlässliche Methode Wachstumsstimulationen in-vitro zu untersuchen, um die Mechanismen, die hinter der Pflanzenwachstumsförderung stehen, aufzuklären.

Das Ziel dieser Arbeit war es, ein in-vitro Pflanzenkultivierungssystem zu etablieren, welches erlaubt neu isolierte Rhizobakterien auf ihre Pflanzenwachstumsförderung zu untersuchen und Mechanismen zu charakterisieren, die für die Pflanzenwachstumsförderung verantwortlich sind. Neben zahlreichen Rhizobakterien, die im Rahmen des EU-RHIBAC Projektes isoliert worden sind ist *Raoultella terrigena* TFi08N ein Bodenbakterium welches im Rahmen des EU-Micro-N-Fix Projektes isoliert worden ist und bisher nicht als PGPR charakterisiert wurde.

Um einen Einfluss anderer Mikroorganismen auszuschließen und um das Wurzelwachstum zu untersuchen, wurden vorgekeimte *Arabidopsis thaliana* Pflanzen auf vertikal-ausgerichtete Agarplatten transferiert, zu denen kurz vor dem Festwerden des Agars PGPR inokuliert wurden. In der Regel wurde 2 Wochen später Spross- und Wurzelwachstum analysiert.

Im ersten Teil dieser Arbeit wurden verschiedene Bakterienstämme hinsichtlich ihrer Pflanzenwachstumsförderung untersucht. Eine deutlich erhöhte Trockenmasseproduktion von Spross und Wurzel wurde für Pflanzen gefunden, die mit *Raoultella terrigena* TFi08N, *Azospirillum brasilense* SP245, *Bacillus megaterium* M3 oder *Bacillus subtilis* OSU142 inokuliert worden sind. Dies war nicht der Fall bei Inokulation mit *Pseudomonas fluorescens* C139. Da die Inokulation mit *Raoultella terrigena* TFi08N eine starke Pflanzenwachstumsförderung aufwies und da dieser Stamm ein bisher wenig charakterisiert ist, wurden an ihm Mechanismen untersucht, die für die Pflanzenwachstumsförderung verantwortlich sein könnten.

Es wurde festgestellt, dass das Wachstum von *Arabidopsis thaliana* am stärksten gefördert wurde, wenn *Raoultella terrigena* TFi08N aus einer Kultur mit einer optischen Dichte (OD) zwischen 1.0 – 2.5 und einer Konzentration von 10^7-10^8 cfu mL^{-1} inokuliert wurde. Des Weiteren zeigte sich eine Abhängigkeit der Pflanzenwachstumsförderung vom pH-Wert des Mediums und der verwendeten N-Form. Während sich Arabidopsispflanzen auf Nitrat-

haltigem Nährmedium als einzige Stickstoffquelle gut entwickelten, nicht aber auf Ammonium-haltigem Nährmedium, konnte in Ammonium-ernährten Pflanzen die Wachstumsrepression durch eine Inokulation mit *Raoultella* revertiert werden, wenn Pflanzen auf ungepuffertem Nährmedium oder Nährmedium mit niedrigem pH-Wert kultiviert wurden. Eine ähnlich starke Stimulation der Lateral- und Primärwurzellänge, höheres Trockengewicht und höhere Stickstoffkonzentration des Sprosses wurden nicht nur bei Pflanzen beobachtet worden, die auf Ammonium kultiviert wurden, sondern auch auf Harnstoff, auf dem eine physiologische Versauerung der Rhizosphäre nicht auftritt. Eine Förderung des Pflanzenwachstums konnte beobachtet werden, wenn die Pflanzen auf Histidin, Arginin oder Glutamin, nicht aber auf Glutamat kultiviert wurden.

Im zweiten Teil dieser Arbeit wurden Mechanismen untersucht die für die Stimulation des Pflanzenwachstums verantwortlich sein können. Hinweise auf eine kompensierende Änderung des Rhizosphären pH-Wertes, eine Nitrifikation von angebotenem Ammonium oder eine Abgabe von wachstumsfördernden volatilen Substanzen durch *Raoultella* wurden nicht gefunden. Im Hinblick auf die genetische Konstitution von Arabidopsis wurde beobachtet, dass vier untersuchte Akzessionslinien unterschiedlich auf die Inokulation mit *Raoultella* reagierten und dass die Expression der Plasmamembran H^+-ATPase AHA2 in der Wurzel für die Wachstumsstimulation benötigt wird.

Analysen von Auxin- und Cytokinin-Reporterlinien zeigten, dass Inokulation mit *Raoultella* zu einer veränderten Phytohormon-Homöostase führt. Da *Raoultella* in der Lage ist Auxin zu produzieren, könnte die Stimulation des Pflanzenwachstums auf ein Auxin-induziertes vergrössertes Lateralwurzelwachstums und eine Auxin-stimulierte Protonenabgabe an der Wurzelplasmamembran zurückzuführen gewesen sein, welche die Nährstoffaufnahme und das Wurzellängenwachstum verbessern. Dies wird durch die Beobachtung unterstützt, dass *Raoultella* auch das Pflanzenwachstum unter P-Mangel verbesserte.

Obwohl in dieser Arbeit der Mechanismus, der für die Förderung des Pflanzenwachstums verantwortlich ist, noch nicht vollständig geklärt werden konnte, trägt diese Arbeit dazu bei, Mechanismen, die bei förderlichen Assoziationen zwischen Pflanzen und Bakterien wirken können, besser zu verstehen.

2 Introduction

Over the past 50 years the input of mineral fertilizers increased drastically and agriculture was intensified due to developments in mineral fertilization and increasing world population. Mineral fertilizers, in particular nitrogen fertilizers contribute to a number of environmental problems, such as nitrogen volatilization, leaching and eutrophication. Improved crop varieties and production systems with enhanced nutrient use efficiency are needed to reduce the input of mineral fertilizers (FAO, 2006). An opportunity to contribute to this is the use of biofertilizers. Biofertilizers are substances, which contains living microorganisms, which colonize the plant and promote plant growth (Vessey, 2002).

In terrestrial ecosystems a large variety of microorganisms live in the soil, which may inhibit, suppress or stimulate plant growth or result in no influence. In contrast to a lot of bacteria, which cause negative effects, there are bacteria, which are beneficial and promote plant growth. Such bacteria have been termed as "Plant Growth Promoting Rhizobacteria" (PGPR) (Kloepper et al., 1989). Beneficial bacteria can be further distinguished into symbiotic, associative or free-living bacteria. The best known example for symbiotic bacteria are Rhizobia, which are known to fix atmospheric nitrogen when being in symbiosis with legumes. These bacteria live in symbiosis with legumes and form nodules on plant roots. They release ammonium or N-metabolites and obtain metabolites from the host plant. In contrast to symbiotic, associative bacteria do not undergo morphological changes during their association with plant roots. Their energy source is root exudates from the host plant. They colonize the root surface or live in intercellular and cellular spaces of the plant tissue but without being pathogenic for the plant (Marschner, 1995).

Beijerinck discovered in 1925 that the bacterial strain *Spririllum lipoferum*, as it has a high lipid content, is able to increase the nitrogen content in culture (Beijerinck, 1925). Nearly 50 years later, Johanna Döbereiner re-described *Spirillum lipoferum as Azospirillum lipoferum* (Döbereiner and Day, 1976), which motivated scientists to investigate growth promotion in plant-bacterial associations. Several bacterial genera have now been reported to be associated with plant, like *Azotobacter*, *Azospirillum*, *Bacillus*, *Paenibacillus*, *Azoarcus*, *Herbaspirillum*, *Pseudomonas*, *Burkolderia*, *Klebsiella*, *Enterobacter*, *Beijerinckia* (Holl et al., 1988, Mrkovacki and Milic, 2001, Boddey et al., 1986, Omar et al., 1996, Hurek et al., 2002, James et al., 2002, Baldani et

al., 2000). As it became evident that nitrogen fixation is not solely responsible for plant growth stimulation by associative bacteria, different mechanisms were assessed to explain the growth promotion effect exerted by such bacteria.

2.1 Mechanism of plant growth promotion

At the beginnings of the discovery of PGPR N_2 fixation was in the fore. Soon it was discovered, that this it not the only mechanism for growth promotion by bacteria. The production of phytohormones and enzymes, an enhanced uptake of mineral nutrients and a role of associative and free-living bacteria species in biotic and abiotic stress control are further mechanisms contributing to plant growth promotion.

2.1.1 Direct mechanisms

2.1.1.1 Biological Nitrogen Fixation (BNF)

The variability in the absolute amounts of fixed N by bacteria in differential systems is determined by the bacterial species, the plant genotype and environmental conditions. The largest amount of N is fixed in symbiotic systems, which is in the range of 50-400 kg N ha $^{-1}$ y^{-1} for legumes and between 20-300 kg N ha^{-1} y^{-1} for non-legumes, while this amount is lower in non-symbiotic associations with 10-200 kg N ha^{-1} y^{-1}. Free-living bacteria have been estimated to fix approximately 1-2 kg N ha^{-1} y^{-1} if they are heterotroph, but 10-80 kg N ha^{-1} y^{-1} if they are autotroph (Marschner, 1995).

Biological N_2 fixation is sensitive to oxygen, as the key enzyme nitrogenase is extremely sensitive to oxygen. Nitrogenase, the key enzyme complex consists of two nonheme iron proteins. The smaller Fe-protein consists of two subunits and a single Fe_4S_4 cluster, while the larger MoFe-protein consists of four subunits and contains 30 Fe and 2 Mo atoms. In some diazotrophic bacteria vanadium replaces molybdenum. Energy is supplied in form of ATP (Yates, 1976).

Different methods are used to examine nitrogen fixation. In the acetylene reduction assay (ARA) acetylene is reduced to ethylene by the enzyme nitrogenase. Ethylene can be measured by gas chromatography. The advantage of this system is the high sensitivity of the detection of ethylene (acetylene is reduced to ethylene), the disadvantage of this method is, that it cannot be determined, if fixed nitrogen is incorporated into the plant (Boddey and Döbereiner, 1994, Okon, 1985). This disadvantage can be circumvented by the ^{15}N isotope dilution and ^{15}N abundance techniques (James, 2000). For the ^{15}N isotope dilution technique it is necessary to grow

plants in a defined ^{15}N-labelled soil. Plant material is measured for its ^{15}N content by isotope-ratio mass spectrometry. Plants obtaining unlabelled nitrogen from the air have a lower ^{15}N enrichment than soil-derived nitrogen. The disadvantage of this technique is its use in field experiments, because soil is difficult to label in an uniform way (Boddey and Döbereiner, 1994).

In the last decades several agricultural crops and their associated bacterial strains have been investigated for plant growth promotion. For several bacteria evidence exist, that the growth promotion of inoculated plants is related to their ability to fix nitrogen. This was proven for *Azospirillum*, inoculated to maize, rice or wheat (Cohen et al., 1980, Garcia de Salamone et al., 1996, Malik et al., 1997, Boddey et al., 1986). The same held true for *Azoarcus* sp. (Hurek et al., 2002), *Azotobacter* sp. (Mrkovacki and Milic, 2001), *Bacillus polymyxa* (Omar et al., 1996), *Burkholderia* sp. (Baldani et al., 2000), *Gluconacetobacter diazotrophicus* (Boddey et al., 2001) and *Herbaspirillum* sp. when inoculated to grass, wheat, rice or sugar cane (James et al., 2002). However, the quantitative determination of nitrogen fixation yielded varying results. Often biological N_2 fixation was demonstrated in pure culture under optimal conditions with regard to pH, temperature, O_2 concentration and energy source (Elmerich, 2007, Okon et al., 1976). In sugar cane a significant contribution of nitrogen fixation of up to 80 % N was reported, but in dependence of sugar cane varieties and environmental conditions (Lima et al., 1987, Boddey et al., 1991). In a more recent study and by use of the ^{15}N natural abundance technique Boddey et al. suggested for sugar cane that up to 60 % of N was conferred by biological nitrogen fixation (Boddey et al., 2001). BNF contributions up to 27 % were determined for *Azospirillum amazonense* Y2 inoculated to rice (Rodrigues et al., 2008).

A further method to study nitrogen fixation by bacteria is the use of molecular biological techniques. The use of mutants, which cannot fix nitrogen, *nif*$^-$ mutants, can provide evidence, if growth promotion is due to nitrogen fixation or not. The observation that growth stimulation by inoculation with a *nif*$^-$ strain still occurred, indicated that BNF is not the only and sometimes maybe even not the dominant mechanism stimulating plant growth in plant-bacterial associations (Barbieri et al., 1986).

2.1.1.2 Production of phytohormones

Bacteria-mediated changes in root growth and morphology led to the hypothesis that phytohormones released by bacteria play a role in the growth stimulation observed in plant-bacteria associations. Altered root morphologies in inoculated plants may be brought about by the release of phytohormones like auxins, cytokinins or gibberellins (Tien et al., 1979, Osmont et al., 2007).

Phytohormones are small signalling molecules that are involved in cell division, cell elongation, pattern formation, gravitropism, flowering, fruit and seed development and response to biotic and abiotic stresses. Auxins, cytokinins, gibberellins, ethylene and abscisic acid are the most important ones. Phytohormones act in very low concentrations and their production, transport and perception is precisely regulated. In dependence of their concentration in the plant tissue phytohormones can exert promotional or inhibitory effects.

A growth stimulation of inoculated plants by the influence of phytohormones was suggested for several bacterial strains. Analysis of bacteria cultures showed that several bacterial strains were able to produce and release phytohormones like IAA, cytokinins or gibberellins. *Azospirillum brasilense* (Fallik and Okon, 1989, Crozier et al., 1988), *Enterobacter* sp. (Mirza et al., 2001), *Paenibacillus polymyxa* (Lebuhn et al., 1997) have been shown to produce IAA. Cytokinin production has been determined for *Paenibacillus polymyxa* (Timmusk et al., 1999), *Pseudomonas fluorescens* (García de Salamone et al., 2001) and *A. chroococcum* (Arshad and Frankenberger, 1991), while gibberellin production has been reported for Bacillus sp. (Gutiérrez-Mañero et al., 2001) and ABA for *Azospirillum brasilense* Sp 245 (Cohen et al., 2008).

2.1.1.2.1 Auxin

It has been estimated that approximately 80 % of rhizosphere bacteria are able to produce IAA (Patten and Glick, 1996). For example, the Azospirillum *nif*-mutant, still promoted plant growth, suggesting, that the measured IAA production was responsible for the growth promotion effect (Barbieri et al., 1986).

Auxins are chemical messengers, which are produced in one cell or tissue. They modulate cellular processes in another cell by interacting with specific protein receptors. Auxins operate developmental processes like stem elongation, apical dominance, root initiation, fruit development, meristem development and oriented or tropic growth and are assumed to induce proton extrusion of plant cells by activation or

increased synthesis of H^+-ATPases. Auxin biosynthesis takes place in shoot apical meristems and young leaves; other important sites are the root apical meristems. Auxin influx is driven by the proton motive force across the plasma membrane either by passive diffusion of the protonated form (IAAH) across the plasmamembrane or by secondary active transport of the dissociated form (IAA^-) via 2 H^+ - IAA^- - symporters (Taiz and Zeiger, 2007). With regard to plant development, auxin is known to play an important role in root morphology. Particularly auxin is involved in lateral root initiation and lateral root primordium development (Reed et al., 1998, Woodward and Bartel, 2005, Aloni et al. 2006, Fukaki and Tasaka, 2009).

Bacteria are able to synthesize IAA via different biosynthetic pathways. In a single bacterial strain several pathways can be active at the same time (Patten and Glick, 1996, Steenhoudt and Vanderleyden, 2000). Tryptophan is the main precursor for the biosynthetic pathway of IAA. The indole-3-acetamide and the indole-3-pyruvate pathway are the predominant pathways. In addition, Prinsen et al. (1993) reported on a tryptophan-independent pathway in *Azospirillum brasilense*. The indole-3-acetamide pathway is used by pathogenic bacteria like *Agrobacterium tumefaciens*, *Erwinia herbicola* and *Pseudomonas syringae* pv. *savastanoi*, known to cause crown galls. But not only pathogenic bacteria use the indole-3-acetamide pathway. The nitrogen-fixing bacteria *Rhizobium* and *Bradyrhizobium* use this pathway, too, as well as *Azospirillum brasilense* (Patten and Glick, 1996, Spaepen et al., 2007, Steenhoudt and Vanderleyden, 2000). For many associative bacteria IAA production was demonstrated, like for *Azospirillum* spp. (Crozier et al., 1988, Fallik and Okon, 1989), *Azotobacter*, *Acetobacter diazitrophicus* (Gonzalez-Lopez, 1985, Bastián et al., 1998), *Herbaspirillum seropedicae* (Bastián et al., 1998), *Paenibacillus polymyxa* (Lebuhn et al., 1997), *Klebsiella pneumoniae*, *Bacillus amyloliquefaciens* FZB42 (Idris et al., 2007), *Gluconacetobacter diazotrophicus* (Lee et al., 2004), *Pseudomonas putida* (Patten and Glick, 2002) or *Pantoea agglomerans* (Sergeeva et al., 2007). To demonstrate plant growth promotion by IAA, mutants have been used. A Tn5-induced mutant of *Azospirillum brasilense* Sp6 with a very low production of IAA did not enhance root development any longer (Barbieri and Galli, 1993). On the other hand an *Azospirillum* IAA-overproducing mutant showed a stronger stimulation of root hair development compared to the wild type, but in dependence of the bacterial concentration (Harari et al., 1998). Bioassays with IAA could mimick the effect of bacteria on root morphology. Exogenously applied combinations of IAA, gibberellin

and kinetin led to an increase in number of lateral root and root hairs comparable to Azospirillum-inoculated roots (Tien et al., 1979). Further studies reported on an increase in number and length of lateral roots of wheat seedlings in response to *Azospirillum brasilense* Sp6 (Barbieri et al., 1986). Indolepyruvate decarboxylase (key enzyme in the indolepyruvic acid and the tryptophan-independent pathway) mediates IAA production in *Azospirillum brasilense* and is upregulated by auxin (Costacurta et al., 1994, Zimmer et al., 1997, Vande Broek et al., 1999). An *ipdC* knockout-mutant of *Azospirillum brasilense* produced only 10% of the wild type IAA level (Spaepen et al., 2007). Another *Azospirillum brasilense* strain SM showed a 50 % decrease in IAA level and stimulated root development much less as the wild type strain (Malhotra and Srivastava, 2007).

2.1.1.2.2 Cytokinin

Cytokinins are N6-substituted aminopurines, which are synthesized in roots and translocated to the shoots in the xylem and play a role in cell division, plant growth and development. Many microorganisms produce and secrete cytokinins or cause plant cells to synthesize them like *trans*-zeatin, [9R]iP, *cis*-zeatin and their ribosides. Cytokinins stimulate tissues to divide or to form special structures like they are known for mycorrhiza. Cytokinin and auxin are known to interact antagonistically in plant development (Swarup et al., 2002, Werner et al., 2003, Laplaze et al., 2007, Perilli et al., 2010). A few bacteria produce cytokinins and auxins, which influences the balance of both hormones in plants. Pathogenic bacteria like *Agrobacterium tumefaciens* stimulate plant cells to divide and cause crown gall. PGPR known to produce cytokinins are *Pseudomonas* sp. (Nieto and Frankenberger, 1989, García de Salamone et al., 2001), *Azotobacter chroococcum* (Nieto and Frankenberger, 1990), *Azotobacter* sp. (Barea and Brown, 1974, Nieto and Frankenberger, 1990), *Bacillus* sp. (Gutiérrez-Mañero et al., 2001, Arkhipova et al., 2004), *Paenibacillus polymyxa* (Timmusk et al., 1999) and *Bacillus megaterium* (Ortíz-Castro et al., 2007). In these cases, cytokinins were determined by imunoaffinity chromatography, high performance liquid chromatography-UV, gas chromatography-mass spectrometry or radioimmunoassay (RIA). A growth dependent production of isopentenyladenine in defined media was described for *Paenibacillus polymyxa* (Timmusk et al., 1999). Zeatin riboside was measured in bacterial cultures of *Bacillus subtilis*. Analysis of lettuce plants revealed a stimulation of shoot and root growth of approximately 30 % with increased cytokinin

contents in shoots and roots when inoculated with *Bacillus subtilis* (Arkhipova et al., 2005). Nieto and Frankenberger determined growth promotion of *Raphanus sativa* inoculated with *Azotobacter* c*hrooconum*. when the cytokinin precursors adenine and isopentyl alcohol were added, which suggested a microbial production of cytokinins (Nieto and Frankenberger, 1990). De Salamone et al. (2001) reported on a *Pseudomonas fluorescent* strain G20-18, which produced in pure culture the three cytokinins isopentenyl adenosine, trans-zeatin ribose and dihydrozeatin riboside in the stationary growth phase. Two selected mutants had a reduced capacity to synthesize cytokinins (de Salamone et al., 2001). Ortíz-Castro et al. reported about the use of Arabidopsis mutants lacking cytokinin receptors. When inoculated with *Bacillus megaterium* these mutant plants were no longer stimulated in growth. This revealed that cytokinin perception is important for the growth promotion of *Bacillus megaterium* inoculated plants (Ortíz-Castro et al., 2007).

2.1.1.2.3 Gibberellins

Gibberellins are tetracyclic diterpenoids. At least 136 naturally occuring GAs have been identified (MacMillan, 2002, www.plant-hormones.info/gibberellin_nomenclature.htm). GA_1 is the most active GA in plants (Davies, 1995). Gibberellins are involved in division and elongation of plant cells. Gibberellins regulate growth, show effects on stem elongation, promote flower, pollen, seed and tube development and germination (Taiz and Zeiger, 2007). The biosynthesis of gibberellin occurs in young parts of shoots and developing seeds and linked to the terpenoid pathway. In the 1950s, the first plant gibberellin was identified by Macmillan and Suter (1958) in *Phaseolous coccineus* seeds, and years later for bacteria (Atzorn, 1988), but to date there is no known role for gibberellins in bacteria (Bömke and Tudzynski, 2009). Several authors reported on the production of gibberellins by associative bacteria. Production of GA was demonstrated for *Azospirillum lipoferum* (Bottini et al., 1989), *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastián et al., 1998), *Azotobacter vinelandii* and *Azotobacter beijerinckii* (Azcón and Barea, 1975), *Azotobacter paspali* (Barea and Brown, 1974), *Bacillus pumilus* and *Bacillus licheniformis* (Gutiérrez-Mañero et al., 2001). Most of the analyses had been done on chemically-defined medium. Furthermore, associative bacteria modify GAs and GA precursors, for example *Azospirillum brasilense* and *Azospirillum lipoferum* hydrolyzed conjugates of GA_{20} and metabolized the resulting aglycones to GA_1 in rice mutants (Cassán et al., 2001). Bottini

et al. (1989) reported on the identification of GA_1, GA_3 and Iso-GA_3 in aseptic cultures of *Azospirillum lipoferum* (Bottini et al., 1989) and that the gibberellin status of corn seedling roots was affected by *Azospirillum lipoferum*. For *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* GA_1 and GA_3 were found in chemically-defined medium (Bastián et al., 1998). However, there is little evidence on the gibberellin content of inoculated plants. Using a bioassay Fulchieri et al. (1993) determined root parameters and GA concentrations in axenically grown plants inoculated with *Azospirillum* strains or cultured under supply of GA. All *Azospirillum* strains enhanced root growth, while plants grown on GA_3 experienced a similar improvement of growth (Fulchieri et al., 1993). This was explained by gibberellin production by *Azospirillum*, 3ß-hydroxylation of 3-deoxy GAs or deconjugation of gibberellin-glucosyl conjugates. Deconjugation of gibberellin-glucosyl conjugate was demonstrated by Piccoli et al. (1997). An enhanced root growth of maize seedlings, inoculated with *Azospirillum lipoferum* was suggested to be due to deconjugation and production of GA. As a further mechanism, bacteria are able to activate inactive 3-deoxy GAs by 3ß-hydroxylation to release GA_3, GA_1 and GA_4 (Piccoli and Bottini, 1994; Piccoli et al., 1996; Cassán et al., 2001). Evidence that GA_3 plays a role in bacterial inoculation was provided by the use of a dwarf gene of rice. Inoculation of this mutant with *Azospirillum* sp. promoted plant growth. The use of Prohexadione-Ca, an inhibitor of gibberellin-biosynthesis confirmed this result, as no reversal of dwarfism was observed and no GA_1 was produced. As gibberellin biosynthesis in bacteria is not well investigated so far no bacterial mutants in GA synthesis are available.

2.1.1.2.4 Ethylene

The phytohormone ethylene is known as the “ripening hormone”, as it is involved in fruit ripening. Furthermore, ethylene is involved in lateral cell expansion, the ability to break seed and bud dormancy, and in leaf senescence. Also the induction of root formation is modified by ethylene. In the ethylene biosynthetic pathway 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of ethylene. The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, hydrolyzes ACC and degrades the ethylene precursor ACC. Ethylene and ACC deaminase production was found in bacteria that colonize roots (Belimov et al., 2001, Perrig et al., 2007, Madhaiyan et al., 2006, Weingart and Völksch, 1997, Onofre-Lemus et al., 2009). Employing ACC deaminase, ACC is degraded to ammonia and alpha-ketobutyrate,

which reduces the levels of ethylene and liberates ammonia as a nitrogen source for bacteria. Thus, ACC-expressing PGPR can promote plant growth by lowering ethylene levels in plants (Glick, 2005). *Variovorax paradoxus* 5C-2, an ACC deaminase producing rhizosphere bacteria stimulated plant growth of plants under stress conditions. A transposon mutant strain with a lower ACC deaminase activity did not confer growth promotion (Belimov et al., 2008). Moreover, the expression of the ACC deaminase gene *acdS* from *Enterobacter cloacae*, in *Azospirilum brasilense* Cd, a strain which produces no ACC deaminase, led to an enhanced growth stimulation of roots from tomato and canola seedlings (Holguin and Glick, 2001).

Root morphology in relation to phytohormone production by bacteria

In many experiments it was observed that the root architecture of plants inoculated with PGPR was changed (Tien et al., 1979, Okon and Kapulnik, 1986, Hadas and Okon, 1987, Mantelin et al., 2006). An increased root surface can improve the uptake of mineral nutrients and thus indirectly lead to positive effects on plant development. Tien et al. reported on changes in root morphology of *Pearl millet* roots when inoculated with *Azospirillum brasilense*, which expressed in an increased number of lateral roots and a higher density of root hairs. The addition of indole acetic acid, gibberellins or kinetin provoked similar morphological changes in root growth as the inoculation with bacteria (Tien et al., 1979). A higher density of root hairs was observed in wheat plants inoculated with *Azospirillum brasilense* strains (Kapulnik et al., 1985, Spaepen et al., 2008). In this case, the bacterial concentration appeared to be important for changes in root morphology. Bacterial concentrations of 10^5-10^6 cfu mL^{-1} enhanced root development, whereas higher concentrations of 10^8-10^9 cfu mL^{-1} inhibited root development (Kapulnik et al., 1985). These morphological changes in inoculated roots have been attributed to the bacterial production and release of phytohormones. Dobbelaere et al. demonstrated in a bioassay a relation between IAA production and altered root morphology when inoculated with Azospirillum. Increasing bacterial concentrations led to decreased root length and enhanced root hair formation. The addition of IAA to plant roots mimicked this root phenotype (Dobbelaere et al., 1999). Patten and Glick isolated the ipdC gene from *Pseudomonas putida* GR12-2 and constructed an IAA-deficient mutant by insertional mutagenesis (Patten and Glick, 2002). They demonstrated that canola roots treated with the mutant had 35 to 50 %

shorter roots compared to inoculation with wild type Pseudomonas strain or to non-inoculated control plants.

The influence of *Phyllobacterium brassicacearum*on on the root architecture of *Arabidopsis* was not explained by released IAA, but by an altered endogenous IAA homeostasis in the plant via modulation of a key step in the auxin signal transduction pathway (Contesto et al., 2010).

Taken together, phytohormones and in particular auxin are produced by many PGPRs and have a strong influence on root morphology.

2.1.2 Indirect mechanisms

2.1.2.1 Increased uptake of mineral nutrients

It was proposed, that PGPR enhance the uptake of mineral nutrients, in particular of nitrogen, phosphorous, potassium or micronutrients like iron (Lin et al., 1983, Okon et al., 1986, Murty and Ladha, 1988, Biswas et al., 2000). Several authors reported on elongated root systems of inoculated plants, being responsible for enhanced mineral uptake. Lin et al. demonstrated that *Azospirillum* inoculation enhanced the uptake of nitrate, potassium and phosphate in corn roots and suggested that this is caused by an improved nutrient efficiency (Lin et al., 1983). Increased NH_4^+ and PO_4^- uptake has been determined for *Azospirillum lipoferum*-inoculated plants (Murty and Ladha, 1988). A combination of an enhanced number and length of root hairs and of a stimulation of NO_3^- uptake has been reported for *Brassica napus* when inoculated with *Achromobacter* (Bertrand et al., 1999).

On the other hand, doubts have been raised whether nutrient uptake is reliably stimulated in successful plant-bacterial associations. Barea et al. (1983) did not find an increased nitrogen content in *Azospirillum brasilense* inoculated plants and Bashan et al. who determined K, P, Ca, Mg, S, Na, Mn, Fe, B, Cu and Zn, could not find consistent patterns of enhanced mineral element contents in inoculated plants, so that growth promotion could not be explained by an enhanced of mineral element uptake (Bashan et al., 1989). Likewise, no clear prediction was made whether growth promotion effects were due to enhanced nitrogen uptake or to nitrogen fixation. Although C_2H_2 reduction activity was measured for plants inoculated with *Azospirillum brasilense*, no effect on nitrogen concentration in shoots was determined (Barea et al., 1983).

In particular under varied nitrogen supply, root development is different. As ammonium acts as a physiologically acid N form, rhizosphere pH drops and root development is inhibited (Britto and Kronzucker, 2002, Marschner, 1995). Plants grown under ammonium nutrition develop short lateral roots and stimulate higher-order lateral root branching (Lima et al., 2010). Plants grown on nitrate have longer lateral roots and less higher-order lateral roots. By buffering the growth medium ammonium toxicity can be overcome and plants are not inhibited in growth anymore (Britto and Kronzucker, 2002). So far, it is not clear how PGPR interfere with the physiological acidification or alkalization induced by ammonium or nitrate, respectively.

Solubilization of phosphates

Phosphorus is an essential element for plants, and phosphate solubilisation is a possible mechanism related to plant growth promotion by rhizobacteria (Gyaneshwar et al., 2002). Solubilisation of phosphates is important for plants, as P is mainly present in soils in insoluble forms. Phosphorus occurs in soils as organic and inorganic P. Inorganic phosphorus cannot be taken up by plants, when it is precipitated. Organic P from organic matter needs to be mobilized to become a P source for plants. Phosphorus is mainly taken up as $H_2PO_4^-$ at physiological pH. Conversion of insoluble phosphorus to an accessible form like orthoposphate for plants has been shown for rhizobacteria. Several bacterial species are known to solubilize phosphates in pure culture, such as *Bacillus*, *Pseudomonas*, *Rhodococcus, Arthrobacter*, *Serratia*, *Enterobacter* (Barea et al., 1976, Laheurte and Berthelin, 1988, Chen et al., 2006). However analyses of inoculated plants are variable with regard to an enhanced uptake of P. De Freitas et al. reported a significantly increase in plant height and pod yield of *Brassica napus* inoculated with Bacillus strains, but no increase in P uptake was found (de Freitas et al., 1997).

Bacteria strains such as *Pseudomonas*, *Bacillus* and *Rhizobium* (Paul and Sundara Rao, 1971, Rodríguez, and Fraga, 1999) are known to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite or rock phosphate. Phosphorus from organic compounds must be hydrolyzed to inorganic P to become available for plants. The following enzymes can hydrolyze organic P: nonspecific phospatases, phytases and phosphonatases and C-P lyases. The main roles play acid phosphatase and phytase (Rodríguez et al., 2006). Associative bacteria are able to release acid phosphatase like *Enterobacter*, *Bacillus*

(Skrary et al., 1998) or *Pseudomonas* (Gügi et al., 1991). *Klebsiella*, *Pseudomonas* and *Bacillus* are known to release phytase (Greiner et al., 1997, Gügi et al., 1991, Idriss et al., 2002).

A contribution to growth promotion under P limitation due to an enhanced phytase activity after inoculation with *Bacillus amyloliquefaciens* FZB45 has been reported by Idriss et al. (2002). Although it has been often reported that bacteria have the ability to solubilize P in in-vitro cultures no growth promotion or no enhanced P uptake was found when these bacteria were inoculated to plants (de Freitas et al., 1997, Taurin et al., 2010). Until now there is no clear evidence for phosphorus solubilization as mechanism for plant growth promotion, most bacteria investigated so far provoked phytohormonal effects at the same time (Barea et al., 1976).

2.1.2.2 Proton efflux

Acidification of the rhizosphere by proton efflux via the plasmamembrane of root cells is a method by which plants enhance the availability of nutrients. Proton extrusion has been found in several plant species to mobilize in particular phosphorous or iron (Marschner, 1995). Enhanced proton extrusion from roots has been reported for plants inoculated with *Azospirillum*. Wheat seedlings inoculated with *Azospirillum brasilense* Cd significantly increased proton efflux (Bashan et al., 1989, Bashan, 1990). A stronger acidification of the rhizosphere and plant growth promotion has been reported for cardon seedlings, when inoculated with *Azospirillum brasilense* Cd on ammonium. The authors explained the observed rhizosphere acidification by a bacterial production of auxins, which may stimulate the plasmamembrane ATPase and lead to the release of protons (Carrillo et al., 2002).

2.1.2.3 Abiotic stress control

Abiotic stress conditions, like drought stress, salt stress or temperature are a limitation for plant growth and crop yield. Plants confronted with abiotic stress conditions have been observed to be less stressed when inoculated with PGPR. Bacterial strains like *Azospirillum* or *Achromobacter piechaudii* enhanced drought stress tolerance of plants (Creus et al., 2004, Mayak et al., 2004). While inoculation with *Azospirillum brasilense*, *Pseudomonas* sp. or *Achromobacter piechaudii* improved salt stress tolerance (Nabti et al., 2007, Nadeem et al., 2007, Mayak et al., 2004). By inoculation with *Burkolderia*

phytofirmans or *Serratia liquefaciens* a better tolerance of plants to temperature stress has been reported (Barka et al., 2006, Zhang et al., 1997).

An improved utilization of soil moisture was observed for dryland grain sorghum inoculated with *Azospirillum brasilense* (Sarig et al., 1988). Field experiments have revealed a 15-18 % increase in grain yield. Higher leaf water potential, lower canopy temperatures and a greater stomatal conductance were found. Determination of total extraction of soil moisture from deeper soil layers resulted in approximately 15 % better moisture of inoculated treatments. Inoculation led to an early plant growth and improved utilization of soil moisture due to a better root system of inoculated plants. The observation of a phenotypically enhanced drought tolerance has been confirmed by gene expression data. Increased expression of *ERD15*, a gene identified to be drought stress responsive has been determined for Arabidopsis plants treated with *Paenibacillus polymyxa* (Timmusk and Wagner, 1999).

2.1.2.4 Biotic stress control

Besides promoting plant growth, PGPR can control biotic stress conditions. Different mechanisms of biocontrol mediated by PGPR are known. The competition for an ecological niche and nutrients, the production of allelochemicals and induced systemic resistance (ISR) are mechanisms for biocontrol of biotic stress. Biocontrol mechanisms have mainly been described for *Pseudomonas* and *Bacillus* species.

Aims of the study

The aim of this study was to investigate the influence of rhizobacteria on plant growth and root architecture. To study root architecture at high reproducibility, a gnotobiotic growth system in agar was established, that allowed inoculating plant growth-promoting bacteria (PGPR) and investigating mechanisms leading to plant promotion effect. Out of a collection of PGPR selected in frame of the EU-project RHIBAC, which provided the background of this thesis, the following strains were investigated: *Raoultella terrigena* TFi08N, *Azospirillum brasilense* SP245, *Bacillus megaterium* M3 and *Bacillus subtilis* OSU142 and *Pseudomonas fluorescens* C139.

In the first chapter an agar-based growth system in petri-dishes was established to cultivate *Arabidopsis thaliana* under gnotobiotic conditions and to determine morphological changes of the roots after scanning of the root system and quantitative analysis of root traits by the WinRhizo software. Since inoculation with *Raoultella terrigena* TFi08N provoked a considerable increase of several root parameters, further bacterial strains were tested, including *Azospirillum brasilense* Sp245, *Bacillus megaterium* M3 and *Bacillus subtilis* OSU142 and *Pseudomonas fluorescens* C139, in addition, the action of the two strains *Raoultella terrigena* TFi08N and *Azospirillum brasilense* Sp245 were investigated in more detail by determining the influence of their growth stage and of their inoculation density on plant growth.

The second part of this thesis investigated the dependence of the nitrogen form and the pH of the medium on the growth promotion by bacteria. As *Raoultella terrigena* TFi08N is so far an uncharacterized PGPR strain, it was decided to study this strain in more detail.

The third chapter of the thesis focussed on the possible mechanisms conferring root growth stimulation by *Raoultella terrigena* TFi08N. Particular emphasis was laid on the hypotheses whether this PGPR might change the rhizosphere pH, accelerate nitrification of supplied ammonium-N or the release of growth-promoting volatile substances.

Furthermore, Arabidopsis mutants defective in root-expressed H^+-pumps or amino acid transporters were investigated for their responsiveness to *Raoultella* inoculation. These studies were accompanied by a preliminary study of the influence of *Raoultella* on phytohormonal changes in the root system employing auxin- or cytokinin-responsive reporter lines.

Even though these approaches could not yet uncover the precise mechanism of plant growth stimulation by Raoultella they provide a detailed picture of individual components in this association and their possible contribution.

3 Material and Methods

3.1 Plant material and growth conditions

The following Arabidopsis lines were used: Arabidopsis thaliana Col-0, WS (Wassilewskija), Aa (Aa-0, Aua-Rhön), Col-glabra (Columbia-glabra), *aha2*, *vha1vha2* and *lht1*.

Arabidopsis thaliana Col-0 seeds were surface sterilized with sodium hypochlorite-ethanol solution. 1 mL solution was added to 100 µL seeds and shaken for 13 minutes at 1400 rpm at room temperature. The supernatant was aspired and seeds were rinsed two times with 100 % ethanol and dried over night. Seeds were germinated on petri dishes containing half-strength Murashige and Skoog (MS, Table 1) medium (Duchefa, Harleem, NL), 1 % sucrose and 0.7 % Difco agar (Becton Dickinson) under short day conditions with a day/ night regime of 10 h/ 14 h, a temperature of 22°C/ 19°C, 45 % humidity and a light intensity of 120 µmol photons m^{-2} s^{-1} in a growth chamber (Percival Scientific, Inc.).

After 6 days seedlings with comparable development were transferred on square plates and cultured vertically for 18 days. Cultivation on square plates was continued on self-made half-strength MS except of nitrogen, which was added according to the experimental setup. To buffer the pH of the growth medium sterile filtered MES was used.

Nitrogen was added in form of L-glutamine, L-arginine, L-glutamic acid or L-histidine with a concentration of 1 mM nitrogen to square plates. Aminoacid solutions were sterile filtrated added to the autoclaved cooled-down nutrient medium before pouring the plates.

Like urea sterile filtered PPD with a concentration of 1mg mL^{-1} (Phenylphosphorodiamidate, an urease inhibitor, ACROS Organics) was added to the autoclaved cooled-down nutrient medium before pouring the plates. To all urea treatments nickel was added with a concentration of 10 µL.

3.2 Bacterial culture and preparation of inoculum

The following strains were used: *Raoultella terrigena* TFI08N (Prof. Joseph Strauss, Austria), *Paenibacillus polymyxa* (Micro-N-Fix Project), *Azospirillum brasilense* SP245 (Micro-N-Fix Project), *Pseudomonas fluorescens* C139 (Rhibac project), *Bacillus megaterium* M3 (Prof. Fikrettin Sahin, Turkey) and *Bacillus subtilis* OSU142 (Prof. Fikrettin Sahin, Turkey).

1 mL of an overnight culture of one colony was added to full medium (YEP, LB or L*, in dependence of the bacterial strain, Table 2) and grown to the exponential growth phase (OD_{600} = 3.5, spektralphotometer) was reached. The bacterial culture was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was removed and the pellet was washed two times with phosphate buffer (1.24 g K_2HPO_4, 0.39 g KH_2PO_4, 8.8 g NaCl per L). Bacteria were dissolved in sterile distilled water and added to the autoclaved, that was cooled down to approximately 30°C medium before pouring the plates (1 mL bacterial solution per L medium).

Table 1. Composition of the nutrient solution of axenically cultured Arabidopsis plants

Macronutrients	**Final concentration**
$MgSO_4$	0.75 mM
KH_2PO_4	0.625 mM
$CaCl_2$	1.5 mM
NH_4NO_3	5 mM
KNO_3	50µM
Micronutrients	
$CoCl_2$	0.055 µM
$CuSO_4$	0.05 µM
H_3BO_3	0.05 µM
KI	2.5 µM
$MnSO_4$	0.05 mM
Na_2MoO_4	0.515 µM
$ZnSO^4$	14.955 µM
FeEDTA	75 µM

Table 2. Composition of the bacterial media

YEP medium (1 L), pH 7
10 g yeast extract
10 g peptone
5 g NaCl

LB medium (1 L)
10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl

L* medium (1 L)
10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
2.5 mM $MgSO_4$
2.5 mM $CaCl_2$

3.3 Harvest and measurements of Arabidopsis plants from plates

To investigate nitrogen fixing ability of *Raoultella terrigena*, $(NH_4)_2SO_4$ was labelled with ^{15}N (1 % labelled by 15N).

To determine dry weight and N and $^{15}N/^{14}N$ concentration a single plant was collected in a stannous cartouche, freezed at -20°C and lyophilized. Dry weight was measured and plant material was analyzed for nitrogen with an elemental analyzer (HEKAtech, type Eurovektor, method „Dumas“, Germany) and IRMS (Thermo, type Delta plus Advantage, Germany).

3.4 Quantification of morphological root parameters

To quantify morphological root parameters, roots were transferred to a foil and lateral roots were separated with forceps. Roots were scanned with a resolution of 600 dpi (Epson Perfection V700 Photo). Background shadows were removed using Adobe Photoshop Version 5.0 LE (Adobe Systems). Total root length, total lateral root length, primary root length and the length of primary, secondary and third lateral roots were quantified by using of WinRhizo version Pro2007d Software (Regents Instruments). The number of lateral roots was counted by eye.

3.5 Rhizosphere pH measurements

To measure the pH of roots an antimony electrode was used (Dr. Günter Neumann, University of Hohenheim, Stuttgart). The middle part of the primary root was measured. To determine pH changes in the rhizosphere 1 g bromocresol purple was suspended in 80 ml water and 1 N NaOH was added dropwise and permanent stirring until pH 9 was achieved. Using 1N H_2SO_4 the indicator solution was set to a pH of 6 and filled up to 100 ml. 10 ml of 1 % bromocresol purple solution were added to 1 L agar-containing nutrient solution. A 0.5 % stock solution of bromocresol green was prepared by dissolving bromocresol green in 70 % ethanol. The final pH was adjusted to 6.5 with 1 N KOH. 5 ml l^{-1} was added to the nutrient solution.

6 days old plants were transferred as described above to square plates, which contained the pH indicator in ½ MS medium. Plants without indicator solution were cultivated in parallel to exclude possible negative effects on growth caused by the indicator.

To standardize colour changes, reference pH buffer solutions were prepared for pH 3.0, pH 4.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0. 50 µl of pH buffer solutions were added to 450 µl of the agar solution.

3.6 GUS-assay

Two reporter lines – DR5::GUS and ARR5::GUS – were used to investigate auxin and cytokinin changes caused by *Raoultella terrigena* under sterile conditions on plates to investigate phytohormonal changes of roots. Seeds of Arabidopsis DR5::GUS and ARR5::GUS lines were sterilized, germinated, transferred to square plates and inoculated as described above. For the GUS-assay a solution was prepared containing x-gluc (Applichem) with a concentration of 20 mg ml^{-1}, dissolved in DMS (Dimethylformamid, Roth).

Plants were transferred from agar plates in GUS-buffer solution. Each well of a 6-well plates contained 4 ml assay buffer. One plant was used per well. Plants were 2 times vacuum-filtrated for 1 min. After that, plant material was incubated at a temperature of 37 °C for 2h (ARR5::GUS) or over night (DR5::GUS). To stop the reaction buffer solution was pipett-off and 80 % ethanol was added. Ethanol step was done two times.

Plants were visualized by microscopy (Zeiss).

Table 4. **Composition of GUS-assay buffer**

Assay buffer (1 mL)
20 µl X-Gluc
200 µl NaPi (500 mM, pH 7)
40 µl EDTA (250 mM)
10 µl K-Ferricyanide (50 mM)
10 µl K-Ferrocyanide (50 mM)
1 µl 100 %Triton X
719 µl dest. Water

3.7 Investigations on the tissue localisation of inoculated *Raoultella terrigena* TFi08N

Roots of *Raoultella terrigena*-inoculated plants were surface sterilized in 80 % ethanol for 1 min and grinded with metal balls. 100 µL of sterilized water was added and mixed. 20 µL of the suspension were spread on YEP medium and incubated overnight at 28 °C. Non-surface sterilized roots of inoculated plants were treated in the same way.

4 Results

4.1 Establishment of a system to investigate plant growth promotion by PGPR under sterile conditions

To investigate plant growth promotion and the influence on root morphology by PGPR under gnotobiotic conditions, a growth system was established by using square agar plates and *Arabidopsis thaliana* as a model plant. Arabidopsis plants were pre-cultured on agar plates with standard half-strength MS medium. After 6 days plants were transferred to vertical plates supplemented with bacteria and cultured for 2 ½ weeks. Shoot and root dry weight and root parameters in particular primary root length, total and total lateral root length and mean number of lateral roots were determined. The use of *Arabidopsis thaliana* is of advantage, not only due to its small size but also as there exist many mutants of Arabidopsis, which allow investigations on mechanisms responsible for growth promotion by bacteria. The plate system is easy to handle and a rapid method to investigate bacterial strains for PGPR activity, as results are obtained after 3-4 weeks. To exclude effects of other microorganisms, it was decided to conduct the experiments under sterile conditions.

4.2 Investigations on PGPR in response to *Arabidopsis thaliana*

Investigations on growth promotion effects by bacteria were conducted with different bacteria strains. As this thesis was conducted in frame of the EU-Project RHIBAC several bacteria strains were available, including *Bacillus megaterium* M3, *Bacillus subtilis* OSU142*, Pseudomonas fluorescens* C139, *Azospirillum brasilense* Sp245 and *Raoultella terrigena* TFi08N.

4.2.1 Characterization of the plant growth promotion conferred by inoculation with *Bacillus megaterium* M3

By use of the described agar plate system plant growth promotion by *Bacillus megaterium* M3 became obvious 16 days after inoculation (Fig. 1). Compared to control plants, a marked growth promotion, expressing in elevated shoot and root biomass, was observed. Dry weights of shoots and roots were 2-3-fold higher, respectively (Fig. 2 A and B). Primary and lateral roots were longer than those of to control plants.

Figure 1: Inoculation of Arabidopsis plants with *Bacillus megaterium* M3 stimulates plant growth. Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Bacillus megaterium* M3 (right side). *Bacillus megaterium* M3 was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days.

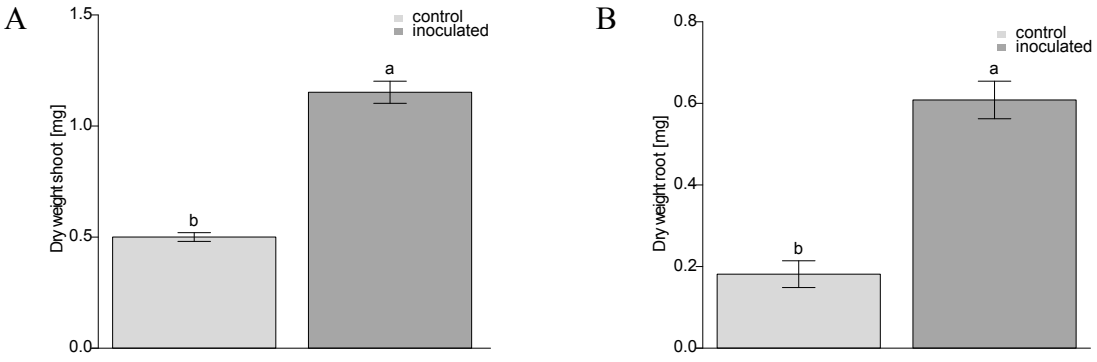


Figure 2: Inoculation with *Bacillus megaterium* M3 increases biomass production in Arabidopsis. Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Bacillus megaterium* M3 and control plants. *Bacillus megaterium* M3 was inoculated at a concentration of 10^7 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.2.2 Characterization of the plant growth promotion conferred by inoculation with *Bacillus subtilis* OSU142

Also the inoculation of Arabidopsis with *Bacillus subtilis* OSU142 resulted in growth stimulation (Fig. 3). Shoot and root dry weights of inoculated plants were increased significantly (Fig. 4 A and B). Plants inoculated with *Bacillus subtilis* OSU142 revealed an elongation of primary and lateral root length compared to control plants. The more bushy appearance of the growth system compared to inoculation with *Bacillus megaterium* M3 was due to the longer time of plant cultivation.

Figure 3: Inoculation of Arabidopsis plants with *Bacillus subtilis* OSU142 stimulates plant growth. Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Bacillus subtilis* OSU142 (right side). *Bacillus subtilis* OSU142 was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 23 days.

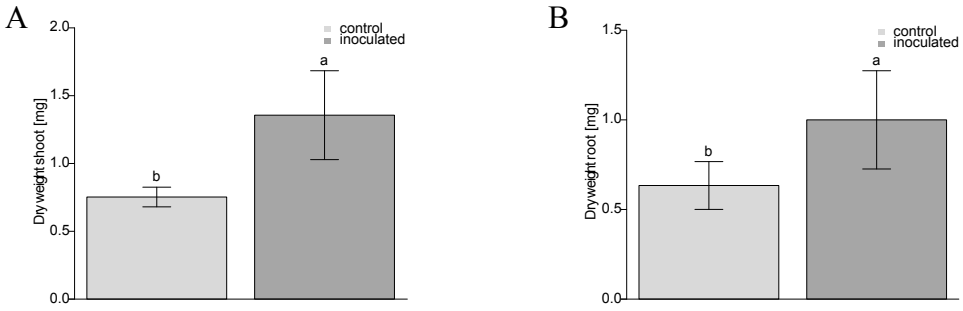


Figure 4: Inoculation with *Bacillus subtilis* OSU142 increases biomass production in Arabidopsis. Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Bacillus subtilis* OSU142 and control plants. *Bacillus subtilis* OSU142 was inoculated at a concentration of 10^7 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 23 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.2.3 Characterization of the plant growth promotion conferred by inoculation with *Pseudomonas fluorescens* C139

It was observed that plants inoculated with *Pseudomonas fluorescens* C139 were reduced in growth under the used conditions (Fig. 5). No significant difference was measured for shoot and root dry weight (Fig. 6 A and B), but an altered root morphology was observed (Fig. 5), as primary root length was reduced and enhanced lateral root growth led to a more bushy appearance of the growth system. Bacteria were harvested in the exponential growth phase and inoculated at a density of 10^7 cfu mL^{-1}. As the inoculation density as well as the growth stage of bacteria is of importance for growth effects, different inoculation conditions were used in subsequent experiments (Chapter 5).

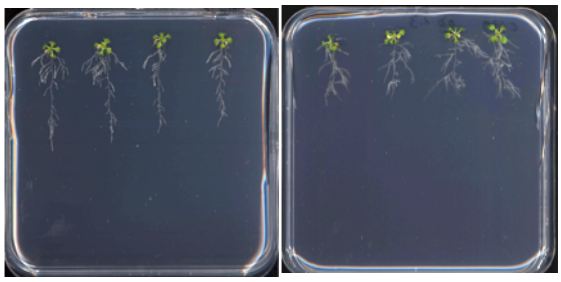


Figure 5: Inoculation of Arabidopsis plants with *Pseudomonas fluorescens* C139 does not stimulate plant growth.
Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Pseudomonas fluorescens* C139 (right side). *Pseudomonas fluorescens* C139 was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days.

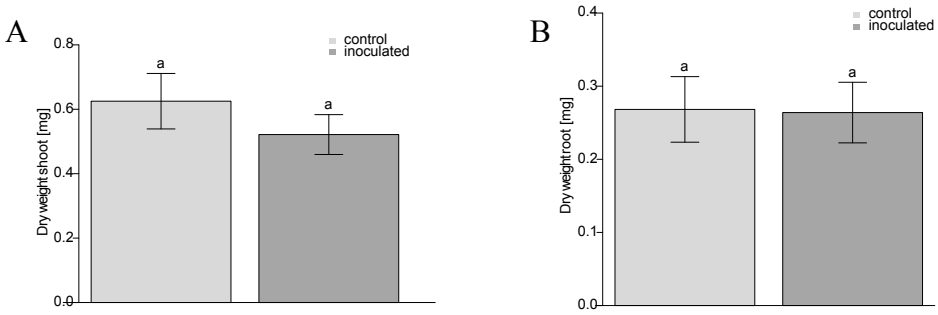


Figure 6: Inoculation with *Pseudomonas fluorescens* C139 does not increase biomass production in Arabidopsis.
Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Pseudomonas fluorescens* C139 and control plants. *Pseudomonas fluorescens* C139 was inoculated at a concentration of 10^7 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.2.4 Characterization of the plant growth promotion conferred by inoculation with *Azospirillum brasilense* Sp245

Plants inoculated with *Azospirillum brasilense* Sp245 clearly underwent growth promotion. This was investigated in dependence of its growth (Fig. 7) and in dependence of different bacterial concentrations of OD 2.0 (Fig. 9).

In dependence of bacterial growth, a two times higher dry weight of shoot was already reached after inoculation with *Raoultella* at OD 1.5 (Fig. 8A). The shoot biomass of plants after inoculation with *Raoultella* between OD 2.0 and OD 4.0 was comparable to inoculation with OD 1.5 (Fig. 9A). Root dry weight followed an optimum response with a maximum at OD 2.5 (Fig. 9B).

Figure 7: Plant growth promotion by *Azospirillum brasilense* Sp245 depends on their growth phase. Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Azospirillum brasilense* Sp245 (right side). *Azospirillum brasilense* Sp245 was harvested at OD´s ranging from OD 1.5 to OD 4.0. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 16 days.

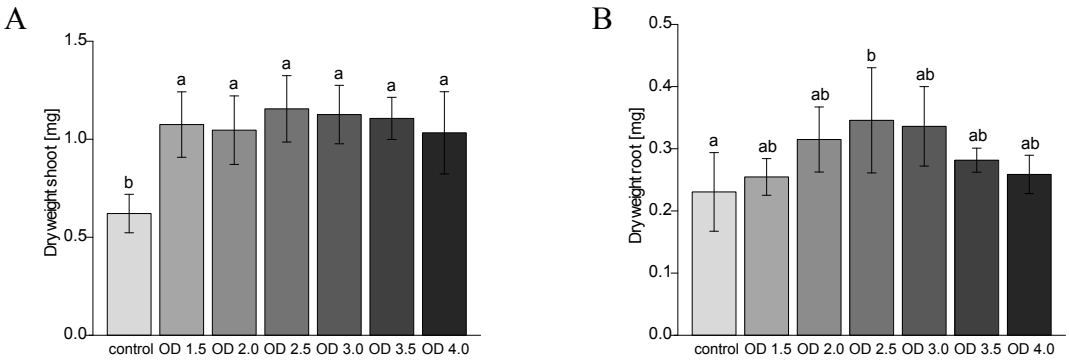


Figure 8: Plant growth promotion by *Azospirillum brasilense* Sp245 depends on their growth phase. Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Azospirillum brasilense* Sp245 and control plants. *Azospirillum brasilense* Sp245 was harvested at OD´s ranging from OD 1.5 to OD 4.0. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 16 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

When inoculated at different bacterial concentrations, shoot dry weight appeared to follow an optimum response curve upon increased bacterial inoculation densities. However, at 10^7 cfu mL^{-1} no increase in shoot and root was observed (Fig. 10 A and B). As this experiment was repeated two times with the same observation, a technical or methodological artifact was unlikely. The reason for this decline remains open.

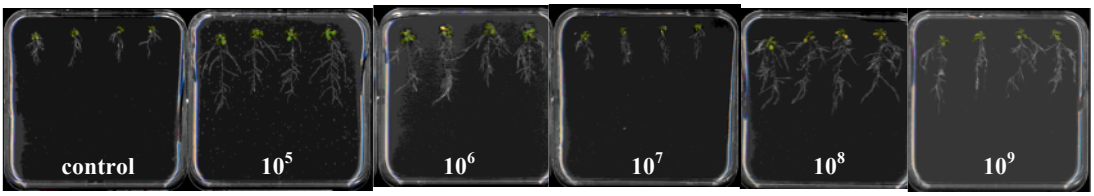


Figure 9: Plant growth promotion by *Azospirillum brasilense* Sp245 depends on the inoculation density.
Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Azospirillum brasilense* Sp245 (right side). *Azospirillum brasilense* Sp245 was harvested at a density ranging from 10^5 cfu mL^{-1} to 10^9 cfu mL^{-1}. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 16 days.

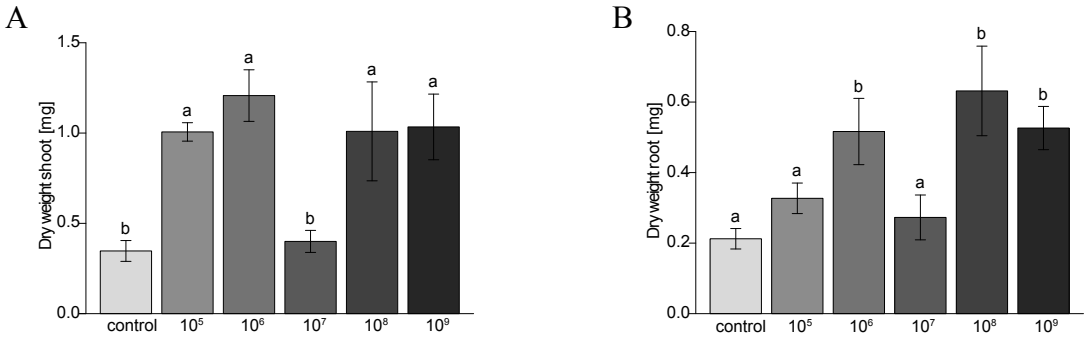


Figure 10: Plant growth promotion by *Azospirillum brasilense* Sp245 depends on the inoculation density.
Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Azospirillum brasilense* Sp245 and control plants. *Azospirillum brasilense* Sp245 was harvested at a density ranging from 10^5 cfu mL^{-1} to 10^9 cfu mL^{-1}. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 16 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.2.5 Characterization of the plant growth promotion conferred by inoculation with *Raoultella terrigena* TFi08N

4.2.5.1 Characterization of the plant growth promotion conferred by inoculation with *Raoultella terrigena* TFi08N in dependence of its growth phase

The effect on plant growth of an inoculation with *Raoultella terrigena* was first determined in dependence of the bacterial growth phase. 20 days after inoculation with *Raoultella terrigena*, a significant increase in shoot dry weight was measured when bacteria were harvested at ODs between 1.0 and 2.5. At higher inoculation densities, growth promotion tended to decrease (Fig. 12 A). Root dry weight, total primary root length and lateral root length (Fig. 12 B, C and D) were all significantly higher for ODs between OD 1.0 to OD 3.0 (Fig. 12 B and C).

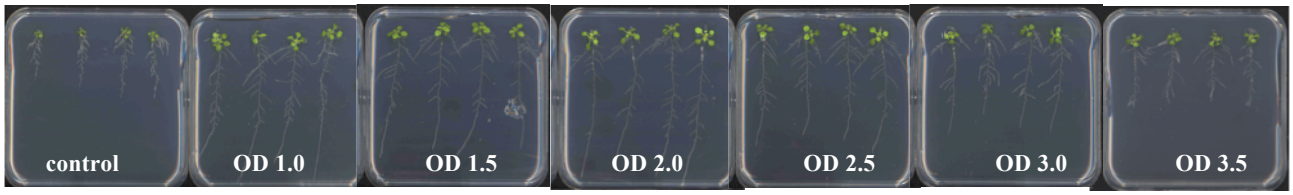


Figure 11: Plant growth promotion by *Raoultella terrigena* TFi08N depends on their growth phase. Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Raoultella terrigena* TFi08N (right side). *Raoultella terrigena* TFi08N was harvested at OD´s ranging from OD 1.0 to OD 3.5. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days.

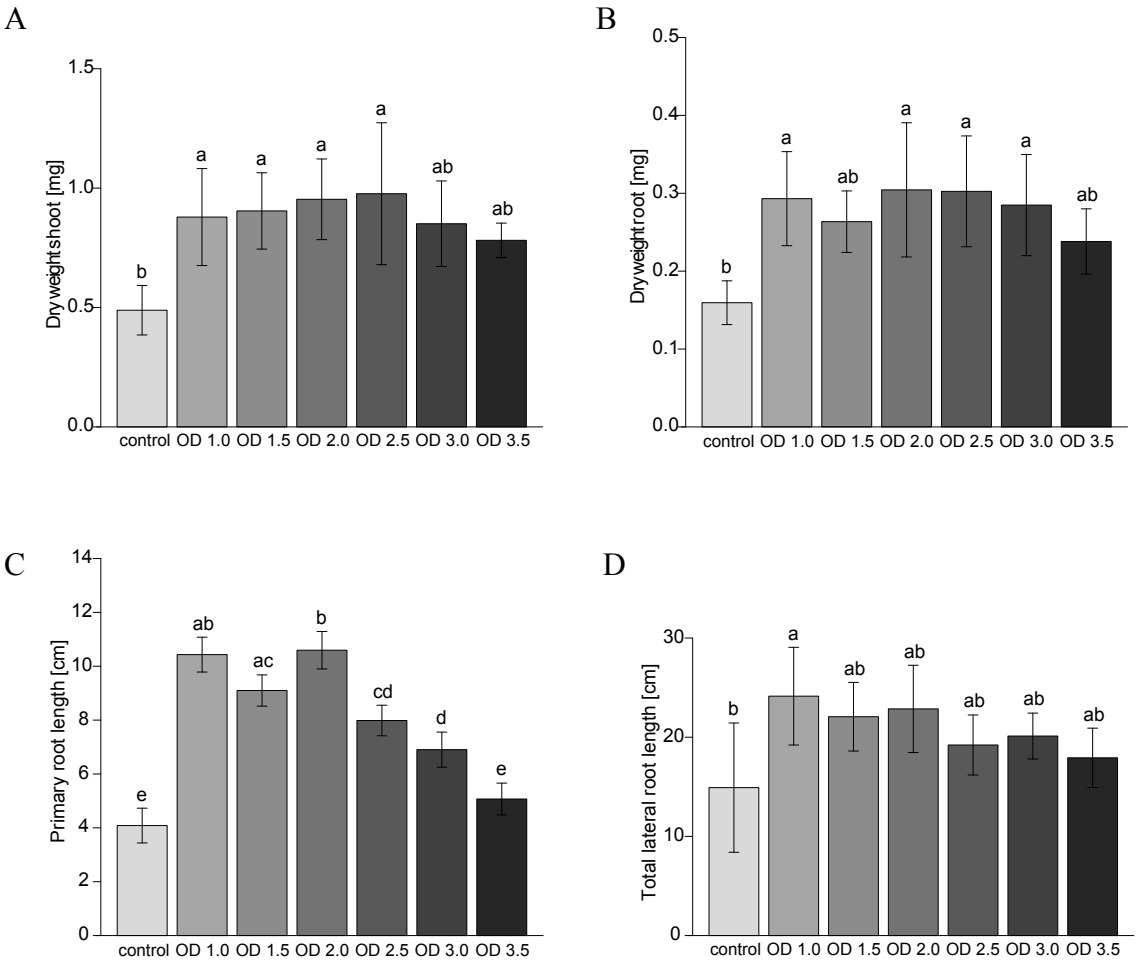


Figure 12: Plant growth promotion by *Raoultella terrigena* TFi08N depends on their growth phase. Dry weights of shoots (A) and roots (B), primary root length (C) and total lateral root length (D) of Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N and control plants. *Raoultella terrigena* TFi08N was harvested at OD´s ranging from OD 1.5 to OD 4.0. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.2.5.2 Characterization of the plant growth promotion conferred by inoculation with *Raoultella terrigena* TFi08N in dependence of the bacterial cell concentration

Raoultella was grown in liquid culture up to OD 2, corresponding to the early exponential growth phase of the bacteria. Cells were harvested and inoculated at different concentrations ranging from 10^5 to 10^9 cfu mL^{-1}. Compared to control plants, a marked growth promotion, expressing in elevated shoot and root biomass, was observed for cell bacterial concentrations between 10^5 and 10^8 cfu mL^{-1} (Fig. 13). While 10^5 and 10^6 cfu mL^{-1} enhanced shoot dry weight by 40%, a concentration of 10^7 and 10^8 cfu mL^{-1} enhanced shoot biomass by 50% (Fig. 14 A). The promotive effect of cell concentration on root biomass was less consistent (Fig. 14 B). Inoculation with a concentration of 10^9 cfu mL^{-1} did not result in plant growth promotion (Fig. 14 A and B).

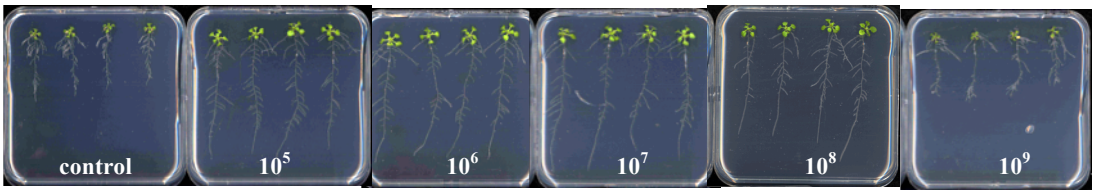


Figure 13: Plant growth promotion by *Raoultella terrigena* TFi08N depends on the inoculation density.
Phenotype of Arabidopsis plants grown under axenic conditions (left side) or inoculated with the bacterium *Raoultella terrigena* TFi08N (right side). *Raoultella terrigena* TFi08N was harvested at a density ranging from 10^5 cfu mL^{-1} to 10^9 cfu mL^{-1}. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days.

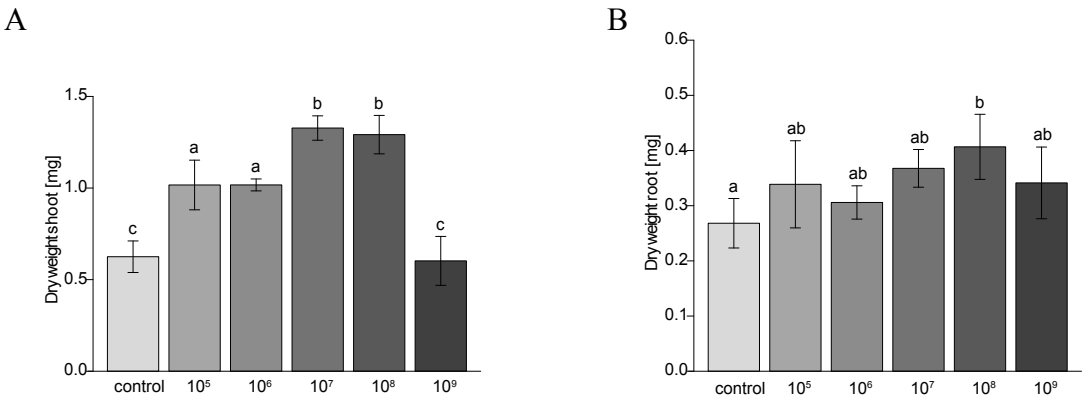


Figure 14: Plant growth promotion by *Raoultella terrigena* TFi08N depends on the inoculation density.
Dry weights of shoots (A) and roots (B) of Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N and control plants. *Raoultella terrigena* TFi08N was harvested at a density ranging from 10^5 cfu mL^{-1} to 10^9 cfu mL^{-1}. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

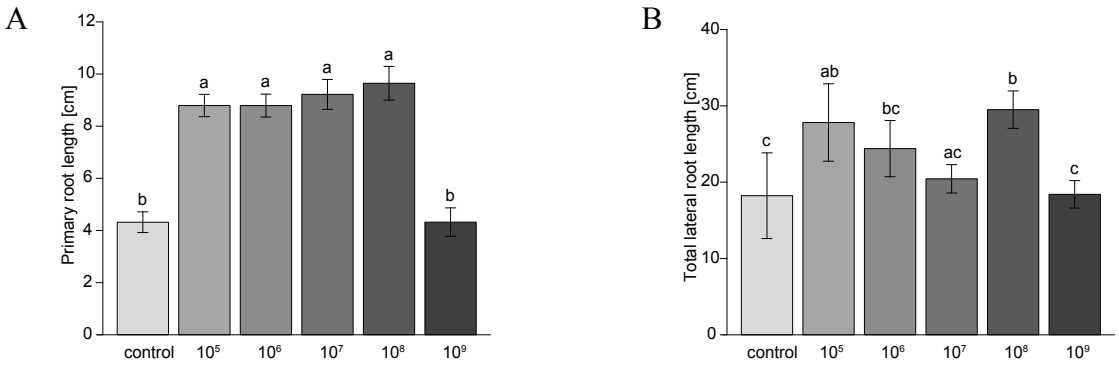


Figure 15: Plant growth promotion by *Raoultella terrigena* TFi08N depends on the inoculation density.
Primary root length (A) and total lateral root length (B) of Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N and control plants. *Raoultella terrigena* TFi08N was harvested at a density ranging from 10^5 cfu mL^{-1} to 10^9 cfu mL^{-1}. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

However, with regard to primary root length, *Raoultella* showed a strong stimulation with a more than twofold increase at cell concentration between 10^5 and 10^6 cfu mL^{-1} (Fig. 15 A). This corresponded to the same concentration, which also stimulated shoot dry weight. By contrast, total lateral root length appeared to be a less reliable measure, since a significant increase was only observed at 10^6 and 10^8 cfu mL^{-1} (Fig. 15 B).

4.3 Impact of *Raoultella terrigena* on root morphology

In frame of the Micro-N-Fix project *Raoultella terrigena* TFi08N was isolated from the rhizosphere soil of wheat plants grown in an agricultural soil. So far, *Raoultella terrigena* TFi08N has not been described as a PGPR. For this reason it was decided to investigate this bacterial strain in more detail and to study the mechanisms underlying its growth promotion effect on Arabidopsis.

4.3.1 Impact of *Raoultella terrigena* inoculation under gnotobiotic conditions on *Arabidopsis thaliana*

Determination of nitrogen concentrations of growth medium for optimal inoculation effects

As the aim of the use of PGPR within this project was to find ways how to minimize the input of mineral fertilizers in agricultural plant production, in particular nitrogen fertilizers, growth conditions were chosen, in which Arabidopsis plants still obtained N supply in order to mimic a basal N provision as it is the case in agricultural systems. A concentration of 1mM NH_4NO_3 had already been shown to allow *Raoultella terrigena* TFi08N to increase shoot and root development (Weishaar, 2007). Varying further the concentrations of NH_4^+ and NO_3^- concentrations (Weishaar, 2007) indicated that a concentration of 1mM NH_4^+ with 100µM NO_3^- yielded optimal results with regard to plant growth promotion. Therefore, this mixture of N forms was also used in the subsequent studies.

4.3.2 Influence of different inorganic nitrogen forms on growth promotion by *Raoultella terrigena* TFi08N

The supplied nitrogen form can influence plant development, in particular root architecture. Therefore, N was supplied to non-buffered half-strength MS-medium either as ammonium-N, nitrate-N or in a concentration ratio of ammonium : nitrate of 90:10. The total N concentration was kept the same.

Nitrate and ammonium were supplied as ^{15}N-labelled substrates to investigate whether these N forms were taken up at different preferences.

Raoultella inoculation had a particularly strong impact on root morphology, which depended on the supplied nitrogen form. Growth promotion was highest under ammonium nutrition (Fig. 16) and clearly visible 16-18 days after inoculation with *Raoultella terrigena* TFi08N. For plants supplied exclusively with nitrate a higher

elongation of lateral roots in particular in the apical root part was observed (Fig. 16). However this differential contribution of *Raoultella* inoculation on root growth was subject to the differential influence of NH_4^+ and NO_3^- on the root development of control plants. While NO_3^- supply allowed a continuous extension of the primary root and of lateral roots leading to a typical root system dominated by the primary root, NH_4^+ supply severely inhibited primary root elongation (Fig. 16, 17 C). This further resulted in a shorter overall length of the total or the lateral root system (Fig. 17 D, E).
By contrast, the number of first, second or third-order lateral roots was higher under ammonium nutrition. This is in agreement with the stimulation effect of NH_4^+ on lateral root branching (Lima et al., 2010). Supplying 10% of the N in the form of NO_3^- did not considerably alter the root phenotype of NH_4^+-grown control plants (Fig. 16, 17 F, G and H).

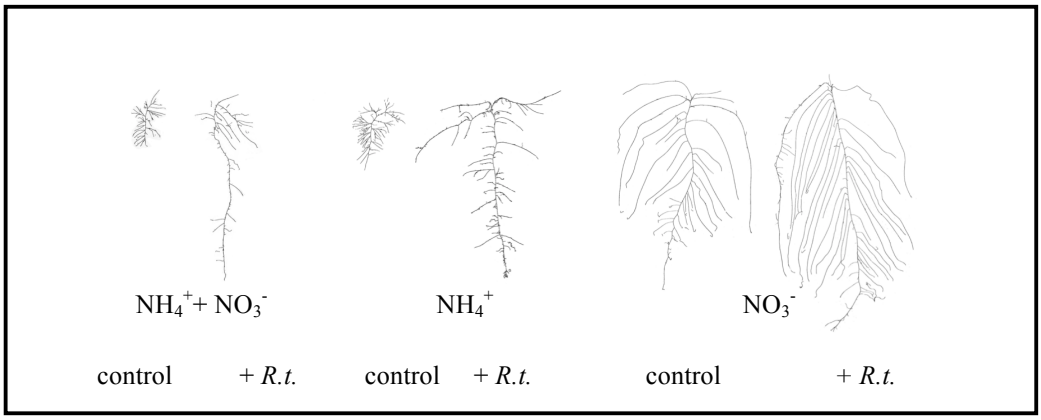


Figure 16: Inoculation with *Raoultella terrigena* TFi08N promotes root growth in dependence of the supplied N form.
Root architecture of Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N (*R.t.*) and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^-, NH_4^+or NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days.

Ammonium-induced growth repression was greatly reduced when plants were inoculated with *Raoultella terrigena* TFi08N. In particular shoot growth and primary root length almost reached the level of NO_3^--grown plants, while lateral root length remained low. Interestingly, the mean number of second and third-order lateral roots went down to approach those of NO_3^--grown plants, too. In all root morphological parameters, NH_4^+-grown plants that were additionally supplied with NO_3^- took in an intermediate position between NH_4^+ and NO_3^- -supplied plants.
Taken together, *Raoultella* inoculation compensated for the NH_4^+-induced repression of root length and stimulated root length also in the absence of NH_4^+.

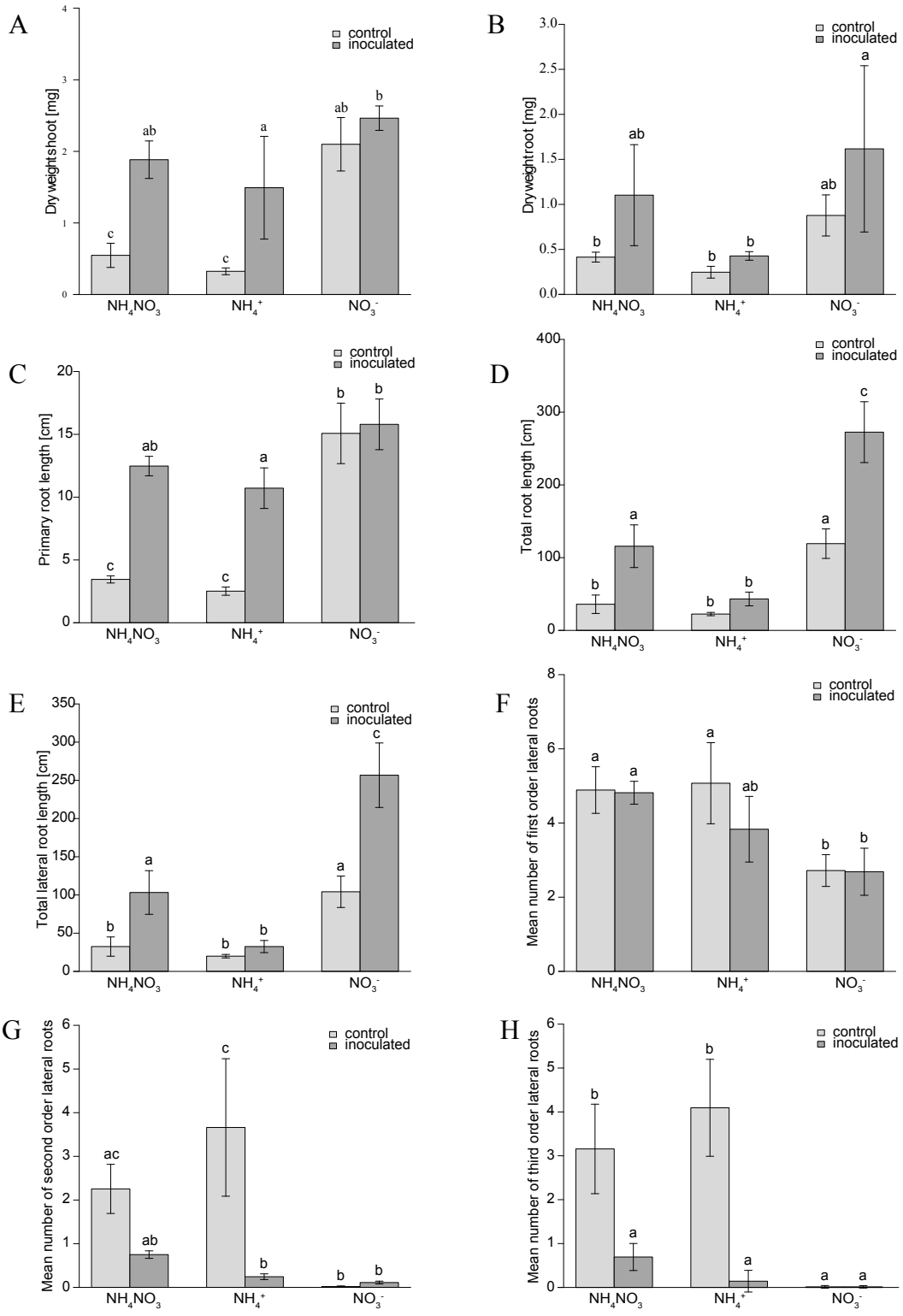


Figure 17: Inoculation with *Raoultella terrigena* TFi08N promotes dry matter production and root growth in dependence of the supplied N form.

Shoot and root dry weights (A and B), primary, total and total lateral root length (C, D and E) and number of first, second and third order lateral roots (F, G and H) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on NH_4^+ with 100µM NO_3^-, NH_4^+ or NO_3^- (1mM N) on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

A determination of total N concentration in shoots and roots did not allow to observe a significant effect of *Raoultella* inoculation, but determining ^{15}N concentration in shoots showed that significant more ^{15}N was accumulating in the presence of *Raoultella* indicating that enhanced lateral root length improved N acquisition and translocation to shoots (Fig. 18 A-D).

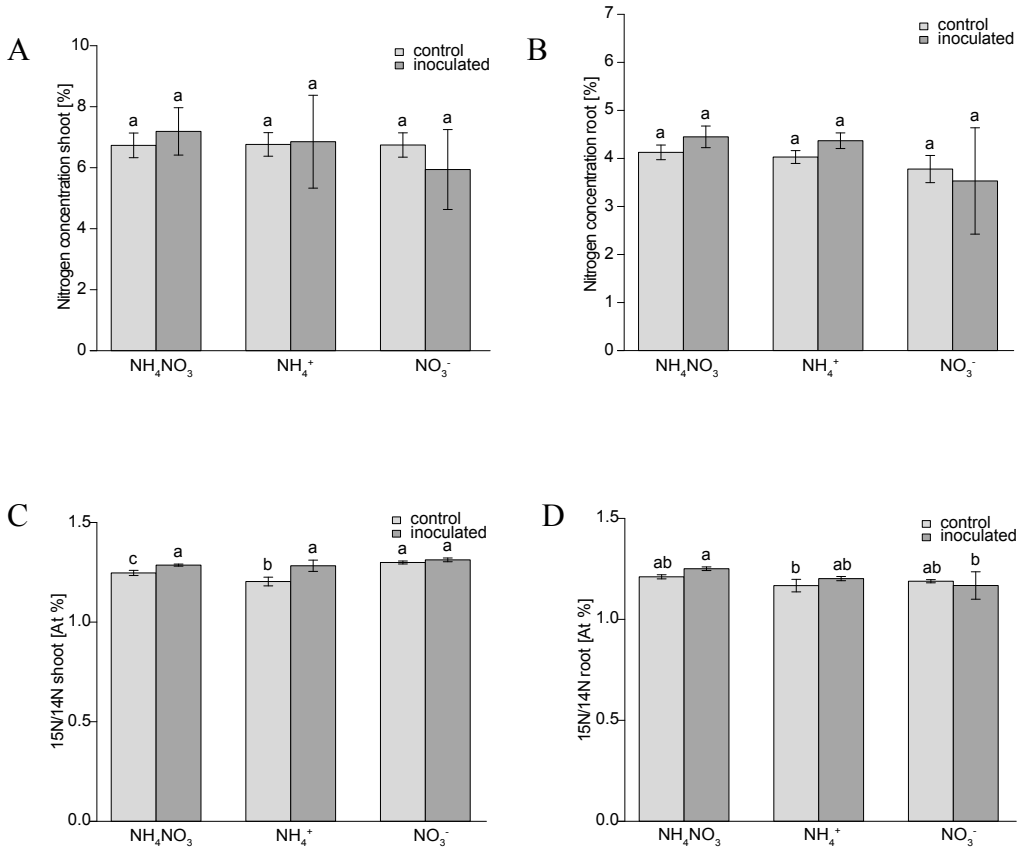


Figure 18: Influence of nitrogen forms and inoculation with *Raoultella terrigena* TFi08N on nitrogen concentrations and $^{15}N/^{14}N$ ratios in shoots and roots.
Nitrogen concentrations in shoots (A) and roots (B) and $^{15}N/^{14}N$ ratios in shoots (C) and roots (D) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on NH_4^+ with 100µM NO_3^-, NH_4^+ or NO_3^- (1mM N) on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.3.3 Influence of different inorganic nitrogen forms on pH buffered medium on growth promotion by *Raoultella terrigena* TFi08N

Under non-buffered conditions the supplied nitrogen form leads to rhizosphere pH changes. Ammonium leads to a physiological acidification of the rhizosphere pH, while nitrate leads to a physiological alkalinization. To avoid this and to investigate the role of *Raoultella* in this process, the pH of the nutrient medium was buffered. Similar to the precedent experiment N was supplied as NH_4^+ or NO_3^-, but this time the pH was buffered to pH 5.5, pH 6.0 and pH 6.5. Dry weights of shoots and roots, root parameters, nitrogen concentrations and $^{15}N/^{14}N$ ratios were determined. Inoculation did not affect shoot dry weight (Fig 20 A), but in the case of ammonium-treated plants inoculation at a pH of 5.5 resulted in a significant increase of root dry weight (Fig. 20 B).

In context to the previous experiment with unbuffered medium, pH buffering allowed better growth of NH_4^+-supplemented roots and shoots. In fact, NH_4^+-supplied plants reached a similar root and shoot dry weight as NO_3^--supplied plants (Fig. 20 A, B), even though NO_3^--grown plants tended to produce more biomass. Under supply of either N form, higher pH values slightly improved plant growth (Fig. 19, 20). Inoculation with *Raoultella* did not further improve shoot biomass production and stimulated root biomass only at pH 5.5 of NH_4^+-grown plants (Fig. 20 A, B). *Raoultella* promoted root growth not only at the level of total lateral root length but also of primary root length (Fig. 20 C, D and E). Although this stimulation became only apparent at pH 5.5, it held true for NH_4^+ and NO_3^- supply. Mean lateral root number tended to be lower under *Raoultella* inoculation, emphasizing the selective stimulatory effect of Raoultella on root elongation rather than lateral root initiation.

Although total N concentration indicated that roots and shoots of all treatments were adequately supplied with N, *Raoultella* inoculation further enhanced total N concentration in shoots of NH_4^+-grown plants at pH 5.5. A significantly different ratio of $^{15}N/^{14}N$ was only observed in the case of NO_3^--supplied plants at pH 6.0, where control plants showed a relative enrichment in ^{15}N compare to inoculated plants (Fig. 21 C, D). No differences between control and bacteria-treated plants were determined for nitrogen concentrations of shoots of the other treatments and of roots for all treatments (Fig. 21 A and B).

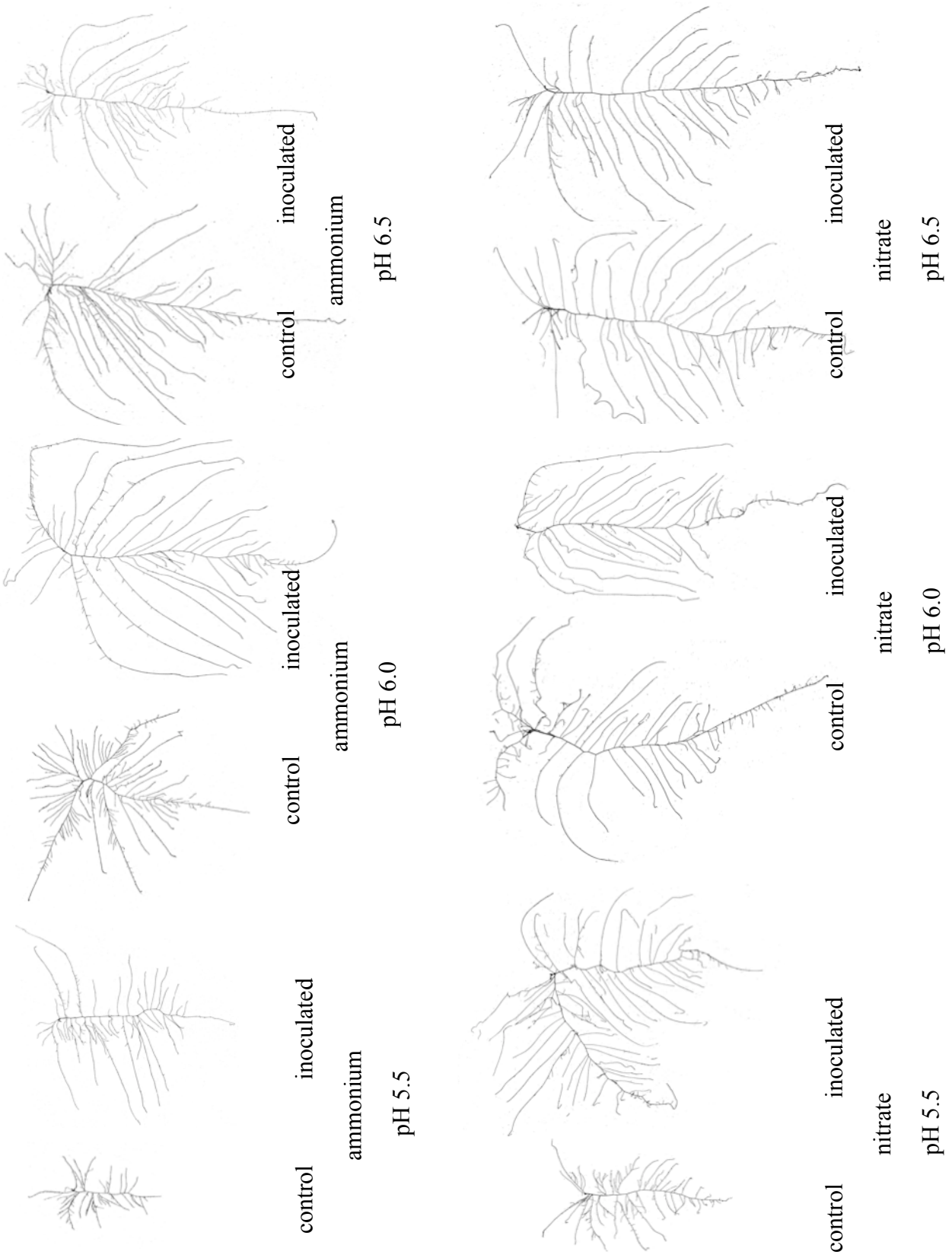


Figure 19: Influence of nitrogen forms, pH and inoculation with *Raoultella terrigena* TFi08N on root architecture.

Representative root architecture of plants cultivated on 1mM NH_4^+ or NO_3^-. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Plants were inoculated with *Raoultella terrigena* at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Six days after germination plants were transferred to treatments and cultivated for 18 days.

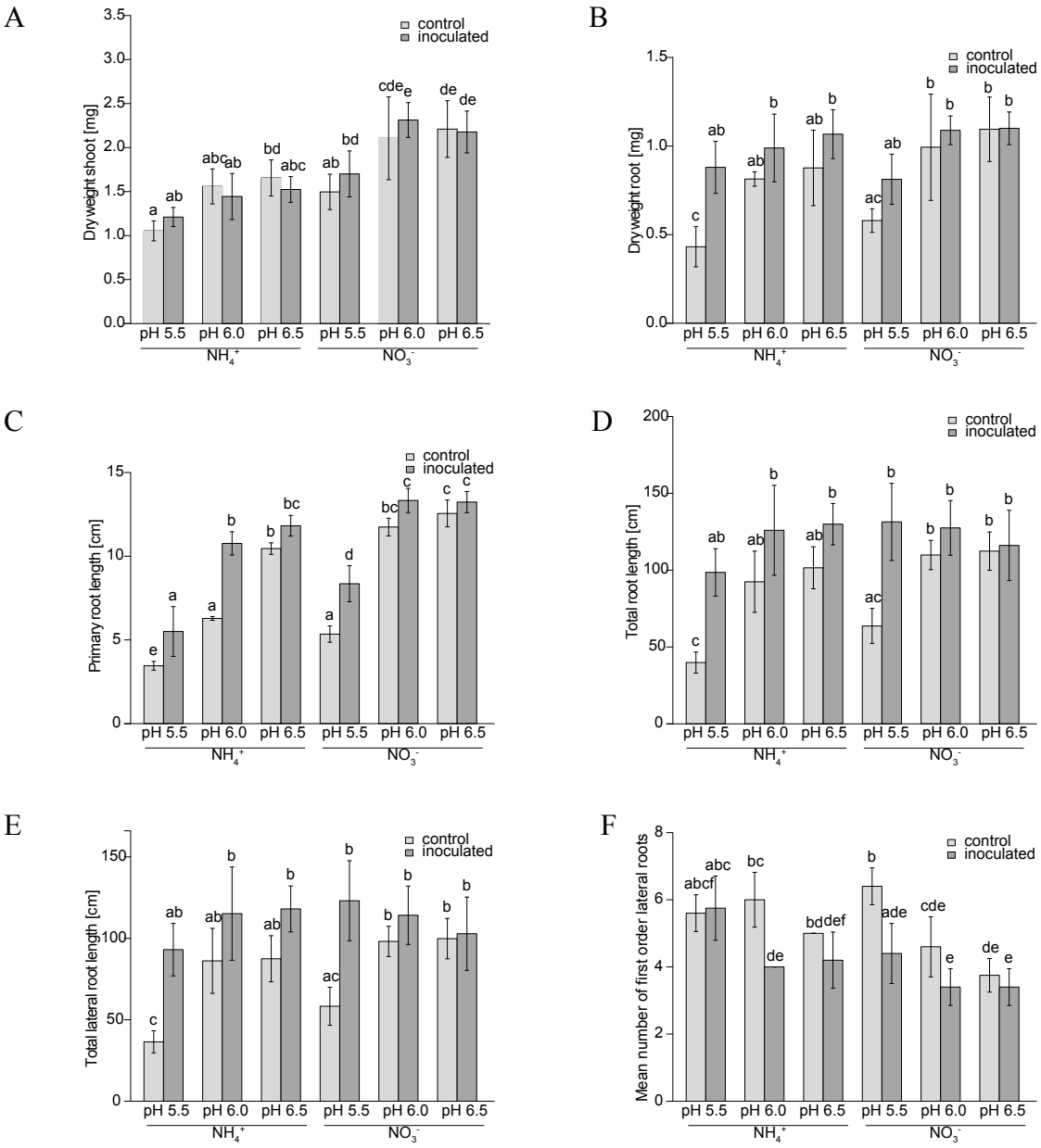


Figure 20: Influence of nitrogen forms, pH and inoculation with *Raoultella terrigena* TFi08N on plant growth.

Dry weight of shoots (A) and roots (B), primary root length (C), total (D) and total lateral root length (E) and mean number of first order lateral roots (F) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ or NO_3^-. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

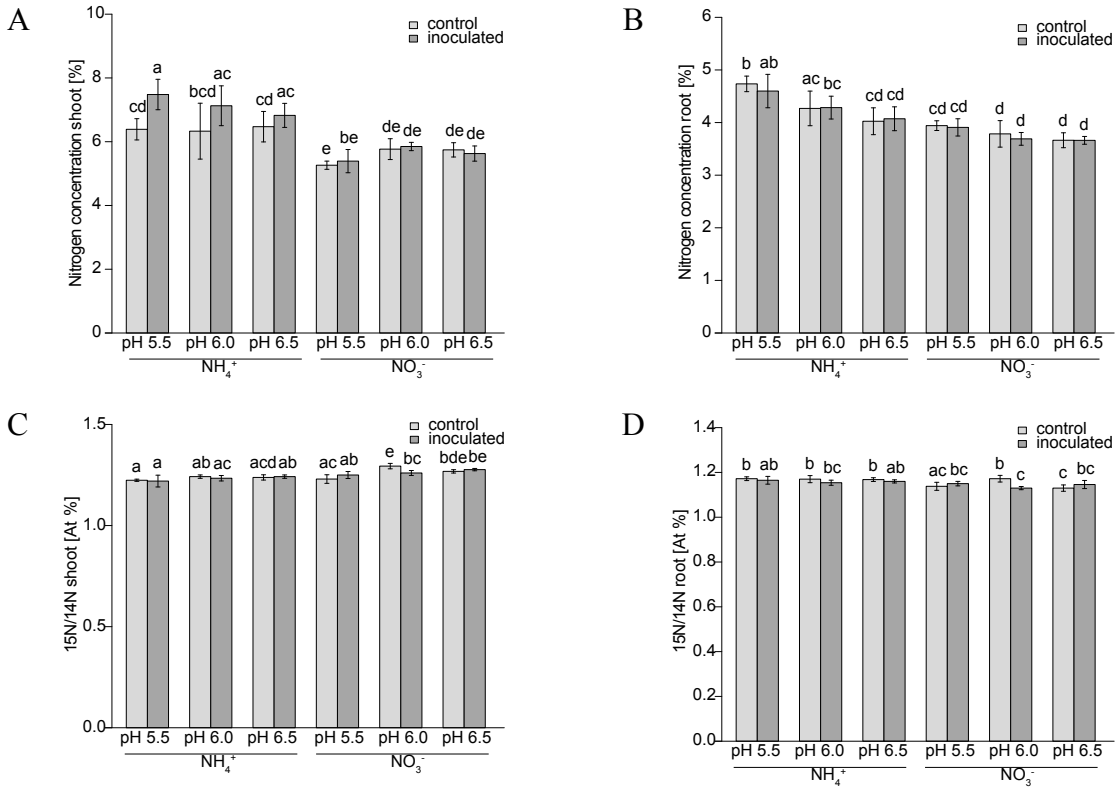


Figure 21: Influence of nitrogen forms, pH and inoculation with *Raoultella terrigena* TFi08N on nitrogen concentrations and $^{15}N/^{14}N$ ratios in shoots and roots.
Nitrogen concentrations in shoots (A) and roots (B) and $^{15}N/^{14}N$ ratios in shoots (C) and roots (D) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ or NO_3^-. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.3.4 Influence of *Raoultella* inoculation on Arabidopsis growth under supply of the organic N form urea

To investigate whether the growth promotion by *Raoultella terrigena* TFi08N can also be observed under supply of an organic N form, nitrogen was added in the form of urea under non-buffered conditions. Growth promotion effect by inoculation was strong (Fig. 22). A three times higher shoot dry weight was measured for inoculated plants compared to control plants (Fig. 23 A). Root dry weight of inoculated plants was four times higher (Fig. 23 B), which was due to a significant elongation in primary root length (Fig. 23 C), total lateral root length (Fig. 23 D) and total root length (Fig. 23 E). The enhancement of total lateral root length was not due to a higher number of lateral roots (Fig. 24 F) as the number of laterals significantly decreased in inoculated plants compared to control plants.

Figure 22: **Influence of nitrogen form and inoculation with *Raoultella terrigena* TFi08N on plant phenotype.**
Representative phenotype of plants cultivated on 1mM urea on non-buffered medium. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Six days after germination plants were transferred to treatments and cultivated for 18 days.

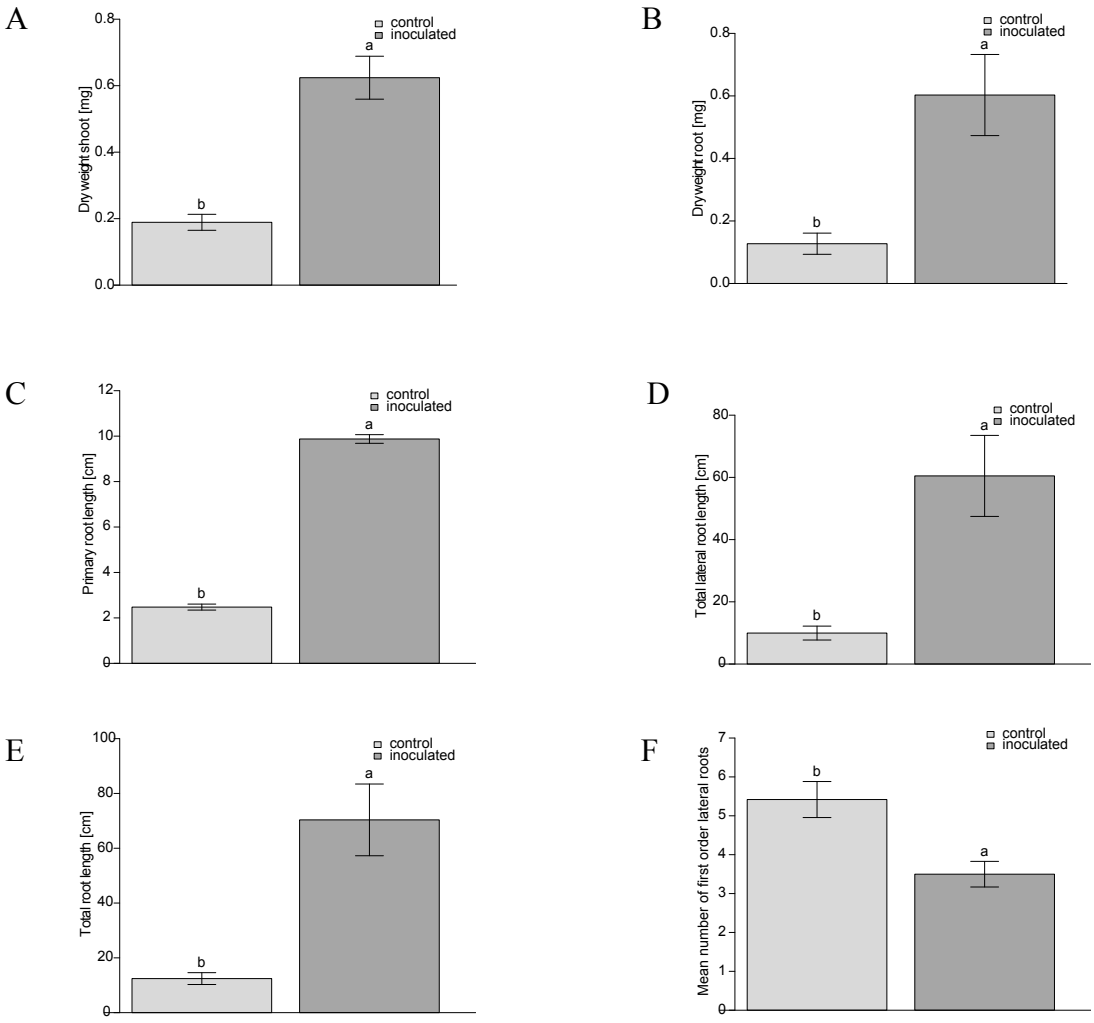


Figure 23: Influence of nitrogen form and inoculation with *Raoultella terrigena* TFi08N on shoot and root growth.
Dry weight of shoots (A) and roots (B), primary root length (C), total lateral root length (D), total root length (E) and mean number of first order lateral roots (F) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

Analyses of nitrogen concentration in roots and shoots resulted in a significantly higher value in shoots of bacteria-treated plants (Fig. 24 A) but this was not the case for roots (Fig. 24 B).

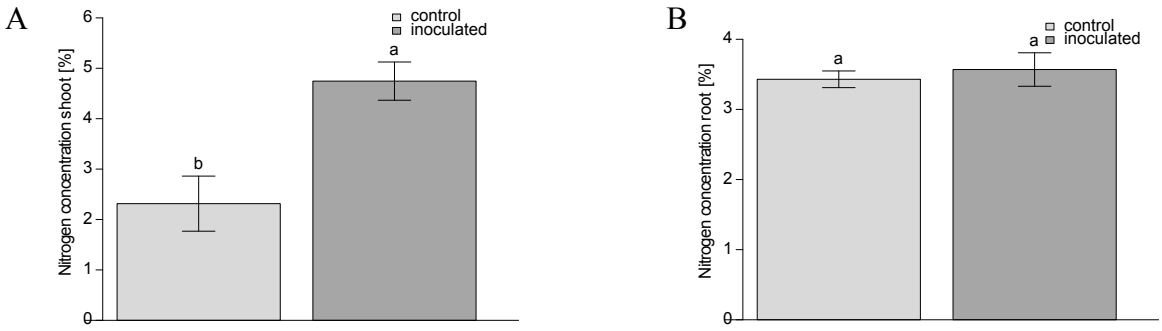


Figure 24: **Influence of nitrogen form and inoculation with *Raoultella terrigena* TFi08N on nitrogen concentrations in shoots and roots.**
Nitrogen concentrations in shoots (A) and roots (B) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.3.5 Influence of *Raoultella* inoculation on Arabidopsis growth under urea supply on pH buffered medium

When plants were grown on NH_4^+ or NO_3^-, pH buffering had a strong influence on root and shoot growth since the physiologically acidic and basic effect of these N forms could be balanced. In the case of the organic N form urea, however, no pH change in the rhizosphere is expected, as long as urea is taken up as a neutral molecule. Indeed, elevating the pH from 5.5 to 6.5 did not considerably improve root and shoot biomass production of control plants (Fig. 25 and 26 A and B). There was only an exception for primary root length, which further elongated with increasing pH (Fig. 26 C). Inoculation with *Raoultella* enhanced root and shoot biomass as well as primary and lateral root length at any pH, but the growth-promoting effect of *Raoultella* appeared to be highest at pH 6.0.

Analyses of nitrogen concentrations showed no difference between control and inoculated roots, but significantly higher values were measured for shoots of bacteria-treated plants at any pH (Fig. 26 E and F).

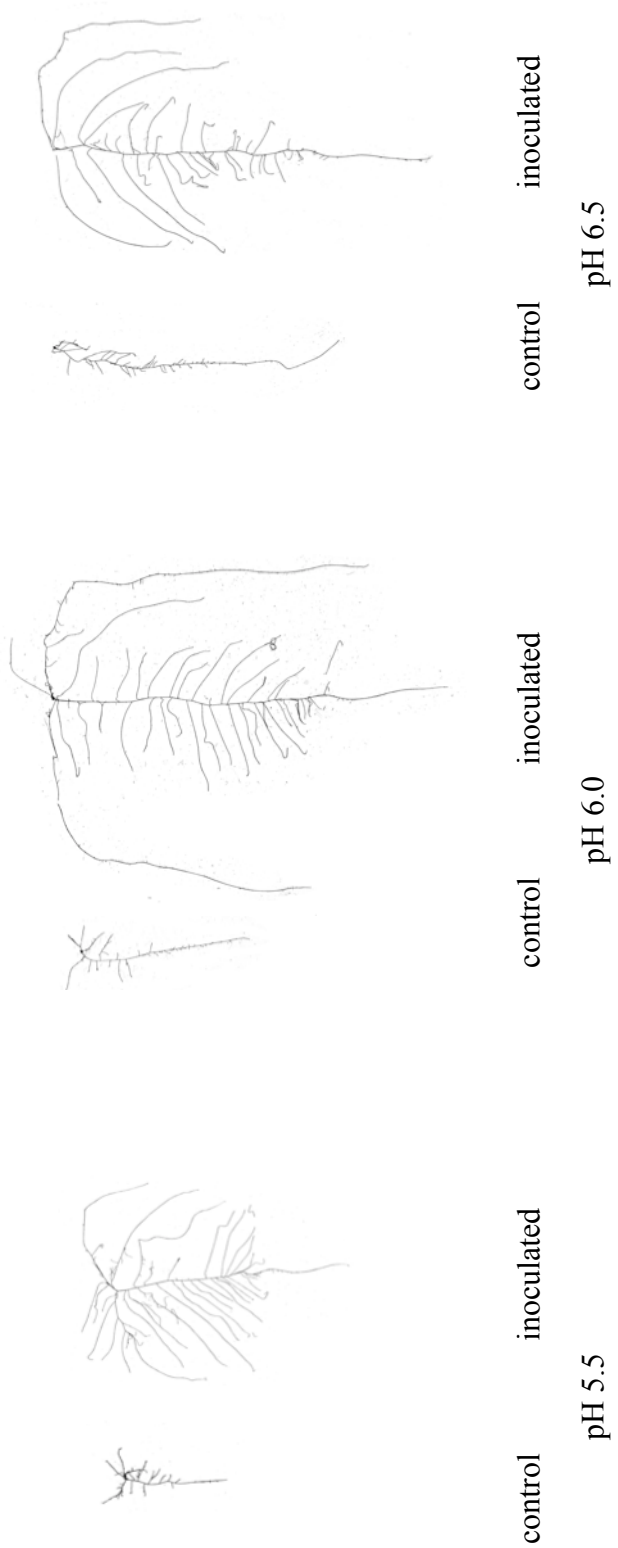


Figure 25: Influence of nitrogen form, pH and inoculation with *Raoultella terrigena* TFi08N on root architecture.
Representative root architecture of plants cultivated on 1mM urea. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Plants were inoculated with *Raoultella terrigena* at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Six days after germination plants were transferred to treatments and cultivated for 18 days.

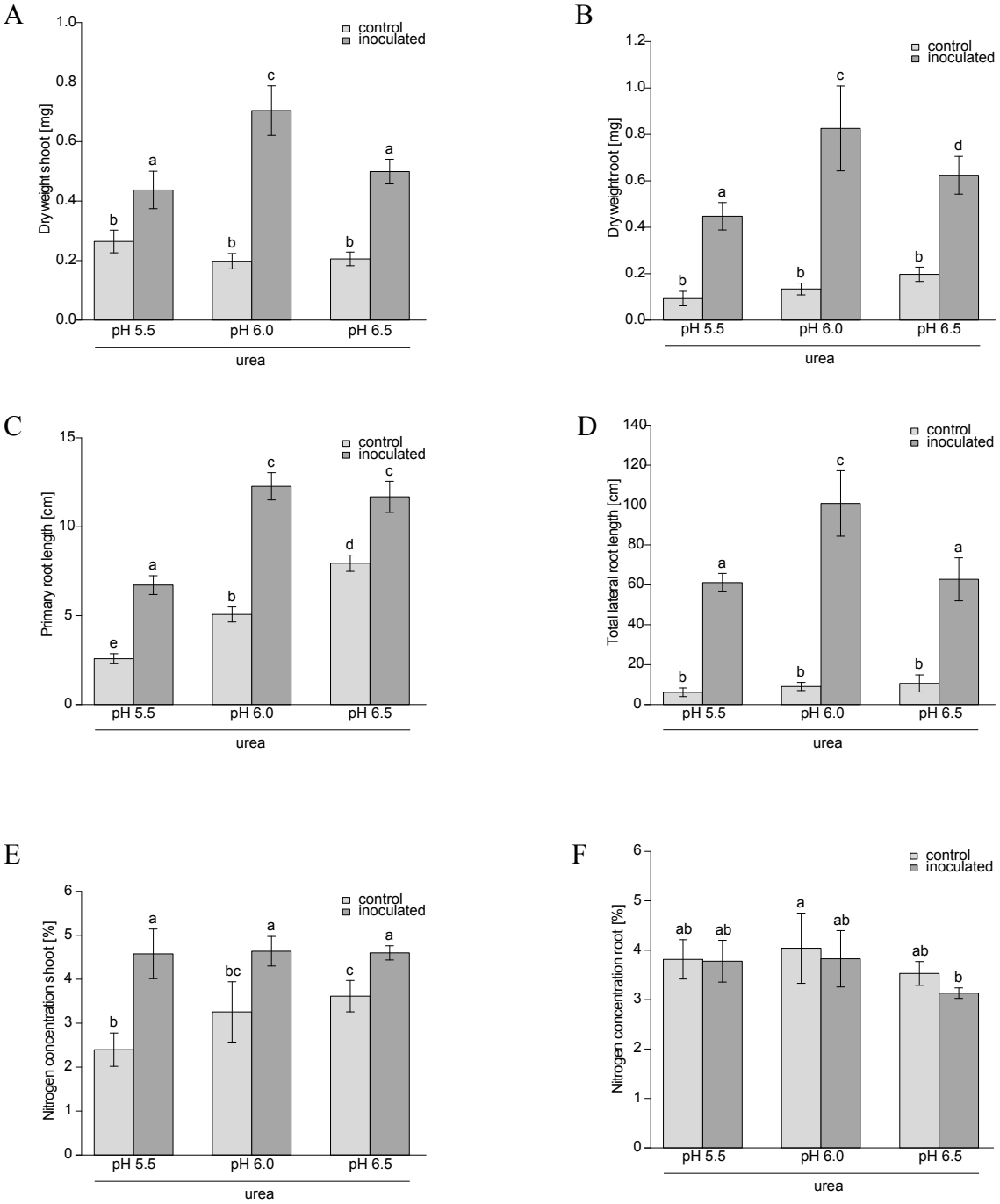


Figure 26: Influence of nitrogen form, pH and inoculation with *Raoultella terrigena* TFi08N on plant growth and nitrogen concentrations of shoots and roots.
Dry weight of shoots (A) and roots (B), primary root length (C), total lateral root length (D) and nitrogen concentrations of shoots (E) and roots (F) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.3.6 Influence of *Raoultella* inoculation on Arabidopsis growth under amino acid supply

To further investigate the role of the supplied nitrogen form on inoculation efficiency, a series of experiments was conducted in which the unbuffered growth medium was supplemented with amino acids as a sole nitrogen source. The amino acids histidine, glutamine, arginine and glutamate influenced control and inoculated plants in different ways. As expected, plant development was inhibited in plants supplied with glutamate (Walch-Liu et al., 2006). Compared to the other amino acids, glutamate strongly decreased shoot dry weight as well as all root parameters like root dry weight, primary, total and total lateral root length (Fig. 27 and 28) when cultivated on arginine, histidine and glutamine. Interestingly inoculation with *Raoultella* could not alleviate this glutamate-mediated repression of root and shoot growth. Compared to NH_4^+ or NO_3^- shoot and root development was weaker, but developed relative to plants cultured on urea (Fig. 17, 23).

Shoot dry weight increased significantly in inoculated plants compared to control plants (Fig. 27 A). Root development differed in dependence of applied amino acid. While root dry weight significantly increased after inoculation of plants grown on histidine and arginine (Fig. 27 B), this was not the case for glutamine. Plants cultivated on glutamine did not show significant increase in root dry weight and elongation of primary root length after inoculation (Fig. 27 B and 28 A), but elongated lateral roots (Fig. 28 B), which was not due to an increase in the number of first or second order lateral roots (Fig. 28 E and F).

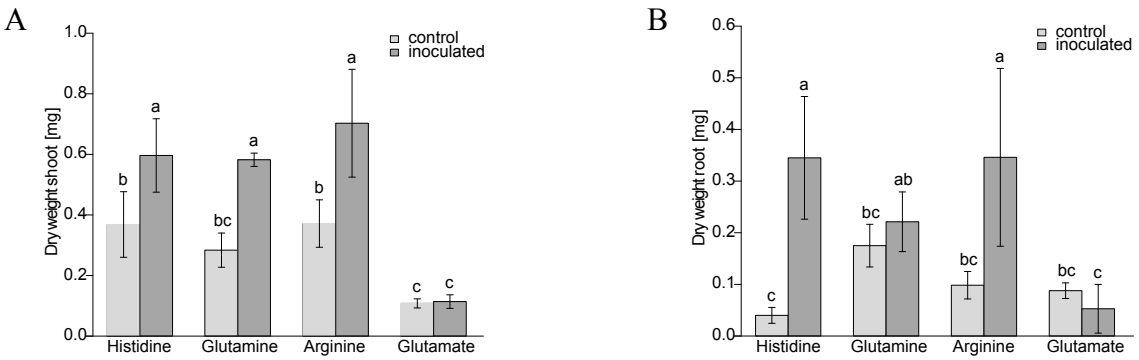


Figure 27: Inoculation with *Raoultella terrigena* TFi08N influences dry matter production in dependence of the supplied amino acid.

Shoot and root dry weights (A and B) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on histidine, glutamine, arginine or glutamate as a sole nitrogen source (1mM N) on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

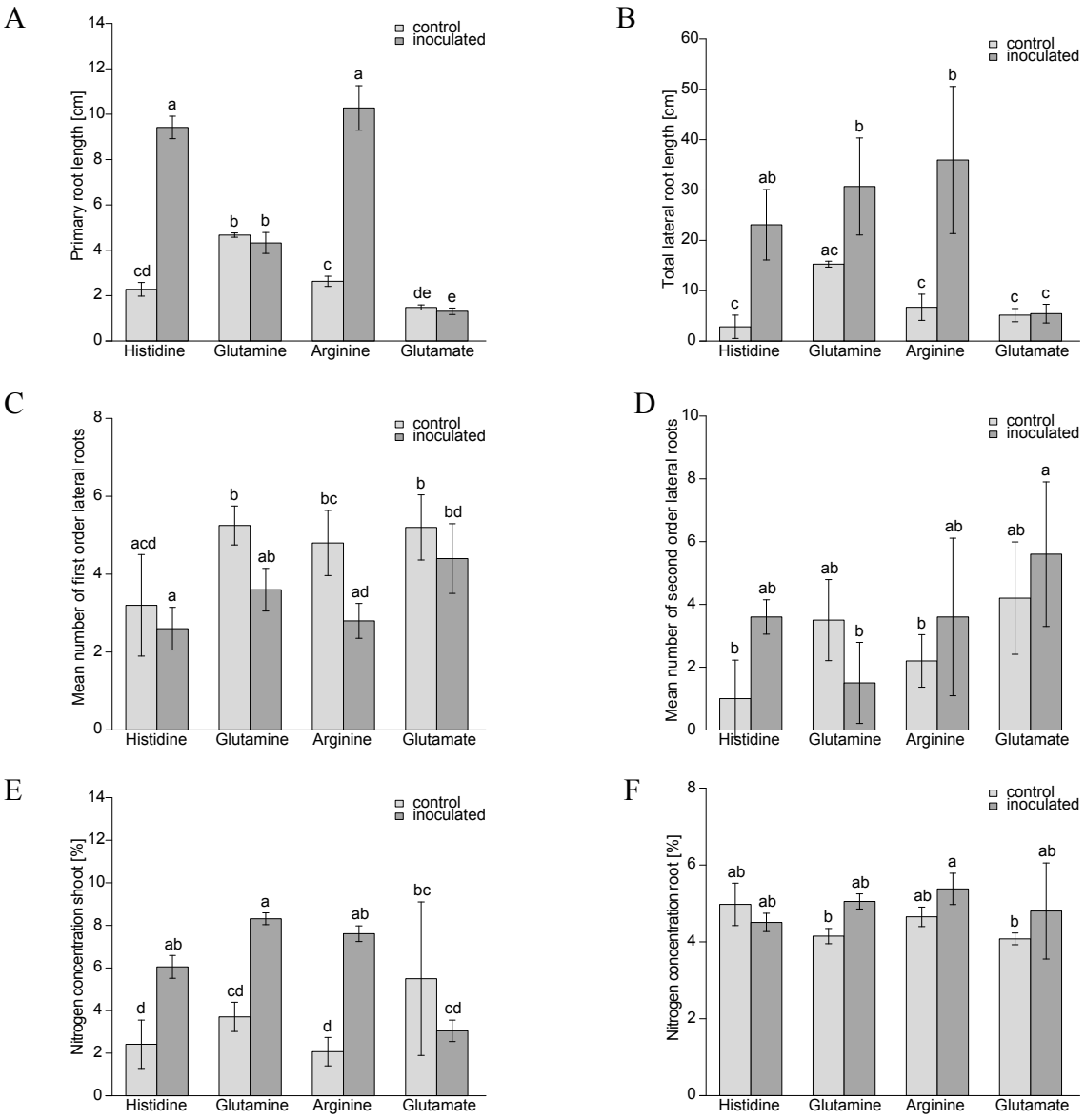


Figure 28: Inoculation with *Raoultella terrigena* TFi08N influences dry matter production in dependence of the supplied amino acid.
Primary root length (A), total lateral root length (B), number of first (C) and second (D) order lateral roots and nitrogen concentrations of shoots (G) and roots (H) of Arabidopsis plants inoculated with *Raoultella terrigena* and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on histidine, glutamine, arginine or glutamate as a sole nitrogen source (1mM N). Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

Analyses of nitrogen concentrations of shoots showed higher concentrations in inoculated compared to control plants when cultivated on histidine, glutamine or arginine (Fig. 28 E and F).

4.3.7 Influence of *Raoultella* inoculation on Arabidopsis growth on glutamate as a sole N source

Plants cultivated on glutamate as a sole nitrogen source and under non-buffered conditions were strongly inhibited in shoot and root development. To verify whether this effect was due to pH changes, the pH of the growth medium was buffered. When cultivated under a buffered pH of 5.5, pH 6.0 or pH 6.5 plants were not inhibited in development anymore (Fig. 29). Shoot dry weights of control plants increased from pH 5.5 to 6.0 (Fig. 30 A). The inhibition of primary root length under non-buffered conditions was overcome by buffering the medium so that primary root length achieved an elongation by raising pH (Fig. 30 B) and lateral roots were elongated significantly when pH was buffered (Fig. 30 C). Inoculation with *Raoultella* led to a significant higher shoot dry weight when cultured under buffered conditions at pH 5.5 but not under non-buffered conditions or buffered conditions at pH 6.0 or pH 6.5 (Fig. 30 A), while the nitrogen concentration of shoot was significantly higher in all buffered treatments (Fig. 30 D). Interestingly, primary root length of inoculated plants did not increase with raising pH like this was observed for other N forms (Fig. 20 C, 26 C, 29 and 30 B). Lateral root length was two times higher for inoculated plants compared to control plants under all buffered conditions (Fig. 30 A) and interestingly increased along the whole root axis (Fig. 29).

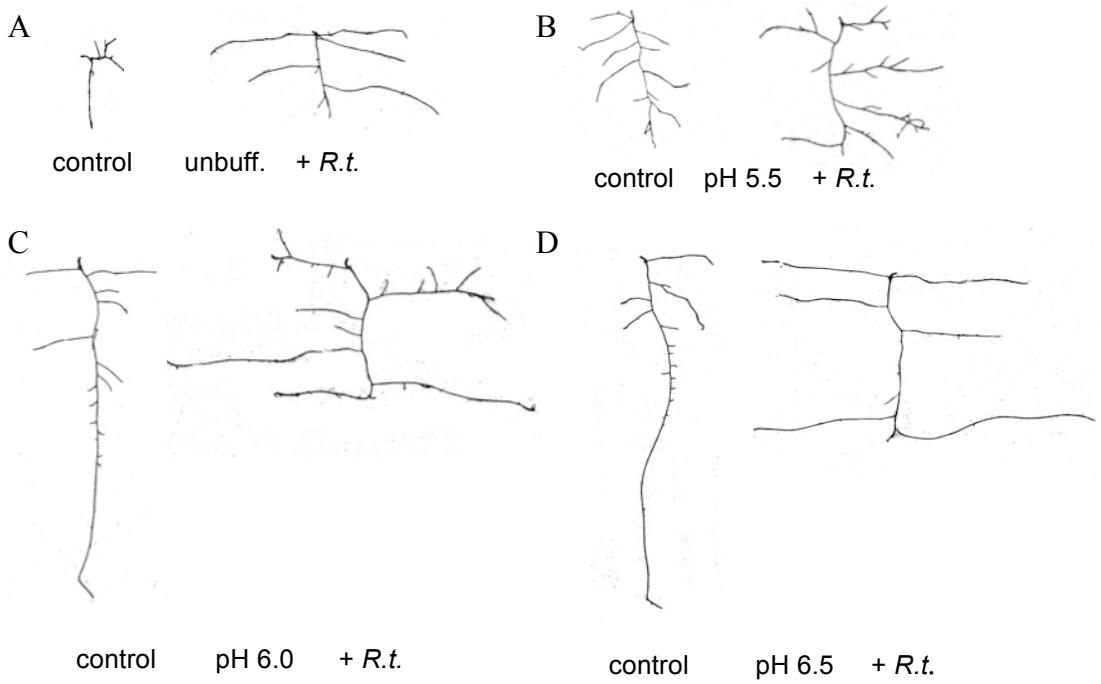


Figure 29: Raoultella affects root architecture in dependence of pH when cultured on glutamate. Phenotype of control roots and roots inoculated with *Raoultella terrigena* TFi08N (*R.t.*). Plants were cultivated on glutamate-supplied medium under unbuffered (unbuff.) conditions (A) and buffered to pH 5.5 (B), pH 6.0 (C) and pH 6.5 (D).

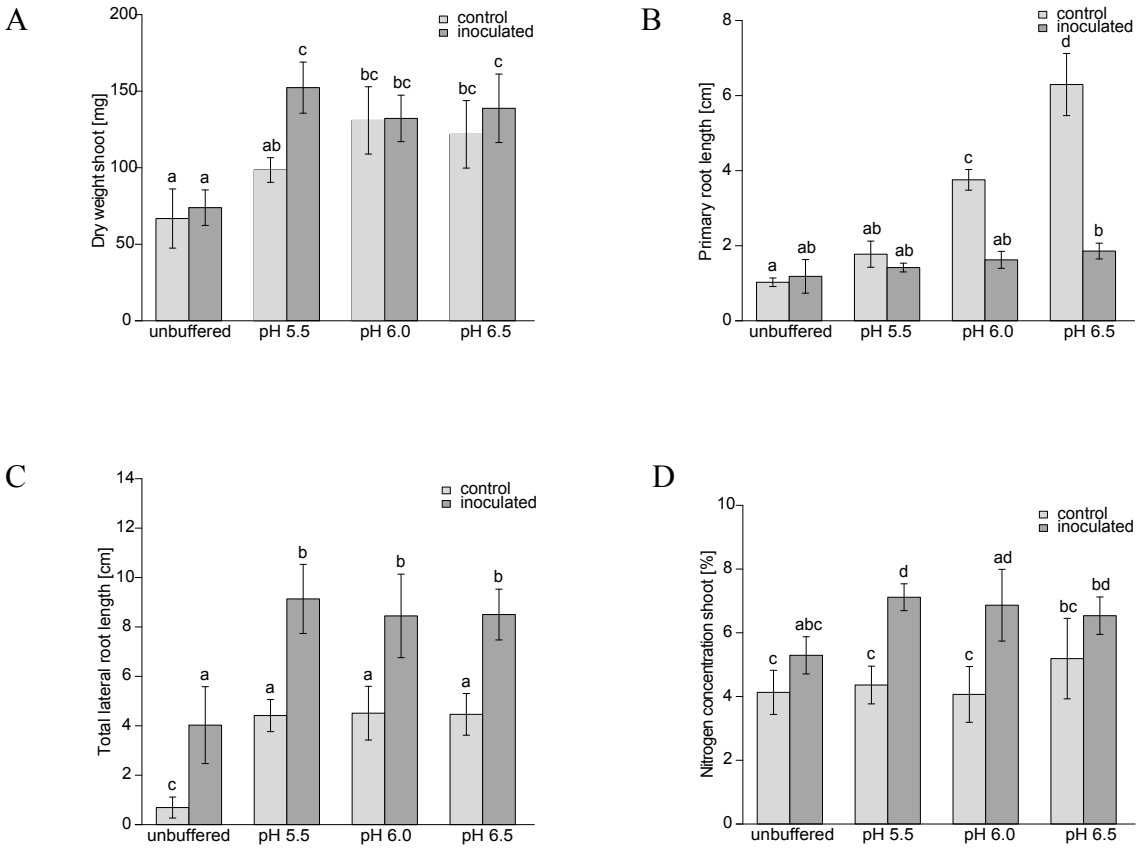


Figure 30: **Influence of glutamate and inoculation with *Raoultella* terrigena TFi08N on shoot dry matter production, root growth and nitrogen concentrations in shoots in dependence of the supplied N form and pH.**

Dry weight of shoots (A), primary root length (B), total lateral root length (C) and nitrogen concentrations of shoots (E) of Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N and control plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM glutamate. The growth medium was buffered to pH 5.5, pH 6.0 or pH 6.5 using MES. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4 Investigations on the mechanism for plant growth promotion by *Raoultella terrigena* TFi08N

4.4.1 Qualitative and quantitative investigations on changes in rhizosphere pH upon inoculation

4.4.1.1 Qualitative investigations of rhizosphere pH changes by application in agar

It was hypothesized that a *Raoultella*-mediated pH change is responsible for plant growth promotion. An agar technique was used, in which a pH indicator was added to investigate rhizosphere pH changes of plants cultivated on NH_4^+, NO_3^- or NH_4^+ with 100µM NO_3^-. Changes in pH were made visible as the pH indicator turned violet at increasing or yellow at decreasing pH.

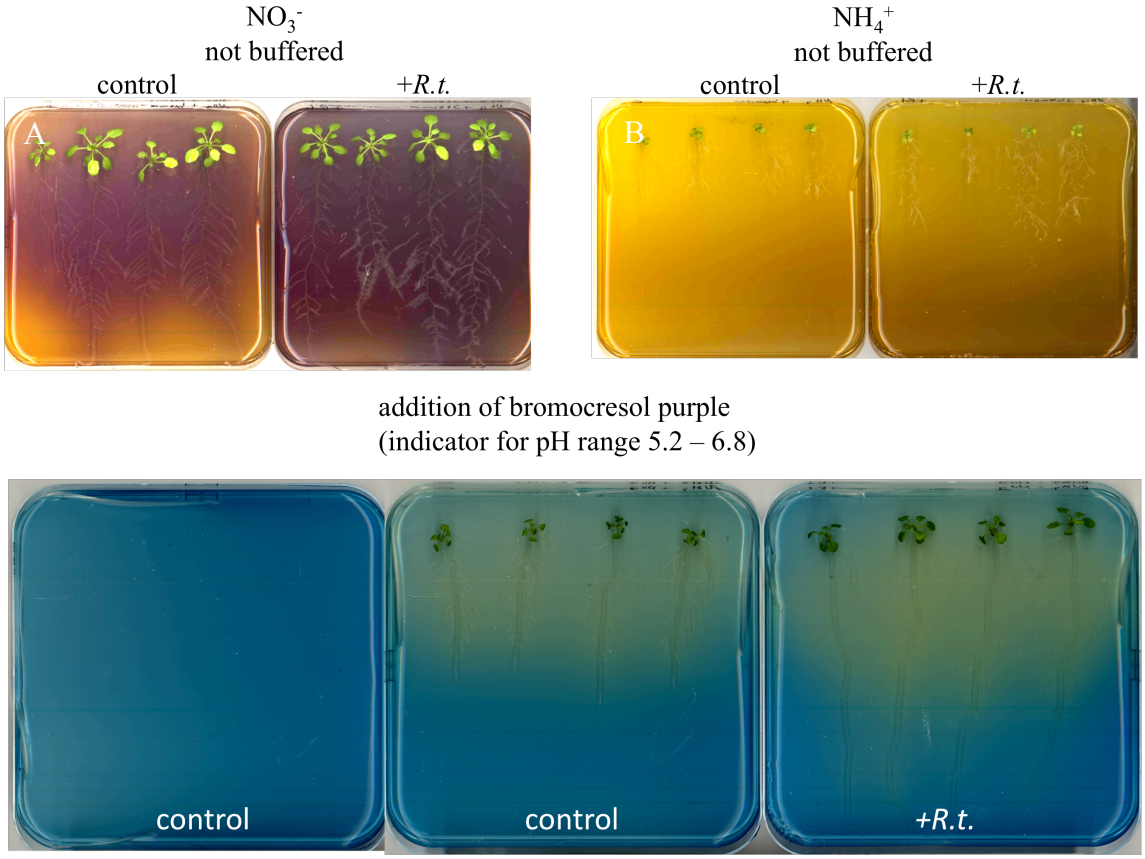


Figure 31: Influence of *Raoultella terrigena* TFi08N on the rhizosphere pH of Arabidopsis plants grown on different N sources.
pH changes of Arabidopsis plants grown on agar medium supplemented with (A) 1mM NO_3^-, (B) 1mM NH_4^+ or 1mM NH_4^+ with 100µM NO_3^- (C) on non-buffered medium. pH changes were made visible by addition of bromocresol purple (A and B) or by addition of bromocresol green (C). Six days after germination plants were transferred to treatments and cultivated for 18 days.

When plants were cultivated on nitrate as a sole nitrogen source, the pH indicator turned violet due to the physiological alkalinization (Fig.31 A). This was the case for control and inoculated plants. The color change to violet was stronger for inoculated plants. This was probably due to a larger root development. No changes in pH were made visible for plants cultivated on ammonium (Fig.31 B). Both treatments - control and inoculation - resulted in a yellowing colour of the pH indicator bromcresol purple, which corresponded to a pH of approximately pH 4. An expected change of the pH indicator to violet in the inoculated treatment, which would correspond to a *Raoultella*-mediated alkalinization, was not observed. When plants were cultivated on ammonium with addition of 10% nitrate, the pH indicator turned from blue to yellow for control and inoculated treatments, which was stronger for inoculated treatments. The observed color change of the pH indicator to yellow was due to the physiological acidification (Fig.31 C). The stronger staining of medium of inoculated treatments was probably due to the better root development of inoculated plants.

4.4.1.2 Quantitative determination of rhizosphere pH change using an antimony electrode

To investigate possible changes in rhizophere pH in more detail, measurements with an antimony electrode were done. Measurements of rhizosphere pH showed a decrease in inoculated plants compared to control plants when these were cultured on NH_4^+ with 100µM NO_3^-. This was not the case for plants grown on NH_4^+ or NO_3^- (Fig. 32).

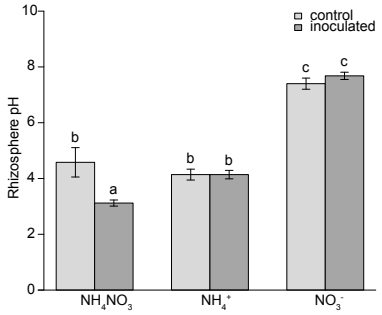


Figure 32: Influence of *Raoultella terrigena* TFi08N inoculation on rhizosphere pH changes of Arabidopsis plants grown on different N sources.
Rhizosphere pH of control plants and plants treated with Raoultella terrigena when cultivated on 1mM NH_4^+ with 100µM NO_3^-, 1mM NH_4^+ or 1mM NO_3^- on non-buffered medium. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Six days after germination plants were transferred to treatments and cultivated for 18 days. Measurements were conducted using an antimony electrode. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4.2 Investigations on the production of nitrite and nitrate by *Raoutella terrigena* TFi08N

In all cases *Raoultella* inoculation improved plant growth considerably under ammonium but much less under nitrate supply, especially when the medium pH was buffered. It was therefore hypothesized, that *Raoultella* may convert ammonium to nitrate by nitrification.

To investigate this, *Raoultella* was grown in a liquid culture assay of minimal medium, supplemented with ammonium; this experiment was kindly conducted by Joseph Strauss. Growth of *Raoultella* in liquid culture resulted in a depletion of ammonium in the early exponential growth phase of *Raoultella*. During the whole growth period of *Raoultella* a conversion of ammonium to nitrite or nitrate could not be observed, which excluded the possibility that *Raoultella* nitrified ammonium (Fig. 34).

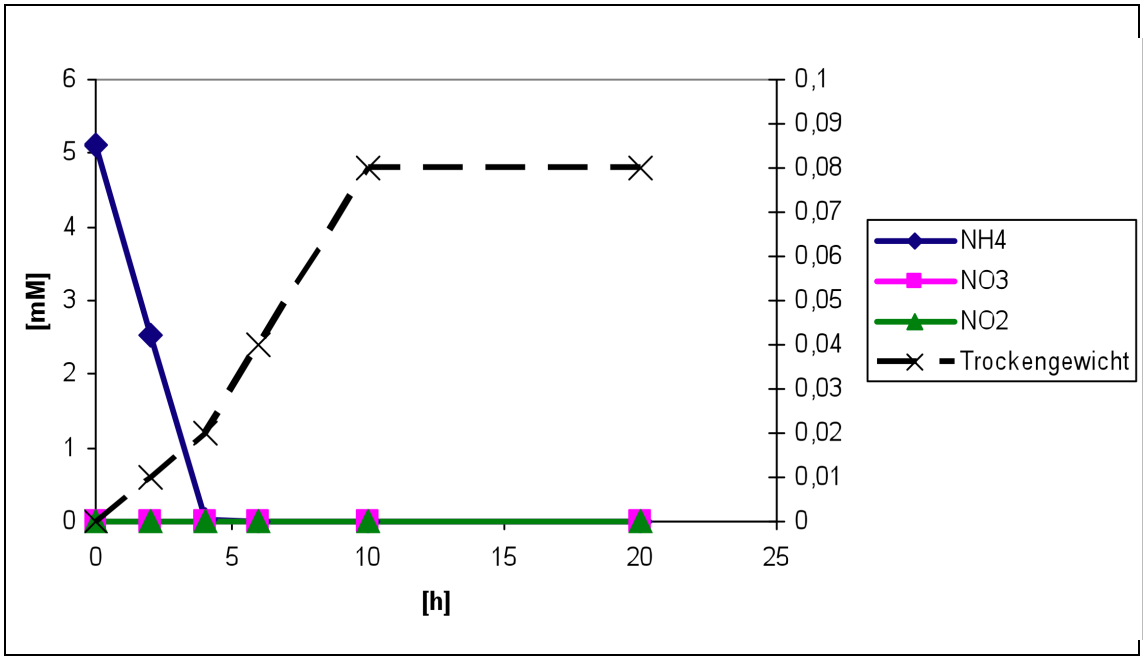


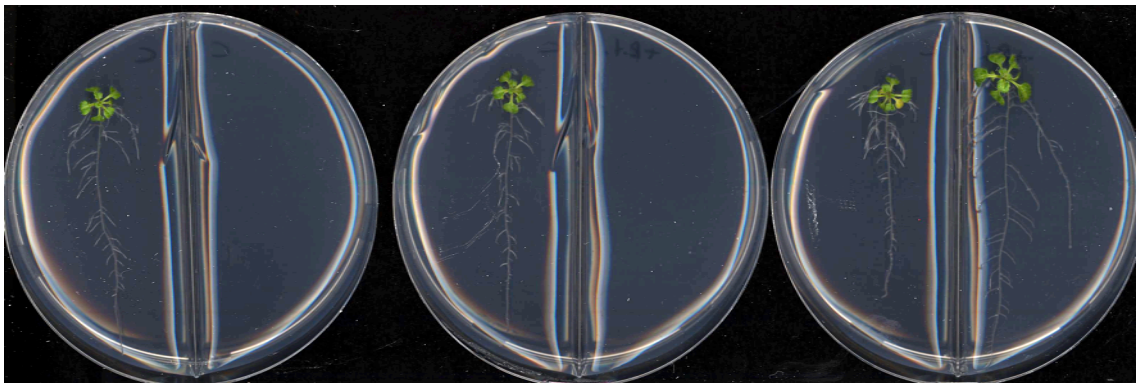
Figure 34: Growth of *Raoultella terrigena* TFi08N in a liquid culture assay.
Concentrations of NH_4^+, NO_3^- and NO_2^- and dry weight of *Raoultella terrigena* TFi08N in a liquid culture. *Raoultella* was grown in a liquid culture assay in minimal medium. Measurements were conducted for 20 hours.

4.4.3 Investigations on the release of volatile substances by *Raoutella terrigena* TFi08N

The hypothesis that the growth promotion by Raoultella is caused by volatile substances was investigated in an indirect way by the use of compartimented petri dishes (Fig.35). Control plants and plants cultivated with Raoultella were grown separately on individual agar patches but in one petri dish so that an exchange of air was possible.

Plants were cultivated on nutrient medium containing 1mM ammonium with addition of 100µM nitrate, a treatment in which *Raoultella* strongly promotes plant growth. Shoot and root dry weight increased significantly only when Arabidopsis plants were in the same compartment as *Raoultella* (Fig. 35 C and Fig. 36 A). The same observation was made for total root length, primary root length and total lateral root length (Fig. 36 B, C and D).

A



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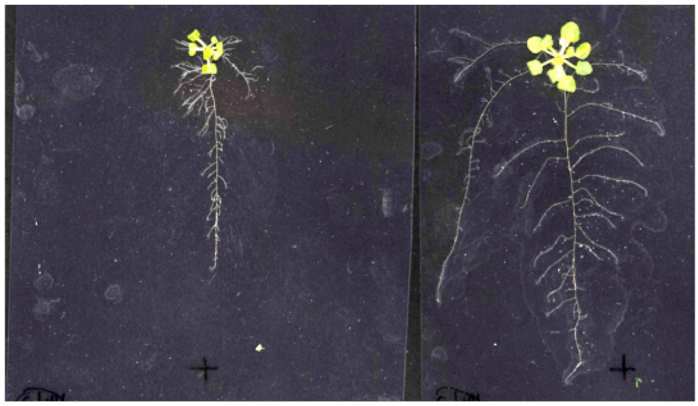


C

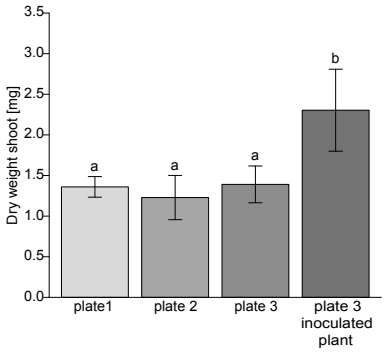


Figure 35: **Influence of the inoculation mode of *Raoutella terrigena* TFi08N on plant growth of *Arabidopsis thaliana*.**
Phenotypes (A and B) and dry weights of shoots (C) of Arabidopsis plants that were not in direct contact with *Raoutella terrigena* TFi08N. The right compartment of the petri dish was either not inoculated (1, left plate), inoculated with *Raoutella terrigena* TFi08N (2, middle plate) or inoculated with *Raoutella terrigena* TFi08N in the presence of *Arabidopsis thaliana* (3, right plate). (B) Direct comparison of plant growth in the left and right compartment of the right plate (3). (C) Shoot dry weights of Arabidopsis plants. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

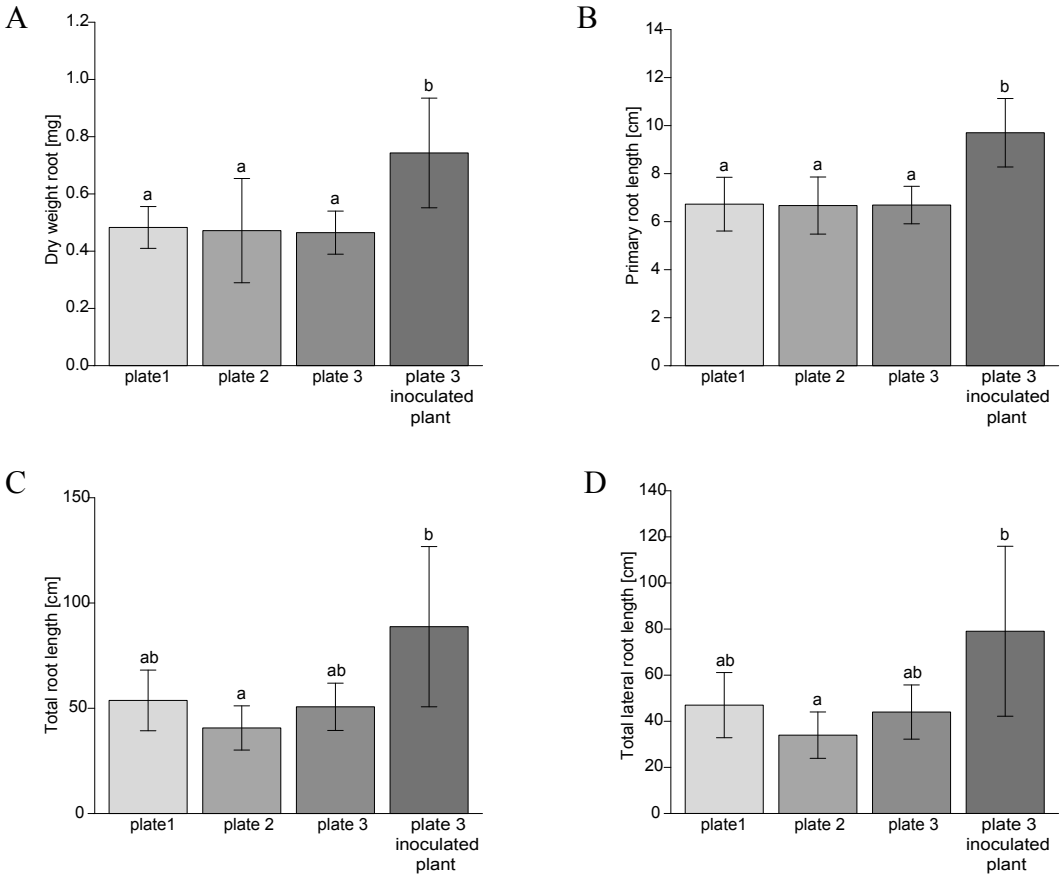


Figure 36: Influence of the inoculation mode of *Raoutella terrigena* TFi08N on plant growth of *Arabidopsis thaliana*.

Dry weights of roots (A), primary root length (B) total root length (C) and total lateral root length (D) of Arabidopsis plants that were not in direct contact with *Raoutella terrigena* TFi08N. The right compartment of the petri dish was either not inoculated (1, left plate), inoculated with *Raoutella terrigena* TFi08N (2, middle plate) or inoculated with *Raoutella terrigena* TFi08N in the presence of *Arabidopsis thaliana* (3, right plate). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

These observations indicated that plants need to be in direct contact with *Raoultella* (Fig.35 A middle plate and plate on the right side) as a growth promotion effect only appeared when plants were cultivated on bacteria-supplemented medium. If the growth promotion by *Raoultella* is caused by volatile substances, a growth promotion should be observed for plants, which did not grow in the same compartment as *Raoultella.*

4.4.4 Influence of the vacuolar and plasmamembrane H^+-ATPase on *Raoultella*-mediated growth stimulation

4.4.4.1 Influence of the vacuolar H^+-ATPase on *Raoultella*-mediated growth stimulation

As *Raoultella* inoculation led to a rhizosphere pH decrease when plants were grown on ammonium-supplemented medium (Fig. 32), it was hypothesized that the acidification capacity of Arabidopsis roots may be stimulated in the presence of these bacteria. Rhizosphere acidification will directly depend on the activity of the plasma membrane H^+ATPase, extruding protons for the cytoplasm into the rhizosphere, or may be indirectly affected by an enhanced cytosolic proton availability, which then may depend on the activity of tonoplast H^+-ATPases.

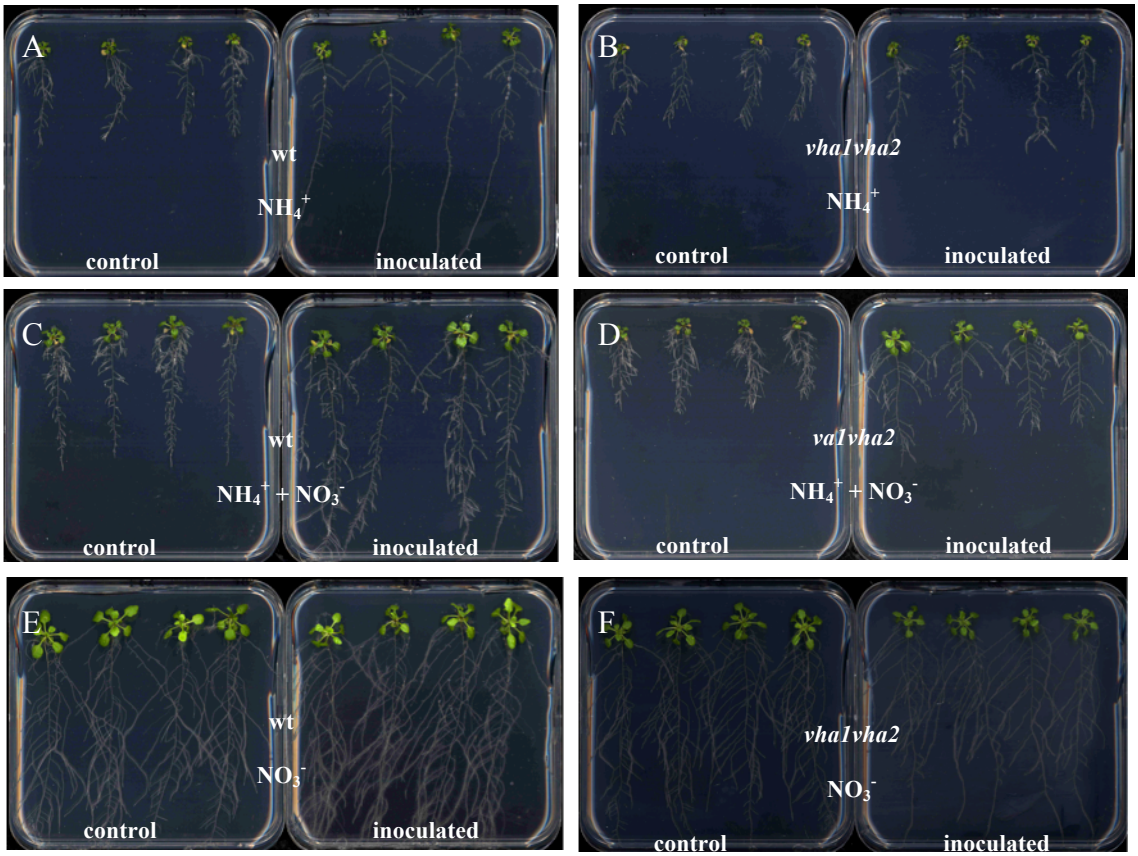


Figure 37: Influence of *Raoultella terrigena* TFi08N on growth Arabidopsis wild type plants and *vha1vha2* mutant plants.
Phenotype of wild type plants (wt) and plants defective in vacuolar ATPase activity (*vha1vha2*). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ (A and B), 1mM NH_4^+ with 100µM NO_3^- (C and D) or 1mM NO_3^- (E and F) on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 21 days.

Therefore, a mutant defective in the expression of two genes that encode subunits of the vacuolar H^+-ATPase (*vha1vha2*) was grown on *Raoultella*-inoculated agar supplemented with different N forms. In this experiment *Raoultella* inoculation tended to inverse shoot dry weight of ammonium-grown wild type plants and significantly increased shoot dry weight and primary root length when ammonium was added together with nitrate (Fig. 38 and 39 A).

A stimulation of shoot dry weight was still observed for ammonium-grown *vha1vha2* mutant plants, while primary root growth was not stimulated by *Raoultella*. Thus, the mutant response to *Raoultella* inoculation was weaker than that of wild type plants.

When plants were supplemented with nitrate, root growth of *vha1vha2* mutant plants was already poorer than that of control plants and inoculation even impaired root growth (Fig. 39 A).

Taken together, only primary root growth may require in part VHA-type vacuolar H^+-ATPases for full stimulation by *Raoultella*.

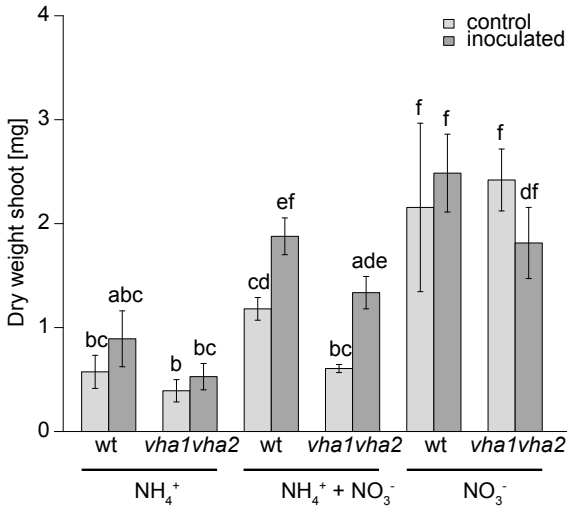
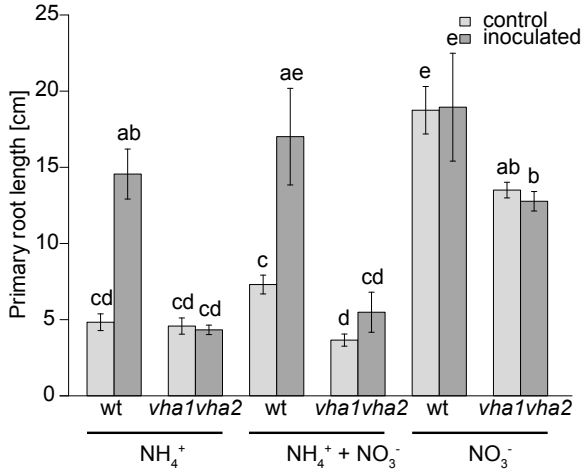


Figure 38: Influence of *Raoultella terrigena* TFi08N on shoot growth of Arabidopsis wild type plants and *vha1vha2* mutant plants.

Dry weights of shoots of wild type plants (wt) and plants defective in vacuolar ATPase activity (*vha1vha2*). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+, 1mM NH_4^+ with 100µM NO_3^- or 1mM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 21 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

A



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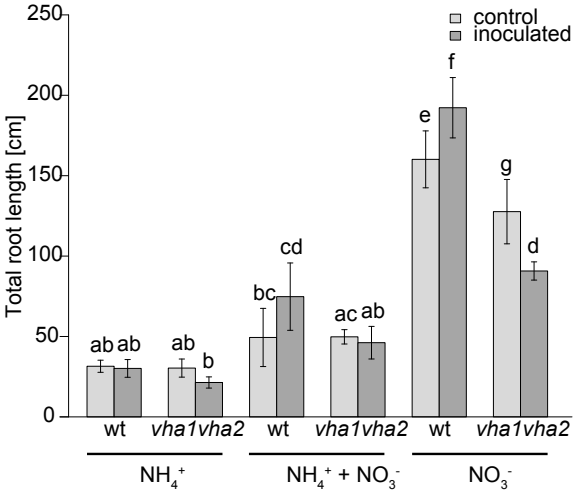

Figure 39: Influence of *Raoultella terrigena* TFi08N on root growth of Arabidopsis wild type plants and *vha1vha2* mutant plants.
Primary root length (A) and total root length (B) of wild type plants (wt) and plants defective in vacuolar ATPase activity (*vha1vha2*). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+, 1mM NH_4^+ with 100µM NO_3^- or 1mM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 21 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4.4.2 Influence of the plasmamembrane H^+-ATPase on *Raoultella*-mediated growth stimulation

To study the role of the plasmamembrane-ATPase in *Raoultella*-mediated growth stimulation, the *Arabidopsis thaliana* mutant *aha2* was used (Fig. 40). This mutant is defective in the expression of the major root plasmamembrane H^+-ATPase AHA2. While none of the root parameters were affected by *Raoultella* inoculation, shoot dry weight tended to increase in the presence of *Raoultella* (Fig. 41 A). However, standard deviations were too large to yield significant differences. Interestingly, N concentrations in shoots and roots of inoculated plants were significantly higher than in the control plants (Fig. 41 E and F). This may indicate that *Raoultella* stimulated N uptake even in the absence of AHA2 while root growth per se could not be stimulated and thus depended on a functional AHA2 protein (Fig. 41 B, C and D).

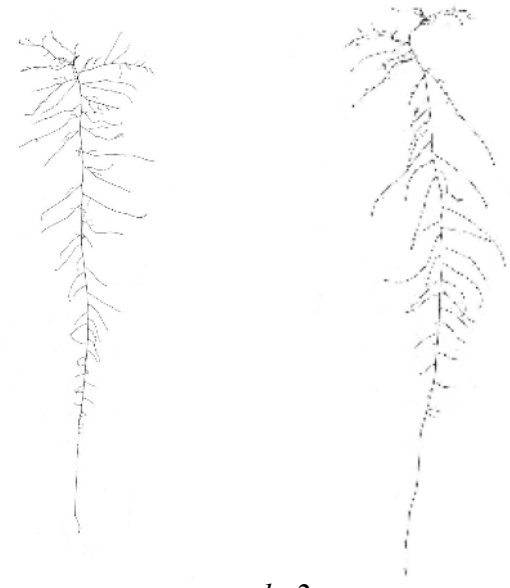

Figure 40: Root architecture of *aha2* mutant plants in dependence of inoculation with *Raoultella terrigena* TFi08N.
Root phenotype of Arabidopsis mutant *aha2*. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 21 days.

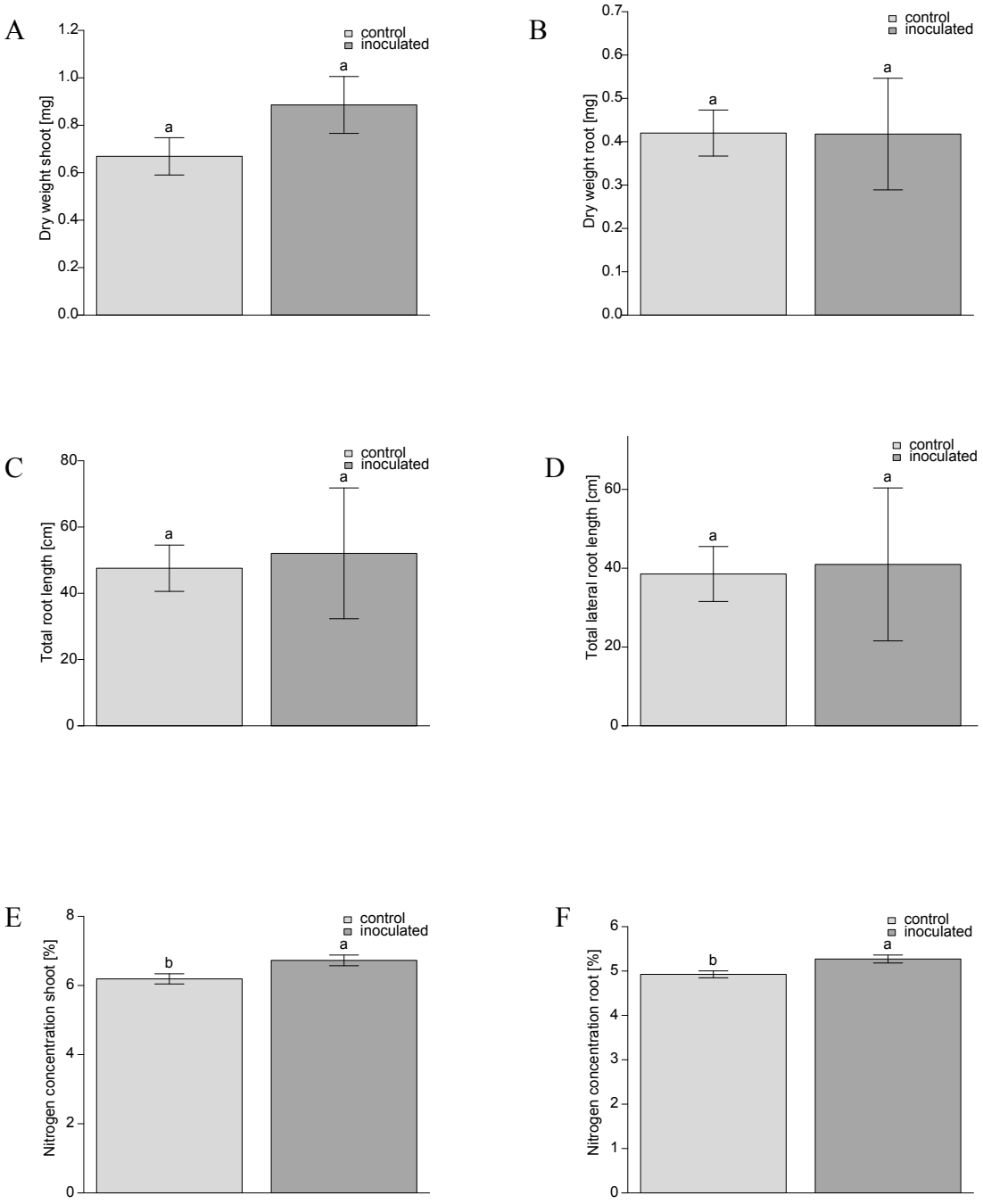

Figure 41: Plant growth of *aha2* mutant plants in dependence of inoculation with *Raoultella terrigena* TFi08N.

Dry weights of shoots (A) and roots (B), total root length (C), total lateral root length (D) and nitrogen concentrations of shoots (E) and roots (F) of Arabidopsis mutant *aha2*. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 21 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4.5 Investigations on the role of the amino acid transporter LHT1 in Raoultella-mediated plant growth stimulation

LHT1 is a high-affinity amino acid transporter expressed in the rhizodermis and the mesophyll of Arabidopsis and responsible for cellular amino acid uptake (Hirner et al., 2006). The LHT1 protein is in particular involved in the uptake and transport of histidine. Since growth promotion by *Raoultella* also occurred, when plants were cultivated on amino acids as a sole nitrogen source, it was of interest to investigate a possible release of amino acids by bacteria and further to this an enhanced uptake of amino acids by the plant. To answer this question, the Arabidopsis mutant *lht1*, defective in expression of the *LHT1* gene, was chosen to study the role of amino acid transport in *Raoultella*-inoculated plants. Figure 42 A shows that inoculated plants underwent a similar growth promotion effect as wild type plants (Fig.16), which suggests that LHT1 has no essential involvement. This was confirmed with significantly higher shoot and root dry weights (Fig. 42 B and C).

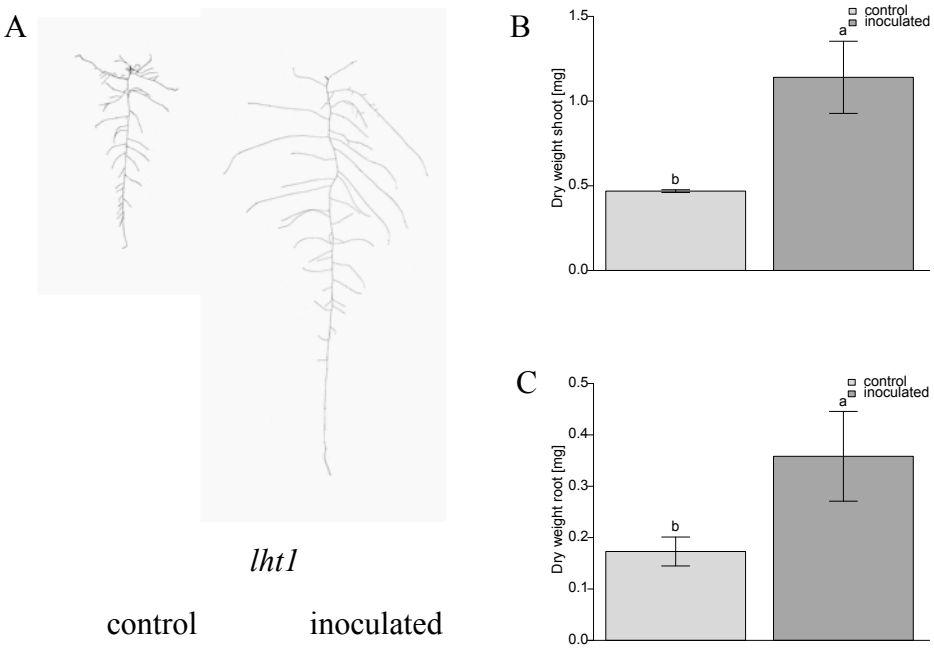

Figure 42: Plant growth of *lht1* mutant plants in dependence of inoculation with *Raoultella terrigena* TFi08N.
Root phenotype (A) and dry weights of shoots (B) and roots (C) of Arabidopsis mutant *lht1*. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4.6 Investigations on the role of urease and the urea transporter DUR3 in Raoultella-mediated plant growth stimulation

Plant growth stimulation by *Raoultella* was also obtained when plants were cultured on urea as a sole nitrogen source. To investigate whether a transport step or rather a metabolic step was involved, the contributions of the urea-hydrolyzing enzyme urease and of the high-affinity urea transporter were examined. By use of PPD, an urease inhibitor, which was supplemented to the growth medium, urea breakdown should be impaired, while by use of *dur3-1*, an Arabidopsis mutant, lacking expression of the urea transporter DUR3 urea uptake should be impaired, and thus growth stimulation by *Raoultella* when plants were grown on urea.

When plants were cultivated on urea-containing agar *Raoultella* inoculation increased shoot dry weight, primary root length and total lateral root length whereas in the presence of the urease inhibitor PPD shoot dry weight and total lateral root length remained at the level of non-inoculated plants (Fig. 43 A and 44 B). Only primary root length was stimulated by *Raoultella* also in the presence of PPD, even though to a lower extent (Fig. 44 A). The lacking growth stimulation by *Raoultella* in PPD-supplemented plants also expressed in lower N concentration (Fig. 44 C).

Exactly the same observation was made when *dur3-1* plants were assayed. This indicated first that the high-affinity urea transporter DUR3 is not required for *Raoultella*-mediated growth stimulation under these conditions, and second, that PPD-sensitive urea degradation is a prerequisite for *Raoultella*-mediated shoot biomass increase and lateral root development, but not or less required for primary root elongation.

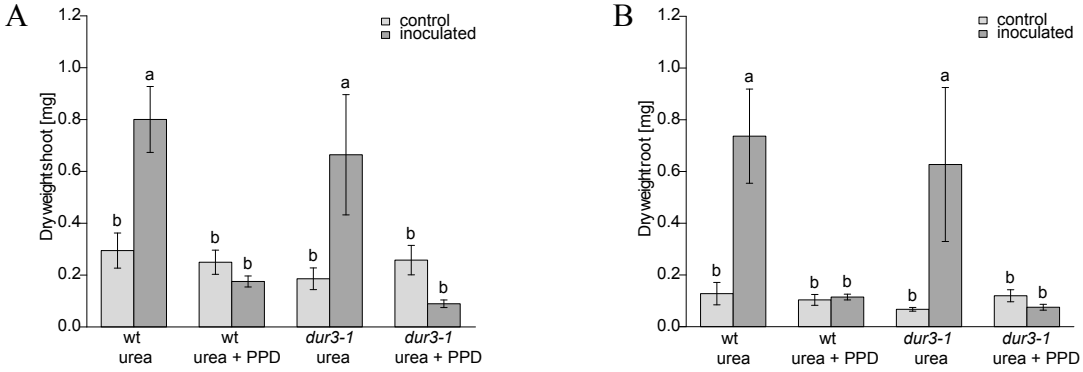

Figure 43: Plant growth of wild type and *dur3-1* mutant plants in dependence of inoculation with *Raoultella terrigena* TFi08N and the presence of PPD.

Dry weights of shoots (A) and roots (B) of Arabidopsis wild type mutant plant *dur3-1*. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea on non-buffered medium with or without PPD. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

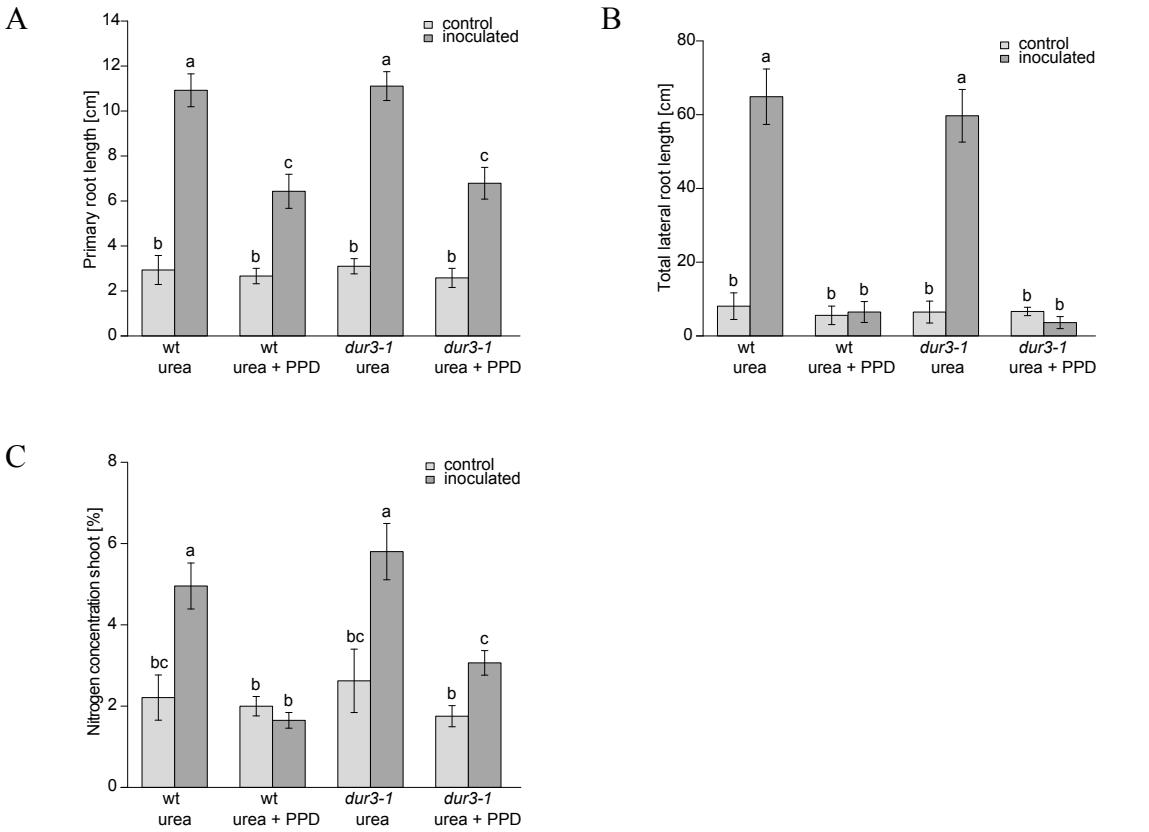

Figure 44: Plant growth of wild type and *dur3-1* mutant plants in dependence of inoculation with *Raoultella terrigena* TFi08N and the presence of PPD.
Primary root length (A), total lateral root length (B) and nitrogen concentrations of shoots (C) of Arabidopsis wild type mutant plant *dur3-1*. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea on non-buffered medium with or without PPD. Six days after germination plants were transferred to treatments and cultivated for 18 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

4.4.7 Investigations on the role of phytohormones in Raoultella-mediated plant growth stimulation

4.4.7.1 Monitoring auxin and cytokinin levels by the use of the auxin- and cytokinin reporter lines DR5::GUS and ARR5::GUS

To investigate the hypothesis that the altered root system of inoculated plants is due to a release of phytohormones by *Raoultella* and further to this mediates a change of the phytohormonal status the plant, an auxin and cytokinin reporter line were used. To monitor auxins, the Arabidopsis reporter line DR5::GUS was cultivated, while for monitoring cytokinins, the Arabidopsis reporter line ARR5::GUS was used. Plants were inoculated with *Raoultella terrigena* and cultivated on 1 mM NH_4^+ + 10 % NO_3^-. Roots of ARR5::GUS plants indicated elevated cytokinin levels in the apical root zones from the tip over the meristematic zone up to the transition zone. In inoculated plants,

cytokinin levels appeared to be slightly lower and the cytokinin reporter was only expressed in the apical root zones, not extending to the transition zone anymore (Fig. 45 A and B). For DR5::GUS reporter lines roots indicated elevated auxin levels in the meristematic zone up to the transition zone, while in inoculated plants the auxin reporter was not expressed (Fig. 45 C and D).

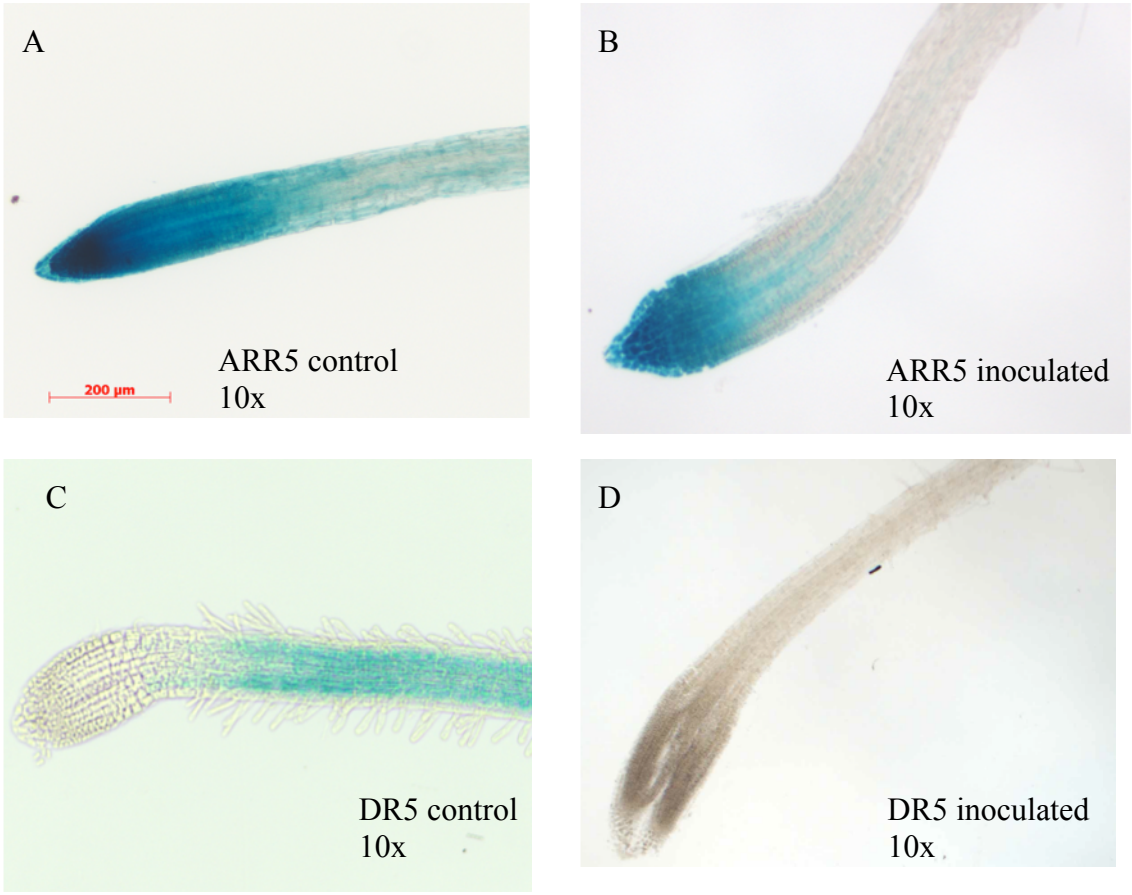

Figure 45: Analysis of auxin and cytokinin reporter lines under inoculation with *Raoultella terrigena* TFi08N.
(A and B) Localization of GUS expression in lateral roots of ARR5::GUS reporter lines under control (A) or inoculated conditions (B). (C and D) Localization of GUS expression in lateral roots of DR5::GUS reporter lines under control (C) or inoculated conditions (D). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM urea on non-buffered medium with or without PPD. Six days after germination plants were transferred to treatments and cultivated for 18 days.

4.4.7.2 Determination of phytohormones in plant material of control and inoculated plants

As Raoultella may stimulate the endogenous phytohormone production, an experiment was conducted to determine phytohormone concentrations in shoots and roots of control to inoculated plants. However, using an radio-immuno assay it was not possible to determine phytohormones, as the amount of plant material was not sufficient for replicated analysis.

4.5 Investigations on the localization of *Raoultella terrigena* TFi08N in roots

4.5.1 Localization studies using GFP-tagged *Raoultella terrigena* TFi08N strains

For the study of PGPR it is important to know in which part of the root PGPR are localized. To investigate root colonization by *Raoultella terrigena* TFi08N Arabidopsis plants were inoculated with GFP-tagged *Raoultella terrigena* TFi08N strains. The strain was kindly provided by the Rhibac project partner Joseph Strauss. The growth response to inoculation with transgenic *Raoultella* was comparable to that of the wild-type strain. Unfortunately, Raoultella could not be localized at the root surface by the available microscopic techniques.

4.5.2 Root colonization assay using *Raoultella terrigena* TFi08N

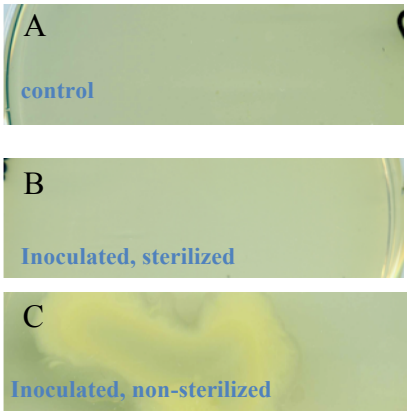

Figure 46: Growth assay of *Raoultella terrigena* TFi08N on agar.
Arabidopsis plants were grown on ½ MS-medium in the absence or presence of Raoultella for 18 days. Roots were harvested, grinded and a dilution of the root suspension was spread on YEP agar. (A) non-inoculated control plants, (B) inoculated plants after surface sterilization of roots, (C) inoculated plants without surface sterilization of roots.

To investigate, if *Raoultella terrigena* colonizes the surface of the intracellular space of root cells, an experiment was conducted in which root extracts of inoculated Arabidopsis plants were plated on agar medium. Arabidopsis seeds were germinated and seedlings were transferred after 6 days on square plates and inoculated with *Raoultella terrigena* as described before. 1mM NH_4^+ + 10 % NO_3^- was added as a nitrogen source. 16 days after inoculation roots were harvested, grinded and spread on YEP medium to test bacterial growth. Grinded root suspension, which was not surface sterilised after harvest showed bacterial growth on YEP medium, while a suspension of

roots sterilized for 1 min with ethanol did not yield bacterial growth anymore (Fig. 46). This suggested that Raoultella was mainly adhering at the surface of Arabidopsis roots. As this is only an indirect method further investigations should be undertaken to monitor root colonization of GFP-tagged *Raoultella* by microscopy.

4.6 Influence of phosphorus supply on *Raoultella*-mediated root growth stimulation

It has previously been shown that PGPR contribute to the solubilization of soil phosphorus. To investigate the influence of *Raoultella* on root growth under P deficiency, Arabidopsis plants were grown on P-deficient nutrient medium. While Arabidopsis plants grew poorly on P-deficient medium and primary roots were severely inhibited in elongation, inoculation with *Raoultella* strongly stimulated primary and lateral root elongation, leading also to slightly enhanced shoot growth (Fig. 47).

Figure 47: Influence of P supply on *Raoultella*-stimulated root growth
Phenotype of Arabidopsis control plants (right side) and Arabidopsis plants inoculated with *Raoultella terrigena* TFi08N (left side) on nutrient medium without phosphorous. *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium without phosphorus. Six days after germination plants were transferred to treatments and cultivated for 18 days.

4.7 Different response to ecotypes of *Arabidopsis thaliana* accession lines to inoculation by *Raoultella terrigena*

Besides Columbia-0 three further ecotypes of *Arabidopsis thaliana* were used for inoculation experiments to investigate the growth response to *Raoultella terrigena* TFi08N in dependence of the plant genotype (Fig. 48 A-C). Non-inoculated plants already differed a lot in their root morphology: WS plants developed a largely extended root system with primary roots reaching almost the bottom of the plate after 20 days. By contrast, Col-gl and in particular Aa plants performed poorly with roots reaching

approximately half of the root length of WS plants. This poor root development of Aa was overcome by inoculation with *Raoultella*. In fact, inoculated Aa plants even reached the highest shoot dry weight of all three genotypes. Thus, *Raoultella* alleviated genetically determined constraints in root development.

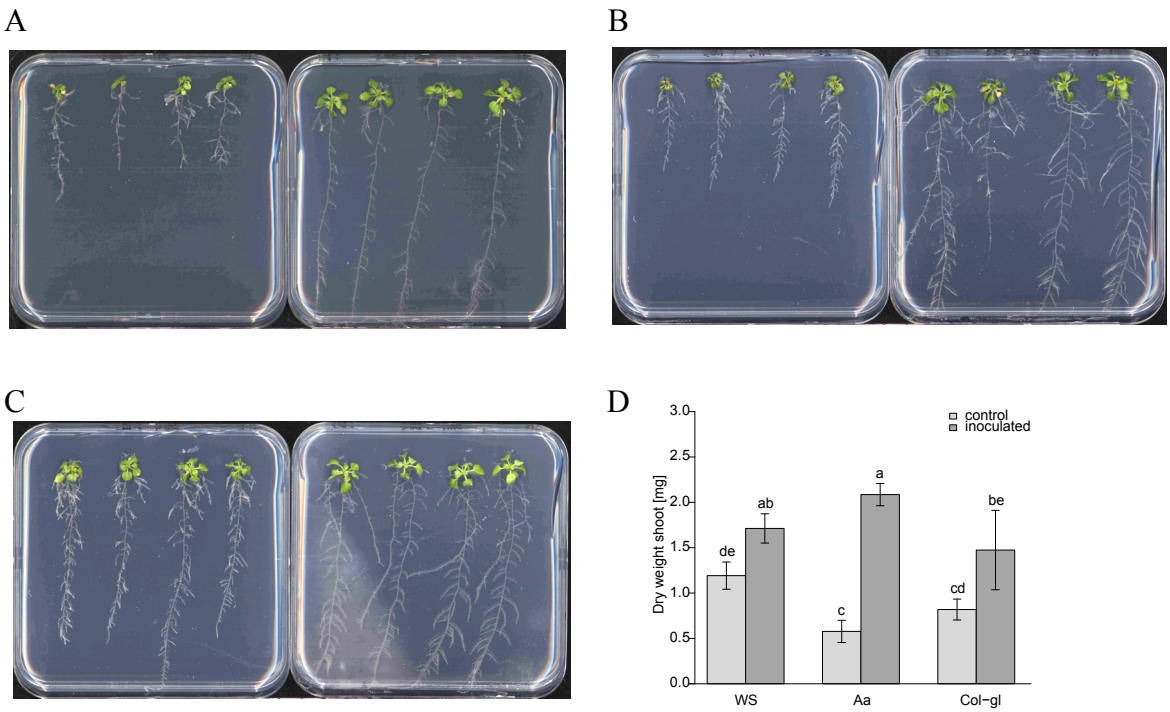

Figure 48: Arabidopsis ecotypes responds different to inoculation with Raoultella
Plant phenotype of Arabidopsis ecotypes Aa (A), Col-glabra (B) and WS (C) and dry weights of shoots (D). *Raoultella* was inoculated at a concentration of 10^8 cfu mL^{-1} after cells were harvested from the exponential growth phase. Plants were cultivated on 1mM NH_4^+ with 100µM NO_3^- on non-buffered medium. Six days after germination plants were transferred to treatments and cultivated for 20 days. Bars represent means +/- SD; n=5. Significant differences at $p \leq 0.05$ are indicated by different letters.

5 Discussion

To substitute or complement mineral fertilizer application by PGPR, a reliable action of bacteria is of importance and thus it is necessary to determine factors disturbing the plant-bacterial associations and to understand the mechanisms, which are behind the growth promotion effect. To investigate plant-bacterial-associations and mechanisms responsible for plant growth promotion by bacteria, it is of advantage to have an easy, fast and reliable system that allows investigating bacterial plant growth promotion effects. A few decades of field experiments with associative PGPR, have resulted in approximately 60-70 % of success with an average yield increase of 5-30 % by inoculation (Okon and Labandera-Gonzalez, 1994). Many greenhouse and field experiments were conducted with varying results (Lucy et al., 2004, Bashan et al., 2004, Kennedy et al., 2004, Baldani and Baldani, 2005) and the mechanisms for plant growth promotion are still not fully understood. Besides nitrogen fixation, the release of phytohormones and enzymes by bacteria, enhanced mineral element uptake, biotic and abiotic stress control are under discussion (Lin et al., 1983, Steenhoudt and Vanderleyden, 2000, Compant et al., 2005). However, agro-ecosystems are complex systems and therefore site-specific factors may influence plant-bacterial-associations from case to case.

In this thesis a model growth system has been established in which Arabidopsis plants are cultured in the presence of PGPR to investigate mechanisms responsible for growth promotion. As it was decided to conduct the experiments under axenic conditions to exclude disturbing effects of co-existing bacteria, an agar plate system was chosen. In particular for the verification of newly isolated bacteria, the plate system used in this thesis offers a good possibility to describe their action on plant growth, which would eventually be not detected due to disturbing conditions when investigated in greenhouse or field experiments. For different reasons *Arabidopsis thaliana* was used as a model plant. Arabidopsis is easily cultivated under sterile conditions due to its small size. A further advantage of Arabidopsis is the large amount of mutants, which allow investigations on the mechanisms for growth promotion. Arabidopsis is easy to handle and the agar plate system is a relatively rapid approach as results are obtained after 3-4 weeks. In a further step, after being evaluated as positive, these bacteria should be tested in agricultural crops and under non-sterile conditions in greenhouse or field trials, as the final aim is to use PGPR for agricultural crops as biofertilizers.

In frame of the Rhibac project several bacterial strains were studied and growth promotion effects were determined and confirmed for the following strains: *Bacillus subtilis* OSU142, *Bacillus megaterium* M3, *Azospirillum brasilense* and *Raoultella terrigena*. For the two species *Pseudomonas fluorescens* C139 and *Paenibacillus polymyxa*, plant growth promotion was not observed. Further investigations should be undertaken to characterize these bacterial species, as it is still possible that they are potential PGPR but may require different growth conditions or inoculation densities. As *Raoultella terrigena* is a newly isolated bacterium (in frame of the Micro-N-Fix project) and has been positively tested for its ability to fix atmospheric nitrogen using by the acetylene reduction assay and the expression of a nitrogenase gene, it was decided to investigate this bacterial strain as a possibly new PGPR more in detail and to study mechanisms of its growth promotion.

5.1 Plant growth stimulation by *Raoultella terrigena* TFi08N is dependent on its growth phase

At any growth stage between OD 1.0 and OD 2.5, *Raoultella terrigena* TFi08N, mediated an increased dry weight of shoots and roots, and an improved root morphology, as described in enhanced total and primary root length (Fig. 12). Higher OD´s, i.e. inoculation with *Raoultella terrigena* harvested at later growth stages, did not result in significant growth stimulation (Fig. 12). In contrast, inoculation with *Azospirillum* yielded an enhanced shoot biomass formation at any of the tested OD´s (Fig. 8). Reasons for plant growth stimulation is dependence of the bacterial growth stage may be found in their phytohormonal production, which may occur at later growth stages only (Timmusk et al., 1999). Actually, this also held true for auxin production by *Raoultella*. Some publications suggest that the plant only can profit from inoculation, when the bacteria are dead and when nutrients become available for the plant after mineralization of bacteria (Okon et al., 1983).

Independent of the growth phase of inoculated bacteria it is of particular interest, when bacteria are applied under field conditions. As a farmer, who has neither laboratory equipment nor time and knowledge of cultivating bacterial strains, the application procedure should be as easy as possible. A method to obtain an easy application is to lyophilize bacteria. Lyophilized bacteria can be handled like mineral fertilizers, which is of advantage when no growth conditions of bacteria have to be considered, but their viability should be taken for granted and should remain reliable.

5.2 Plant stimulation is dependent on bacterial cell concentration of *Raoultella terrigena* TFi08N

Inoculation with different bacterial concentrations of *Raoultella* resulted in shoot growth stimulation, when bacteria were applied at concentrations of 10^7 and 10^8 cfu mL^{-1} (Fig. 14 A). With regard to a concentration of 10^9 cfu mL^{-1} no effect due to inoculation was observed. It was already reported that a successful inoculation requires an optimal bacterial concentration and that in case of a concentration between 10^5–10^6 cfu mL^{-1} can result in success (Bashan, 1985, Puente et al., 2009). However, inoculation experiments are difficult to compare, as greenhouse and field experiments differ regarding inoculum application (i.e. seed or seedling application). The optimum bacterial cell concentration can be related to phytohormone production by bacteria, like it has been reported for bacterial growth (Timmusk et al., 1999). Furthermore phytohormones act in very low concentrations and affect plant growth in a positive or negative manner. This was observed in a bioassay, when IAA was added to the growth medium of Arabidopsis plants. By increasing concentrations of IAA root development was inhibited. A shortening of primary roots was observed, while lateral root growth was enhanced (Weishaar, 2007). The effect on wheat root development by inoculation of Azospirillum could be mimicked with an addition of Trp which serves as a precursor for bacterial auxin production (Dobbelaere et al., 1999). It can be expected that large numbers of bacteria produce large amounts of IAA and then may lead to inhibitory effects on plant development.

5.3 *Raoultella terrigena* TFi08N stimulates plant growth in dependence of the inorganic nitrogen form and pH

As root development differed when roots were cultivated on ammonium or nitrate supplemented medium (Fig. 16), a series of experiments was conducted to investigate the role of the supplied nitrogen form in bacteria-stimulated plant growth. Inoculated plants cultivated on ammonium, nitrate or ammonium with addition of nitrate responded positively to bacterial inoculation with *Raoultella terrigena* (Fig. 16). But the response was strongest, when plants were cultivated on ammonium. The fact, that *Raoultella*-treated plants cultivated on ammonium showed a strong response to inoculation suggested that *Raoultella* is able to overcome ammonium toxicity. No differences in the growth of *Raoultella* in dependence of the nitrogen form were determined when *Raoultella* was grown in liquid culture (data not shown), suggesting that Raoultella may

develop and amplify on agar plates irrespective of the supplied N form. In control plants, the nitrogen form led to differences in root architecture of control plants, in particular when cultured under non-pH buffered conditions due to a physiological acidification by plants of ammonium-supplied medium or physiological alkalinisation of nitrate-supplied medium (Britto and Kronzucker, 2002, Marschner, 1995). In the absence of *Raoultella* plants cultured on ammonium were inhibited in their growth (Fig. 16). Primary root length was shorter compared to plants cultivated on nitrate and lateral roots developed first-, second- and third-order lateral branches (Figs. 17 C, F, G and H). Lateral root initiation has been shown to be stimulated under local ammonium supply, while lateral root elongation has been related to nitrate supply (Lima et al., 2010, Zhang et al., 1999, Remans et al., 2006). Plants supplied with nitrate developed only first-order lateral roots, but the length of their lateral roots was much higher compared to ammonium-supplied plants. Inhibited root development is a part of the ammonium-toxicity symptom and has been explained by Britto and Kronzucker (2002).

To exclude that the observed plant growth stimulation by *Raoultella* is due to a pH effect, the nutrient medium was buffered. A range between pH 5.5 and pH 6.5 was chosen and proved to be suitable for plant growth promotion effects (Fig. 19). Control plants, which were supplied with ammonium and buffered to pH 6.5 were able to develop in a similar way to plants supplied with nitrate under non-buffered conditions. Thus, whenever plants reached an optimal growth, under control conditions without inoculation no growth promotion due to inoculation was observed (Fig. 20 A). Regarding root dry weight of ammonium-grown plants only at a pH, buffered to 5.5 a significantly higher root dry weight was measured in inoculated plants compared to non-inoculated plants (Fig. 20 B). With respect to other root parameters significant elongation of primary root length and total root length of inoculated plants were measured in case of both nitrogen forms at pH 5.5 (Fig. 20 C-E). Different hypotheses were generated to explain the growth promotion effect under ammonium nutrition after inoculation with *Raoultella terrigena*. It was proposed, that *Raoultella terrigena* is able to convert ammonium to nitrite or nitrate. This was investigated in a separate culture experiment with *Raoultella* (chapter 5.6). Furthermore it was suggested, that *Raoultella terrigena* is able to increase rhizosphere pH. This was studied by determination of rhizosphere pH and by use of Arabidopsis mutants defective in expression of the plasmamembrane or vacuolar ATPase (see chapter 5.10). Furthermore it was hypothesized, that *Raoultella terrigena* is able to release gaseous substances like

ammonia or the phytohormone ethylene. This was investigated in an indirect experiment by using separated agar plates (see chapter 5.6). The obtained results suggested that when plants are cultivated under suboptimal conditions, like this is the case for plants cultivated under ammonium under unbuffered conditions, *Raoultella* is able to overcome plant growth suppression.

5.4 *Raoultella* stimulated plant growth when cultivated on urea

The cultivation of plants on the organic nitrogen source urea leads to a strong growth promotion by inoculation after Raoultella (Fig. 22). Dry weights of shoots and roots (Fig. 23 A and B), root parameters (Fig. 23 C-F) and nitrogen concentrations of shoot (Fig. 24 A) were significantly higher in inoculated plants compared to control plants. The enhanced total root length resulted from elongated primary and lateral roots and not from an increase in the number of lateral roots, as the mean number of first order lateral roots of inoculated plants was lower compared to non-inoculated plants (Fig. 23 F). As urease is a Ni-containing urea hydrolase Ni was added to the medium, so that an ineffective utilization of urea-N could be excluded. Compared to plants cultivated on inorganic nitrogen sources, plant development of urea grown control plants was poorer. To exclude pH effects, urea was supplied to nutrient medium buffered to pH 5.5, pH 6.0 or pH 6.5. Like for unbuffered conditions a strong growth stimulation of inoculated plants was observed (Fig. 25), which was strongest when plants were cultivated at a pH of 6.0. As it was the case for inoculated plants cultured on ammonium *Raoultella* was able to overcome urea-suppressed plant growth. In addition, the improved plant development may have also resulted from higher nitrogen concentrations in inoculated shoots (Fig. 26 E). A higher root uptake or hydrolysis, assimilation or translocation of urea within the plant might have been involved and responsible for the higher nitrogen concentration in the shoots. As no differences between inoculated and non-inoculated plants were determined for roots, the assimilation and translocation of urea-N are most likely to explain increased nitrogen concentrations of the shoots and thus for enhanced plant growth.

To investigate whether urea transporters or the enzyme urease were involved in the improved uptake and use efficiency of urea, the corresponding Arabidopsis mutants were employed. However, the high-affinity urea/H^+ symporter DUR3 did not play a role in *Raoultella*-mediated plant growth promotion (Fig. 43), since the Arabidopsis mutant *dur3-1* showed the same response to *Raoultella* as wt plants (Fig. 43), which indicates,

that already a hydrolysis of urea in the growth medium could be involved, like this could be due to a release of the enzyme urease by Raoultella.

The role of urease in plant growth stimulation was evaluated in another experiment. PPD, a urease inhibitor was used to investigate the role of the hydrolysis of urea. No growth promotion was observed anymore in inoculated plants when PPD was added (Fig. 43). Primary root length of inoculated plants was elongated, but not as much as in the absence of the inhibitor (Fig.43). These results may suggest that *Raoultella* is also inhibited in growth after addition of PPD to the medium and therefore not able to stimulate plant growth. To exclude a growth inhibition of *Raoultella*, *Raoultella* should be investigated in liquid culture supplemented with PPD. It is expected, that *Raoultella* produces the enzyme urease, which may allow a better and faster assimilation and transport of urea in the plant. PGPR and most other bacteria are known to produce urease and *Azospirillum* has been shown to modify soil urease activity (Todd et al., 1986, Perotti and Pidello, 1999).

5.5 Influence of bacteria on root development in dependence of amino acids

When inoculated with *Raoultella*, plants cultivated on the amino acids arginine, histidine or glutamine as a sole nitrogen source led to growth stimulation, (Fig 27). These results indicated that *Raoultella* is able to overcome amino acid-dependent growth suppression as it was observed for ammonium or urea under non-buffered conditions.

Interestingly by cultivation of plants on glutamate in pH-buffered medium primary root growth inhibition was overcome by *Raoultella* (Fig. 29). Shoot growth responded positively to inoculation when medium was buffered to 5.5 (Fig. 30 A), and an interesting observation was, that primary root growth of inoculated plants was inhibited when plants were cultivated on medium buffered to pH 6.0 or pH 6.5 (Figs. 30 B). The Arabidopsis mutant *lht1*, lacking expression of the LHT1 transporter was inoculated with *Raoultella* to investigate the role of the amino acid uptake capacity in plant growth stimulation. The development of control plants was comparable to wild type plants (Hirner et al., 2006). A growth promotion effect was observed for inoculated *lht1* plants. The transporter is expressed in both the rhizodermis and mesophyll of Arabidopsis (Hirner et al., 2006). An influence on plant growth due to a release of amino acids by bacteria, which could be part of the promotion effect on plant growth could not be shown, as a strong plant growth stimulation was observed in *lht1* plants, suggesting that

amino acids released by bacteria play no role. However further investigations should be undertaken, by measuring bacterial culture and plant material for amino acid concentrations as well as culturing of *lht1* plants on arginine, as LHT1 cannot use arginine as a sole nitrogen source.

5.6 No evidence for the production of volatile plant growth-promoting substances by *Raoultella*

Theoretically, plant growth stimulation of inoculated plants cultivated on ammonium may be due to the release of substances by *Raoultella* to feed plants. This possibility was investigated in an indirect approach by the use of separated petri dishes. No growth promotion effect was observed for Arabidopsis plants, which were not in direct contact with *Raoultella* (Fig. 35). This indicated, that the mechanisms of growth stimulation by *Raoultella* is probably not caused by volatile substances ammonia or the phytohormone ethylene, even through this requires further investigations, e.g. by measurements of ethylene and ammonia in bacterial cultures. In the liquid culture assay, which was kindly conducted by a project the partner J.Strauss *Raoultella terrigena* produces neither ammonium nor nitrate or nitrite (Fig. 34).

5.7 No evidence for nitrogen fixation in *Raoultella*-dependent plant growth promotion

To investigate if the growth promotion effect can be explained by nitrogen fixation, the plant medium was labelled with ^{15}N (^{15}N-isotope dilution technique). If symbiotic or associative bacteria fix atmospheric nitrogen, which is not enriched with ^{15}N, plants will have a lower ^{15}N concentration. The ^{15}N isotope dilution technique suggested that plants inoculated with *Raoultella terrigena* did not release ^{14}N to the plant. The ability for nitrogen fixation was demonstrated for *Raoultella* by an acetylene reduction assay and by expression of the nitrogenase gene. N_2-fixation was one of the first mechanisms suggested to promote the growth of plants inoculated with PGPR. However results of the last decades have generated controversy, as some investigations proved that growth stimulation could be explained by the determined nitrogen fixation, measured as a net transfer of $^{15}N_2$. On the other hand nitrogen fixation has been shown to be very low (Kapulnik et al., 1985, Boddey et al., 1986). Using the acetylene reduction assay a contribution of nitrogen fixation to plant growth promotion has been reported in several studies (Döbereiner et al., 1972a, Döbereiner et al., 1972b, Day et al., 1974), but the

acetylene reduction assay can easily overestimate nitrogen fixation and should be used carefully and results should be confirmed by ^{15}N tracer studies (Gaskins et al., 1985, Boddey and Döbereiner, 1988). For inoculated *Paspalum notatum* plants, it was estimated that nitrogen fixation is up to 90 kg N ha^{-1} a^{-1}. *Azotobacter paspali* was isolated from the rhizosphere of Paspalum, but it has not been proven that there is a transfer of fixed nitrogen from Azotobacter to the plant (Döbereiner et al., 1972). Rodrigues et al. reported that the N_2 fixation by several Azospirillum strains, as expressed in dry matter of grain, panicle number and increased nitrogen accumulation, can be up to 11-18 %, which they measured by ^{15}N dilution technique (Rodrigues et al, 2008). However, PGPR need specific conditions for nitrogen fixation, which may be a reason why no contribution of nitrogen fixation was determined in any of the present experiments. For instance, the energy source (organic acids), temperature, O_2 content, pH or the nitrogen source affect nitrogen fixation efficiency (Döbereiner et al., 1972, Day and Döbereiner, 1975, Okon et al., 1976). Thus although *Raoultella* is able to fix atmospheric nitrogen under certain growth conditions, this may not have happened in the present experiments.

5.8 *Raoultella* may increase nitrogen concentrations in plants

When Arabidopsis plants were cultivated on ammonium-N at pH 5.5, an enhanced nitrogen concentration in the shoots was found (Fig. 21 A). No differences were determined for shoots or roots under non-buffered or buffered conditions for the nitrogen forms ammonium, nitrate or ammonium with addition of nitrate. By contrast, inoculation of plants cultivated on urea, irrespective of pH buffering, led to significantly higher nitrogen concentrations in shoots (Figs. 24 A and 26 E), which pointed to an improved assimilation and translocation of urea-N under inoculation. This was also observed for plants, cultivated on the amino acids arginine, glutamine or histidine (Fig. 28 E), as well as for glutamate-cultivated plants, when the medium was buffered to pH 5.5 or pH 6.0 (Fig. 30 E).

Several reports have shown an increase in total nitrogen content in PGPR-inoculated plants (Boddey et al., 1986, Kapulnik et al., 1983, Nur et al., 1980, James et al., 2002). Although the nitrogen requirement of a plant cannot be completely covered by PGPR, at least nitrogen fertilization can be reduced. Gunarto (1999) reported that nitrogen supply was reduced by 80 mg N pot^{-1} due to inoculation of an *Azospirillum* strain, as the same shoot dry weight was obtained by inoculation and fertilization with 160 mg N pot^{-1}

compared to non-inoculated treatments at a fertilization of 240 mg N pot^{-1}. The conducted experiments of this thesis revealed that the nitrogen form determines the increase in nitrogen concentrations of inoculated plants, which provided hints to a differential assimilation or translocation of the supplied nitrogen form after bacterial inoculation. To confirm this, analyses of ammonium, nitrate, urea and amino acids in the plant material should be conducted.

5.9 Phytohormones

Besides nitrogen fixation as a major mechanism to promote plant growth, the production of phytohormones by PGPR has been proposed to confer plant growth promotion (Tien, 1979). Auxin is the best investigated phytohormone released by PGPR. Phytohormones, in particular auxin and cytokinins play an important role in plant growth and development, in particular in root growth and a release of such phytohormones could explain the improved root development after inoculation with PGPR. Furthermore, it has been shown that bacteria are able to release ACC deaminase, which leads to a decreased the level of ethylene (see chapter 3).

Experimental evidence for a release of phytohormones by *Raoultella terrigena* was obtained only in liquid bacterial culture. IAA production in liquid culture by *Raoultella* was determined by the project partner J. Strauss.

As no IAA- mutant of *Raoultella* was available to date, it was not possible to investigate whether plant growth promotion still occurs. As it has been demonstrated for *Azospirillum* the use of mutants, which are impaired of the biosynthetic pathway of IAA is a convenient way to study the contribution of IAA to plant growth promotion. The alternative approach, measuring phytohormones by RIA failed, because too little plant material was available to obtain reliable data in a sufficient number of replicas. Moreover, a MS-based determination was not yet available.

Several publications report on an altered root morphology of PGPR-inoculated plants and bioassays for phytohormones, the analysis of phytohormones in bacterial culture and the use of mutants with altered root morphology have indicated that bacteria release phytohormones (Tien et al., 1979, Loper and Schroth, 1986, Asghar et al., 2002, Ribaudo et al., 2006, Remans et al., 2008).

Phytohormones are known to be involved in lateral root development, in particular a major role of auxin in lateral root initiation has been well described. In addition, cytokinins and ethylene are known to influence processes that alter root architecture

(Péret et al., 2009, Reed et al., 1998, Aloni et al., 2005, Swarup et al., 2002, Alonso et al., 2003). The phytohormones cytokinin and ethylene interact antagonistically with auxin (Swarup et al., 2002, Stepanova et al., 2007, Swarup et al., 2007, Negi et al., 2008, Ivanchenko et al., 2008, Moubayidin et al., 2009).

GUS-assays of DR5::GUS plants (a reporter line for auxin) revealed that there is a shift of auxin levels in lateral roots of plants inoculated with *Raoultella* (Fig. 45), as the auxin reporter was not expressed in inoculated plants. In ARR5::GUS plants, a reporter line for cytokinin levels a stronger staining was observed in lateral roots when plants were inoculated (Fig. 45). Differences in DR5::GUS expression of plants were also observed for plants inoculated with *Bacillus megaterium*, *Phyllobacterium brassicacearum* or *Serratia marcescens* 90-166, too (López-Bucio et al., 2007, Contesto et al., 2010, Shi et al., 2010). These observations were interpreted as indications for a phytohormone release by PGPR, although it is still possible that PGPR just release substances that modify plant endogenous phytohormone synthesis or release.

A further possibility could be that Raoultella plays a role in endogenous phytohormone homeostasis. Results of DR5::GUS and ARR5::GUS plants indicated that *Raoultella* led to a shift in hormone levels. Contesto et al. suggested that PGPR might affect auxin transduction pathways and influence endogenous phytohormone homeostasis, which lead to altered root morphology (Contesto et al., 2010).

The use of Arabidopsis mutants defective in i.e. IAA production, analysis of plant material and analyses of *Raoultella* cultures for the release of further phytohormones, like cytokinins, gibberellins, abscisic acid and ethylene, would provide useful information to investigate these hypotheses.

Taken together, several lines of evidence suggested that *Raoultella* may either produce or release auxins on its own or modify auxin homeostasis in Arabidopsis: i) *Raoultella* released auxins when cultured in liquid culture in the absence of plants; ii) morphological changes in root system architecture, in particular enhanced lateral root elongation mimicked auxin actions (Fig. 16); iii) DR5::GUS reporter lines indicated that inoculation with Raoultella induces a spatial shift in the auxin levels which may indicate a de-localized auxin accumulation in lateral roots (Fig. 45). Apart from auxin also cytokinins may contribute to *Raoultella*-mediated changes in root morphology as the cytokinin accumulating root zone became smaller, thus allowing auxins to further stimulate lateral root elongation.

5.10 The role of rhizosphere acidification in *Raoultella*-mediated plant growth stimulation

Ammonium-grown Arabidopsis plants were strongly stimulated in growth by *Raoultella*, in particular when the medium pH remained unbuffered (Fig. 16). This suggested that Raoultella may increase the rhizosphere pH and that this compensated for the ammonium-induced acidification of the rhizosphere, which may be detrimental for root growth. However, neither the use of a pH indicator nor pH measurements by an antimony electrode could confirm this assumption. In fact *Raoultella* inoculation increased the surface of an acidified root zone (Fig. 16), however this may also have been caused by an improved plant growth and the resulting higher proton release. On the other hand, pH measurements with electrodes indicated that *Raoultella* decreased the rhizosphere pH (Fig. 32), so that the initially set hypothesis of a pH increase by *Raoultella* had to be rejected.

It has been reported that the plasma membrane H^+-ATPase is stimulated by auxins (Taiz, and Zeiger, 2007). As *Raoultella* produces auxins, it may well be that plasma membrane H^+-ATPases were stimulated and thus proton efflux to enhance the uptake of nutrients thereby leading to improved plant growth. This hypothesis would be in agreement with an enhanced proton efflux by roots of *Azospirillum brasilense*-treated plants, which also showed a stronger acidification of the rhizosphere (Bashan et al., 1989, Bashan, 1990, Carrillo et al., 2002).

For a further verification of this hypothesis the Arabidopsis mutant *aha2*, lacking expression of a major plasma membrane H^+-ATPase was grown in the presence of *Raoultella*. Inoculation of *aha2* plants did not lead to a significant plant growth stimulation with regard to shoot dry weight and morphological root parameters. Higher H^+-ATPase activities may not only promote the uptake of mineral nutrients, but also stimulate root elongation, since cell elongation requires cell wall acidification (Taiz and Zeiger, 2007).

Evidence for an involvement of indirect cellular pH effects, as being dependent on the activity of tonoplast-localized V-type H^+-ATPases was not obtained. The *vha1vha2* double mutant still responded to Raoultella inoculation with an increased shoot growth, even though to a lower extent. However, primary root elongation was not any longer stimulated in *vha1vha2* double mutants, indicating that in particular this morphological root trait requires intact vacuolar acidification.

Conclusions

When *Arabidopsis thaliana* plants were inoculated with *Raoultella terrigena* a positive plant response was obtained in dependence of bacterial concentration, the growth phase of inoculated bacteria, supplied nitrogen forms, media pH and the genetic constitution of Arabidopsis mutants. The weak plant stimulation, which was observed for plants cultivated on nitrate or ammonium under buffered conditions, is most likely due to an already optimal plant development. The strongest growth stimulation was observed for plants cultivated on ammonium with addition of 10 % nitrate. This may indicate that cytokinins, which are synthesized in nitrate-supplied roots only, were required to fully support *Raoultella*-dependent growth stimulation. With regard to the underlying mechanism the present results indicated that *Raoultella* is able to decrease rhizosphere pH and to produce IAA. Thus, the pH decrease in the rhizosphere of inoculated plants most likely results from the stimulation of the plasma membrane H^+-ATPase by IAA and as a consequence, a better uptake of nutrients and improved plant growth. This mode of action would also explain why *Raoultella* improved plant growth under P deficiency.

A stimulation of the plasma membrane P-type H^+-ATPase may also hold true for the growth of inoculated Arabidopsis plants on alternative N sources, in particular for urea and the amino acids histidine and arginine. In these cases, *Raoultella* conferred better growth in particular at lower pH, when the plant plasma membrane H^+-ATPase is less effective in generating a membrane potential required for nutrient uptake, and higher N accumulation in shoots and might also have profited from a more efficient assimilation and translocation of amino-N.

The most prominent morphological effect of *Raoultella* was the strong stimulation of lateral root elongation, which not only occurred under supply of reduced N forms but also under supply of NO_3^-. This may be indicative for a release of auxins by *Raoultella*, which could be confirmed by a bacterial liquid culture assay but in plants only by a shift in the localization of auxin levels in DR5:GUS reporter lines and maybe a decrease in cytokinin levels in lateral roots, as suggested by ARR5:GUS analysis of the reporter lines.

Taken together, the present thesis could not fully elucidate the mechanims of action of *Raoultella* on Arabidopsis growth. However, the systematic analysis of the influences of N forms and medium pH could define the growth conditions that allow *Raoultella*-mediated growth stimulation. It will now be a major goal of subsequent experiments to

employ transcriptome studies and further Arabidopsis mutant and reporter lines to identify target genes and processes in Arabidopsis that are stimulated by *Raoultella*.

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Participation in the training course "Sicherheit im Laborbetrieb" (safety in laboratory administration) for project leaders

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9 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe. Es wurden nur in der Arbeit ausdrücklich benennte Quellen und Hilfsmittel benutzt. Wörtlich und sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.