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Interactions between non-symbiotic N_2-fixing bacteria and plant roots in plant-microbial associations

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Aquest treball està dedicat
a l´Anna, al David i a
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LIST OF ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	adenosine diphosphate
AMT	ammonium transporter
ARA	acetylene reduction assay
ATP	adenosine 5’-triphosphate
BNF	biological nitrogen fixation
CFU	colony forming units
CPS	capsular polysaccharides
cv.	cultivar
d	days
DNA	deoxyribonucleic acid
DW	dry weight
EPS	exopolysaccharides
EtBr	ethidium bromide
GA	gibberellic acid
HATS	high-affinity transport system
HMW	high-molecular-weight
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ISR	induced systemic resistance
LATS	low-affinity transport system
LMW	low-molecular-weight
LPS	lipopolysaccharides
Napp	apparent nitrogen mineralization
MS	Murashige-Skoog medium
Nfert	fertilized nitrogen
Nmin	mineral nitrogen
NR	nitrate reductase
OD_{600}	optical density at 600 nm

OPA	ortho-phthalaldehyde buffer
ORF	open reading frame
PBS	phosphate buffer
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
PHB	poly-ß-hxdroxybutyrate
RH	relative humidity
RNA	ribonucleic acid
R/S	root to shoot ratio
RT	room temperature
SD	standard deviation
SE	standard error
sp.	species (singular)
SPAD	spectral plant analysis diagnostic
spp.	species (plural)
TE	Tris-EDTA buffer
UV	ultraviolet
vs.	versus
v/v	volume per volume
WHC	water holding capacity
WT	wild type

1. SUMMARY – ZUSAMMENFASSUNG

1.1. Summary

The development of biofertilizers on the basis of plant growth promoting rhizobacteria (PGPR) may be a promising approach to partially substitute costly and energy-consuming mineral fertilizers in agricultural plant production and to support agriculture in developing countries. A successful and competitive rhizosphere colonization of PGPR strains has been identified as a prerequisite for the expression of plant growth promoting effects. Apart from a wide range of external factors with an impact on the colonization process, such as soil properties, temperature, soil moisture and fertilization levels, in particular plant-microbial interactions may play an important role for the successful establishment of compatible associations. In this context, certain plant root exudates may act as signals to mediate bacterial responses with importance for root colonisation (e.g. motility and chemotaxis, production of extracellular polysaccharides). On the other hand, the induction of bacterial plant growth promotion may also depend on ability of the host plant to respond to the presence and the activity of the associated bacteria. It was therefore the aim of this thesis to investigate the contribution of putative PGPR to growth and N uptake in wheat plants and characterize the underlying mechanisms in root-bacterial associations.

In the first part of this thesis, the contribution of various non-symbiotic diazotrophic rhizobacteria to plant growth promotion and N nutrition has been studied in a series of greenhouse pot inoculation experiments with wheat (*Triticum aestivum* L.). Different bacterial inoculants, plant genotypes, soil properties, water regimes and N fertilization levels have been varied as factors with potential impact on plant growth promotion by diazotrophs. The contribution of biological nitrogen fixation was assessed by the ^{15}N dilution method. Plant growth and grain yield were influenced by the different N fertilization levels but no stimulation of growth or N uptake was note upon bacterial inoculation. These observations suggested a high degree of specificity or limiting factors, determining a successful plant-microbial association.

The second part describes possible mechanisms that may be involved in the establishment of diazotrophs in the rhizosphere of suitable host plants. As an initial step of the colonization process, a targeted movement of the bacteria to the root surface is required and root exudates may act as attractants. Since dicarboxylic acids are known to exert chemotactic activity on diazotrophic bacteria, seed and root exudates of two graminaceous crops (*Triticum aestivum* L. and *Zea mays* L.) and for comparison also of a non-graminaceous plant species (*Phaseolus vulgaris* L.) were collected in hydroponic

culture with and without N supply, and organic acid profiles in these root exudates were analysed. Bacterial motility assays were conducted with the major carboxylates detected in the root exudates of the selected plant species and compared to glucose and water, using *Brevibacillus reuszeri* as a model bacterium. Pure malate, which was found at high levels in root exudates of bean and wheat, and particularly malonate (bean) and t-aconitate (maize) stimulated the motility of *Brevibacillus reuszeri* as compared with glucose or water. A particularly intense promotion of bacterial motility was recorded in the presence of crude root exudates of wheat and maize plants grown under N limitation, which was not observed for root exudates of bean. However, this was not related with comparable changes of malate or t-aconitate concentrations in the root exudates. In wheat exudates, malate concentrations even decreased in response to N limitation. These findings suggest the presence of specific factors released in root exudates of N-deficient cereals, promoting the rhizosphere colonisation with *B. reuszeri.* For an identification of the respective factors, a more comprehensive profiling of the root exudates is necessary.

In associations with diazotrophic bacteria, host plants are supplied with ammonium by the bacterial partner. This raised the question whether plant ammonium uptake systems have an impact on the efficiency of the association. To address this problem, an antisense approach was conducted with tomato, with characterised ammonium transporters *(LeAMT1;1* and *LeAMT1;2)*. The final goal was the inhibition of the ammonium transporters by production of *LeAMT* antisense lines to study their putative role in plant associations with diazotrophic bacteria. Northern blot analysis revealed a strong repression of *LeAMT1;2* expression in three independent antisense lines associated with a lower ammonium uptake capacity under N-sufficient and N-deficient growth conditions. In contrast, *LeAMT1,1* expression was only weakly repressed in antisense lines and there was no impact on N uptake. A faster decline of chlorophyll in older leaves indicates a physiological function of LeAMT1;1 and LeAMT1;2 in ammonium uptake and retrieval in shoot and root cells. The absence of consistent effects on N acquisition of the investigated antisense lines limited the suitability of this approach for studies on associations with diazotrophic bacteria.

1.2. Zusammenfassung

Die Entwicklung geeigneter Biofertilizer auf der Basis pflanzenwachstums-stimulierender Bakterien (PGPRs) könnte sowohl aus finanzieller Perspektive als auch für die Anwendung in Entwicklungsländern einen vielversprechenden Ansatz darstellen, um Kostenintensive Mineraldünger in der landwirtschaftlichen Pflanzenproduktion einzusparen. Dabei stellt eine erfolgreiche und kompetitive Besiedelung der Rhizosphäre durch PGPRs eine Voraussetzung für wachstumsfördernde Effekte auf Pflanzen dar. Obwohl der Besiedelungsprozess durch zahlreiche externe Faktoren, wie Bodeneigenschaften, Temperatur, Bodenfeuchte und Düngungsniveau beeinflusst werden kann, könnten auch selektive Wechselwirkungen zwischen Wirtspflanze und dem Bakterium eine entscheidende Rolle für das Zustandekommen kompatibler Assoziationen spielen. Hierbei könnten pflanzliche Wurzelabscheidungen eine Signalwirkung auf bakterielle Reaktionen, wie Beweglichkeit, Chemotaxis oder die Produktion von Exopolysacchariden ausüben, die für die Besiedelung der Rhizosphäre von Bedeutung sind. Andererseits könnte auch die Fähigkeit der Wirtspflanze, auf die Gegenwart der besiedelnden Bakterien zu reagieren, eine wichtige Rolle spielen. Es war das Ziel dieser Arbeit den Beitrag von PGPR auf das Wachstum und die N-Aufnahme von Weizenpflanzen zu unterscheiden und dafür verantwortliche Mechanismen in Wurzel-Bakterie-Assoziationen zu charakterisieren.

Im ersten Teil dieser Arbeit wurde in Gewächshausversuchen mit Weizen (*Triticum aestivum* L.) der Beitrag assoziativer diazotropher Bakterien zur Wachstumsstimulierung und N-Aufnahme von Weizenpflanzen untersucht. Verschiedene Bakterienstämme, Weizengenotypen, Bodeneigenschaften, Bewässerungsregimes und N-Düngungsniveaus wurden als externe Faktoren mit potenziellem Einfluss auf die Stimulierung des Pflanzenwachstums durch Diazotrophe untersucht. Der Beitrag der biologischen Stickstoff-Fixierung wurde mit der ^{15}N-Verdünnungsmethode bestimmt. Das Pflanzenwachstum, der Kornertrag und die Ertragsarchitektur wurden durch die verschiedenen N-Düngungsniveaus beeinflusst, doch es zeigten sich keine Behandlungsunterschiede in Varianten mit oder ohne bakterielle Inokulation. Dies weist auf einen hohen Grad an Spezifität bislang unbekannter Faktoren hin, die für das Zustandekommen erfolgreicher bakterieller Assoziationen mit der Wirtspflanze verantwortlich sind.

Im zweiten Teil der Arbeit werden mögliche Mechanismen beschrieben, die eine Besiedelung der Rhizosphäre kompatiblen Wirtspflanzen durch Diazotrophe beteiligt sein könnten. Erster Schritt des Besiedelungsprozesses ist eine gerichtete Bewegung der Bakterien zur Wurzeloberfläche. Dabei können Wurzelexsudate als Signalsubstanzen

fungieren. Insbesondere Dicarbonsäuren haben eine chemotaktische Aktivität auf Diazotrophe. Deshalb wurden Samen-, und Wurzelexsudate von zwei Gramineen (*Triticum aestivum* L.*, Zea mays* L.) und zum Vergleich auch von einer dikotylen Pflanzenart (*Phaseolus vulgaris* L.) gewonnen, die in Nährlösungskultur mit und ohne Stickstoffangebot kultiviert wurden. In den Exsudaten wurden organische Säuren bestimmt. Die chemotaktische Wirkung auf *Brevibacillus reuszeri* als Modell-Bakterium wurde für die dominierenden Carboxylate in den Wurzelexsudaten der untersuchten Pflanzenarten im Vergleich zu Glucose und Wasser untersucht. Malat, das in hoher Konzentration in Wurzelexsudaten von Bohne und Weizen gefunden wurde, und besonders Malonat (Bohne) und t-Aconitat (Mais) förderten die Beweglichkeit von *B. reuszeri* im Vergleich zu Glucose und Wasser. Eine besonders intensive Förderung der bakteriellen Beweglichkeit wurde in Gegenwart einer Gesamtfraktion von Wurzelexsudaten von Weizen und Maispflanzen beobachtet, die unter N-Mangelbedingungen angezogen wurden. Dies galt jedoch nicht für die Wurzelexsudate der Bohne und war auch nicht mit entsprechenden Veränderungen der Konzentrationen von Malat und t-Aconitat verbunden. In den Wurzelexsudaten von Weizen nahm die Konzentration von Malat unter N-Mangel sogar ab. Eine genauere Charakterisierung der betreffenden Faktoren erfordert eine umfassendere Analyse der Exsudatzusammensetzung.

In Assoziationen mit diazotrophen Bakterien werden die Wirtspflanzen mit Ammonium versorgt. Dies wirft die Frage auf, ob auch pflanzliche NH_4^+-Aufnahmesysteme einen Einfluss auf die Effizienz der Assoziation haben. Daher wurde ein Antisenseansatz mit Tomate, die ein charakterisiertes Ammoniumaufnahmesysteme (*LeAMT1;1* und *LeAMT1;2)* hat, durchgeführt. Ziel war es, die NH_4^+-Aufnahme durch Herstellung von Antisenselinien zu hemmen, um so die mögliche Bedeutung für Assoziationen mit diazotrophen Bakterien zu untersuchen. Northern-Blot Analysen ergaben eine starke Repression der Expression von *LeAMT1;2* in drei unabhängigen Antisenselinien, die mit einer verminderten NH_4^+-Aufnahmekapazität sowohl unter N-Mangelbedingungen als auch bei ausreichender Stickstoffversorgung verbunden war. Im Gegensatz dazu zeigten *LeAMT1,1*-Antisenselinien nur eine leicht verminderte *LeAMT1,1*-Expression und keinen Effekt auf die NH_4^+-Aufnahme. Eine beschleunigter Chlorophyllabbau in älteren Blättern weist auf eine physiologische Funktion von LeAMT1,1 and LeAMT1;2 bei der Aufnahme und Rückverlagerung von Ammonium in Sprosszellen hin. Das Fehlen konsistenter Effekte des gewählten Antisenseansatzes auf die N-Aneignung machte daher dessen Einsatz für die ursprünglich geplanten Untersuchungen für Assoziationen zwischen Wurzeln und diazotrophen Bakterien ungeeignet.

2. INTRODUCTION

2.1. Nitrogen nutrition

Nitrogen is the mineral nutrient required in largest quantities by crops. A continuous increase of the world population depends on an enhanced production of food, which, in turn, requires an increased input or an improved utilization of nitrogen fertilizers in plant production. In 1962, 12 x 10^6 t N were applied in the world, on 2005 this amount has been increased by eightfold (95 x 10^6 t N) (EFMA statistics, 2008; http://www.efma.org). The industrial production of N fertilizers via the Haber-Bosch process demands large inputs of fossil energy contributing to global warming. Therefore, alternative fertilization strategies are sought.

The use of plant growth-promoting rhizobacteria (PGPR) in agriculture might represent a more environmentally sound alternative and help to reduce the use of chemical fertilisers and thereby also the investment of farmers (Kennedy *et al.*, 2004). Since graminaceous plant species are still the most important plants for human nutrition (FAO, 2003; http://www.fao.org/), the study of associative interactions between diazotrophs and non-leguminous plant species, together with the understanding of the mechanisms involved merit high attention.

2.1.1 Forms of nitrogen in agricultural ecosystems

The earth's atmosphere contains 78% N, representing the largest pool of N on the earth. Nitrogen is essential for many biological processes; it is a structural component of all amino acids, proteins, and is present in the nucleic acids, such as DNA and RNA, as well as in many secondary plant metabolites (e.g. alkaloids). In most agricultural ecosystems different N forms are found, among which the most important are: nitrate, nitrite, ammonium; gases such as ammonia, N_2, N_2O and NO; and organic nitrogen such as urea, amino acids and peptides.

Ammonium ions are readily immobilized in soils, especially by humic substances and clay minerals. Due to its negative electric charge, nitrate binds less to the soil constituents and can be easily leached out contaminating the ground water. This

contamination is associated with an increased risk of environmental problems, such as eutrophication, a process leading to high algal populations and the death of aquatic life due to an excessive demand for dissolved oxygen (Laegreid *et al.*, 1999).

Five main processes cycle nitrogen through the biosphere, atmosphere, and geosphere: nitrogen fixation, nitrogen uptake by organisms, mineralization, nitrification and denitrification. The movement of nitrogen in different forms is described by the nitrogen cycle (Fig. 2.1-1).

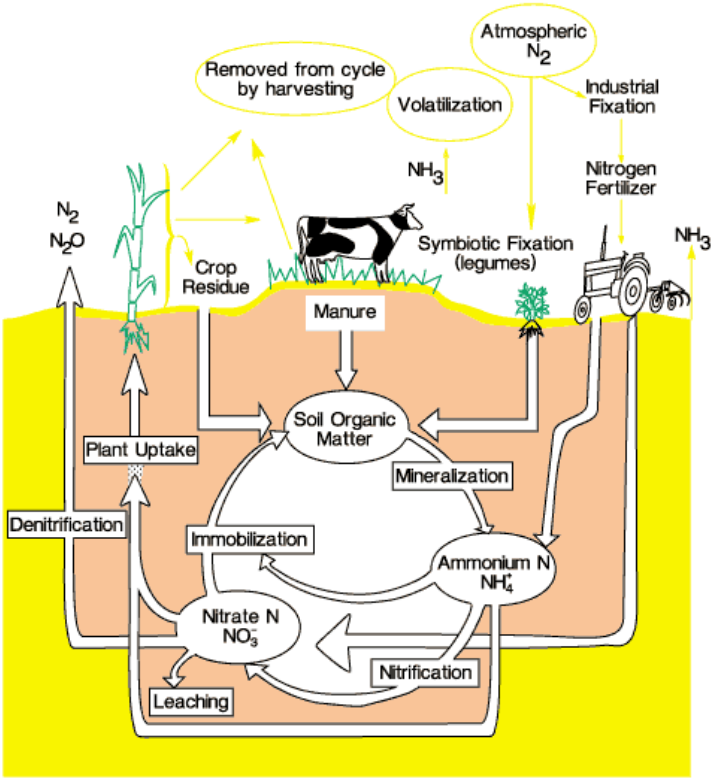

Figure 2.1-1 Representation of the main processes involved in the N cycle (from Mahler, Tindall and Mahler, www.uidaho.edu/wq/wqpubs/cis962.html).

Nitrogen fixation is the process wherein N_2 is converted to ammonium, essential because it is the only way that organisms attain molecular nitrogen directly from the atmosphere. In addition to N_2-fixing bacteria, high-energy natural events such as lightning, forest fires, and even hot lava flows can cause the fixation of smaller, but significant amounts of nitrogen. The global N fixation by volcanism has been estimated to be 10^9 mol of fixed N y^{-1}, similar to other major sources such as

lightning (Mather *et al.,* 2004), which have been estimated to mount up to 5-8% of total fixed N.

After N is incorporated into organic matter, it is converted back into inorganic nitrogen by a process called N mineralization. A significant amount of the N contained within the dead organism in form of proteins, amino acids and nucleic acids is converted to ammonium. Once in the form of ammonium, N is available for use by plants or for further transformation into nitrate (NO_3^-).

The conversion of ammonium to nitrate is performed primarily by nitrifying bacteria, but also by fungi, such as the genera *Aspergillus* (Hora & Iyengar, 1960). The primary stage of nitrification, the oxidation of ammonium (NH_4^+) is performed by bacteria, such as *Nitrosomonas,* which converts ammonium to nitrite (NO_2^-). Other bacterial species, such as *Nitrobacter*, are responsible for the oxidation of the nitrite into nitrate (NO_3^-). This two-step conversion of ammonium into nitrate is called nitrification (Scheffer, 1988). Under anaerobic conditions, oxidized forms of nitrogen such as nitrate and nitrite (NO_2^-) are converted to dinitrogen (N_2) by denitrification, and to a lesser extent, to gaseous nitric oxide (NO) (Scheffer, 1988). Ammonia (NH_3) is also a gas released to the atmosphere, particularly in basic soils by deprotonization of NH_4^+, and during the hydrolysis of urea in areas with intensive animal husbandry (Scheffer, 1988).

2.1.2 Ammonium uptake, assimilation and transport system in plants

Nitrate and ammonium are the prevalent nitrogen sources taken up by roots for growth and development of higher plants (Marschner, 1995). Nitrogen is required in larger amounts than any other mineral nutrient. However, in nature ammonium and nitrate frequently occur at relatively low concentrations and are subjected to wide variations regarding their concentrations in the soil solution. To cope with this situation, plant cells have evolved a repertoire of transporters that enable them to efficiently import ammonium and nitrate over a wide range of concentrations. The nitrate concentration in the solution of most agricultural soils is between 0.5 and 10 mM, while ammonium concentrations are often 10-1000 times lower and rarely exceed more than 50 µM (Marschner, 1995). However, when both N forms are

simultaneously supplied at equimolar concentrations, especially in the micromolar concentration range, higher uptake rates of ammonium versus nitrate were found in hydroponically grown *Arabidopsis* plants (Gazzarrini *et al.,* 1999) and in maize (Xu *et al.*, 1992).

After entering the cell, ammonium is assimilated into organic compounds. In higher plants four amino acids are mainly found, glutamine, glutamate, aspartate and asparagine (Lam *et al.,* 1995). Glutamine is formed in an amidation reaction catalyzed by the enzyme glutamine synthetase, which is condensing ammonium and glutamate into glutamine under consumption of ATP (Lea & Miflin, 1974). A second enzyme, glutamate dehydrogenase may play a role in ammonium assimilation, particularly during photorespiration in leaf cells (Yamaya & Oaks, 1987). Additionally, ammonium is released from catabolic processes within the cells such as nitrate reduction, phenylpropanoid metabolism or amino acid catabolism (Loqué & von Wirén, 2004).

Ammonium uptake across the root plasma membrane has to be tightly regulated since excess in the cytosol might cause toxicity (Britto *et al.,* 2001). The high concentration of ammonium in the cells leads to a constant ammonium leakage from roots. Ammonium is generally supposed to be immediately assimilated in the roots, either after ammonium uptake or nitrate reduction, and not translocated as ammonium to the shoots (Glass & Siddiqui, 1995; Marschner, 1995; Lam *et al.,* 1995). However, it has been shown that concentrations of ammonium of more than 1 mM occur in the xylem sap of ammonium-grown tomato and oilseed rape plants (Husted *et al.,* 2002; Schjoerring *et al.*, 2002). In leaves, large quantities of ammonium are generated by processes such as photorespiration, nitrate reduction, protein degradation and lignin biosynthesis (Joy, 1988; Leegood *et al.,* 1995). Ammonia can easily pass through membranes whenever the pH is high and therefore may get lost in significant amounts to the atmosphere (Husted *et al.,* 2002). Therefore, ammonium retrieval might be of particular importance. Furthermore, ammonium can also enter the cell or cell compartments via potassium or water channels (Howitt & Udvardi, 2000; Loqué *et al.,* 2005).

Different ammonium transporters have been isolated and characterized in yeast (*Saccharomyces cerevisiae*) (Marini *et al.,* 1997), fungi (*Aspergillus nidulans*) (Monahan *et al.,* 2002), mycorrhiza (*Hebeloma cylindrosporum*, *Tuber borchii*) (Javelle *et al.,* 2001; Montanini *et al.*, 2002), bacteria (*Escherichia coli*, *Azotobacter vinelandii*) and animals (*Caenorhabditis elegans*) (von Wirén & Merrick, 2004). Moreover, the human rhesus factors also function as ammonium transporters (Ludewig *et al.,* 2001). Most detailed studies on ammonium transporters in plants have been carried out in *Arabidopsis*, rice and tomato. Those plant species express a different number of AMT genes. To date 6 genes (*AtAMT1;1-AtAMT1;5* and AtAMT2;1) have been found in *Arabidopsis* (Ninnemann *et al.,* 1994; Gazzarrini *et al.,* 1999; Rawat *et al.,* 1999, Sohlenkamp *et al.,* 2002), 10 genes (belonging to OsAMT1-OsAMT4 subfamilies) have been found in rice (Suenaga *et al.,* 2003; Sonoda *et al.*, 2003) and 3 homologue genes (*LeAMT1;1-LeAMT1;3*) and a gene of the LeAMT2 subfamily have been found in tomato (von Wirén *et al.*, 2000b).

In bacteria such as *E. coli* and *A. brasilense*, a N-regulated ammonium transporter (*AmtB*) has been characterized. Under N limiting conditions, *Azospirillum* can take up trace amounts of NH_4^+, suggesting the existence of an ammonium-repressed uptake mechanism (Hartmann & Kleiner, 1982) and an additional role in ammonium retrieval, which might leak out of the cell by NH_3 diffusion through the membrane (Van Dommelen *et al.*, 1997).

In plants, ammonium uptake systems in roots and leaves are relevant not only for primary ammonium uptake into the root, but also for ammonium transport within the plant and for N retrieval in roots and leaves. Concentration-dependent ammonium uptake in roots of various plant species generally shows biphasic kinetics. The high-affinity transport system (HATS) operates in the submillimolar concentration range, approaches saturable kinetics, shows energy-dependence and a depolarization of the plasma membrane. The second phase, named low-affinity transport system (LATS) appears at millimolar concentrations and represents a non-saturable system (Wang *et al.,* 1993). With regard to the relatively low ammonium concentrations in the soil solution (Marschner, 1995), the HATS should be by far more relevant for N nutrition *in planta*. The regulation of

AMT genes is dependent on the plant N status (Shelden *et al.,* 2001) and can vary depending of plant species, which might implicate different physiological functions of the corresponding transporters in plants.

In tomato (*Lycopersicon esculentum* Mill.), LeAMT1;1 and LeAMT1;2 are 75.6% identical at the amino acid level whereas LeAMT1;3 is more distantly related (62.8% identity) (von Wirén *et al.,* 2000b). Whereas all three genes were expressed in leaves, only two of them, *LeAMT1;1* and *LeAMT1;2* were expressed in the roots, preferentially in root hairs (Lauter *et al.,* 1996; von Wirén *et al.,* 2000b).

In roots, *LeAMT1;1* was induced by N deficiency, while *LeAMT1;2* showed inducible expression after supply of ammonium or nitrate (Lauter *et al.,* 1996; von Wirén *et al.,* 2000b). This might indicate a role of *LeAMT1;2* in ammonium retrieval compensating for the constant ammonium efflux from roots derived from amino acid catabolism (Feng *et al.*, 1998). The opposite regulation of *LeAMT1;1* and *LeAMT1;2* by N supply suggested that these two transporters act complementary to meet the plant´s demand under a wide range of ammonium concentrations in soils (von Wirén *et al.,* 2000b).

In leaves, *LeAMT1;2* was found to be diurnally regulated with highest expresion levels during the day, whereas *LeAMT1;3* was up-regulated during darkness. This might indicate that *LeAMT1;2* could be involved in the uptake of ammonium from the xylem to the mesophyll cells or in the retrieval of photorespiratory ammonium (von Wirén *et al.*, 2000b), while *LeAMT1;3* might be required for transport or retrieval of ammonium derived from reactions involving light-repressed enzymes, such as apararagine synthetase and glutamate dehydrogenase (Lam *et al.,* 1998; Melo-Oliveira *et al.*, 1996). Both genes were up-regulated after nitrate re-supply. *LeAMT1;1* in leaves was constitutively expressed a rather low level, irrespective of the N status, suggesting a housekeeping function (von Wirén *et al.,* 2000b).

At the protein level, LeAMT1;1 and LeAMT1;2 present affinity constants of 10 and 60 µM, respectively when assayed by electrophysiological studies in *Xenopus* oocytes (Ludewig *et al.,* 2002; Ludewig *et al.,* 2003) which indicates that these expressed AMT proteins encode functional high-affinity transporters, and their different substrate affinities might allow plants to match their uptake to a wide

range of ammonium concentrations found in the environment. However, for a more direct functional proof, the study of ammonium influx by single AMT proteins is a more suitable approach. Despite the characterization of the regulation and spatial expression of these AMT genes, their physiological function remains unclear. For a more detailed physiological analysis of these tomatos AMT transporters, transgenic lines have been generated that express an antisense construct of these genes for gene silencing.

Plants growing in close association with N_2-fixing bacteria are able to overcome growth limitations in N-depleted soils. The molecular mechanism by which N_2-fixing bacteria promote plant growth is still unclear. It has been shown that inoculation with *A. brasilense* of N-starved tomato plants led to an up-regulation of the expression of the ammonium transporter *LeAMT1;2*, indicating that bacterially fixed and released ammonium might have induced gene expression (Becker *et al.,* 2002). This observation was taken as a starting point to investigate whether the ammonium fixed and released by PGPR might contribute to N nutrition in tomato. It was hypothesized that *LeAMT1;1* or *1;2* antisense lines might profit less from bacterially fixed ammonium and thus suffer more from N deficiency under growth conditions in which the availability of N is poor.

2.1.3 Biological N_2 fixation (BNF)

The biological conversion of N_2 to ammonia performed by diazotrophic bacteria is highly energy consuming. N_2 is reduced to NH_3 under consumption of ATP and redox equivalents, and is associated with the formation of H_2 as a byproduct ($N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$). The enzyme that catalyzes the reaction is called nitrogenase and consists of the dinitrogenase reductase protein (Fe protein) and the dinitrogenase (MoFe protein) which actually catalyzes the reduction of N_2. The formation of hydrogen gas is always accompanied by formation of ammonia and is a wasteful process. Therefore, some microorganisms posses hydrogenases that recover this otherwise lost form of energy.

Three major strategies of N_2 fixation can be differentiated in terrestrial ecosystems, symbiotic, non-symbiotic or associative, and free-living N_2 fixation. Symbiotic

systems contribute with approx. 70%, while non-symbiotic systems contribute with approx. 30% (Peoples & Craswell, 1992). The contribution of free-living diazotrophs is very small, because the majority of these microorganisms are heterotrophic bacteria being subjected to substrate limitation (Marschner, 1995). The terrestrial input (natural origin and human activities) of N from BNF accounts for approximately 240–280 t N $year^{-1}$ (Galloway, 1998), this amount is much higher compared to the 85 t N $year^{-1}$ consumed by nitrogenous fertilizers all over the world in 2002 (FAO, 2005/2006; http://faostat.fao.org/).

2.1.3.1 Symbiotic N_2 fixation

Bacteria of the genus *Rhizobium* and their relatives form important symbiotic interactions with leguminous plants. Depending on the plant species infection by the microsymbiont may occur on developing root hairs, at the junction of lateral roots or at the base of the stem. The first step in host plant infection is the release of phenolic compounds (flavonoids) by the roots, acting as a signal for rhizobia to stimulate chemotaxis and the expression of bacterial nodulation genes. *Nod*-gene induction is required for the production of lectins (*nod*-factors) and the attachment of the bacteria onto root hairs. The next steps are an invasion of the rhizobia through the plant infection thread and the development of the nodule meristem. Inside the dividing nodule rhizobia cells are packed into symbiosomes and transformed into bacteroids. The transformation of the bacteroids is accompanied by the synthesis of hemoglobin, nitrogenase and other enzymes required for N_2 fixation (Rolfe & Gresshoff, 1988).

To perfom N_2 fixation, bacteroids need to obtain sources of carbon and energy from the plant. These are dicarboxylic acids, such as malate and succinate. It has been widely accepted that, in return, bacteroids simply provide the plant with ammonium or ammonia, which diffuses across the peribacteroid membrane and is assimilated into amino acids in the plant cytosol of the nodular tissue. The form in which N is exported from roots to shoots depends on the type of nodules, the indeterminate ones exporting amides (aspargine, glutamine), whereas tropical legumes, which form determinate nodules, export ureides, such as allantoine or citrulline (Mylona *et al.,* 1995).

Actinomycetes of the genus *Frankia*, establish a N_2-fixing symbiosis in root nodules of a large number of non-leguminous woody dicotyledonous plants (e.g. members of the Elaeagnaceae, Rhamnaceae) (Clawson *et al.,* 1998) and with trees (e.g. of the genera *Casuarina* and *Alnus*) (Nazaret *et al.,* 1991). Another N_2-fixing symbiosis is found in cyanobacteria *Nostoc* living on Gunnera plant species or *Anabena* with the fern *Azolla anabaena* (Bergman *et al*., 1992; Peters, 1975).

2.1.3.2 Non-symbiotic N_2 fixation

Besides symbiotic and free-living N_2 fixation, another microbial strategy of BNF is found in terrestrial ecosystems, the association between non-symbiotic diazotrophs and non-leguminous plants. Associative N_2-fixing bacteria colonize close to or in different organs of the host plants without inducing morphological changes (e.g. nodule formation). The habitat of these bacteria may be the rhizosphere, the root surface but also the endodermis, the intercellular spaces of cortex cells, the vascular system (Ramos *et al.,* 2002) and also the leaves (Vlassak *et al.,* 1973). In these associations the host plant provides root exudates as an energy source for N_2 fixation. However, the benefit to the plant is mainly indirect, as approximately 90% of the fixed N becomes only available for the plant after the death of the bacteria (Marschner, 1995).

The first associative diazotroph was reported by Beijerinck in 1925 under the name *Spirillum lipoferum* (now called *Azospirillum*). A major contribution to the research on BNF with grasses was initiated by J. Döbereiner when she joined the research team at the National Centre of Education and Agricultural Research from Brazil in the 1950s. Several genera have been reported to include diazotrophs which are more or less associated with plants, including *Azoarcus, Azospirillum, Azotobacter, Beijerinckia*, *Derxia*, *Enterobacter, Klebsiella, Raoultella, Paenibacillus* and *Pseudomonas.*

A strain isolated from surface-sterilized roots of one crop and subsequently being inoculated onto that same crop has been termed a "homologous" strain; and inoculated on another crop has been defined as “heterologous”. Some endophytic

strains have been reported to improve growth and yield when being inoculated on a homologous host plant, for example *A. brasilense* Sp245 in wheat (*Triticum aestivum*) (Boddey & Dobereiner, 1988); *Azoarcus* spp. in Kallar grass (*Leptochloa fusca*) (Hurek *et al.,* 1994); *Gluconacetobacter diazotrophicus* in brazilian varieties of sugarcane (*Saccharum* spp.) (Cavalcante & Dobereiner, 1988); the cyanobacterium *Anabaena azollae* in the tropical aquatic fern *Azolla* spp. (Peters, 1975); and *Rhizobium leguminosarum* bv. trifolii in rice (*Oryza sativa*) (Yanni *et al.,* 2001). Other non-symbiotic endophytic N_2-fixers are *Herbaspirillum* and *Burkholderia.*

Estimates of the amount of N_2 fixed by associative diazotrophic bacteria vary largely. In cereals (C_3 plants) grown under field conditions in temperate climates, the amount of fixed N_2 is at most 5-10 kg N ha^{-1} y^{-1} (Vinther *et al.,* 1981). Okon *et al.* (1994) reported that in about 60-70% of the inoculation experiments with *Azospirillum*, 5-30% significant yield increases were recorded. Positive effects observed at the beginning of the growth season are not always translated into increased yields, a common feature on heavier and/or well-fertilised soils. Positive effects are generally obtained at low to intermediate N fertilization levels. To obtain increased yields it is important to use an optimal inoculum concentration and the appropriate inoculation methodology (Dobbelaere *et al.,* 2001) for improving the competition of the inoculated against the native rhizosphere bacteria. Sugarcane, a C_4 plant, shows the highest potential for associative N_2 fixation, with up to 60-80% of the plant N being derived from these associations, translating into more than 200 kg N ha^{-1} (Boddey *et al*., 1991). In contrast, often observed growth enhancement effects by inoculation with associative bacteria are due to other causes than N_2 fixation, such as improved root growth by hormonal effects (see below). The amounts of fixed N_2 differ a lot depending on the cultivar, level of N fertilizer, site, water supply, season, temperature, rotation system, intercropping and the cultivation technique, making the estimation difficult the quantity of total fixed N_2. Despite these uncertainties, results from groups in Brazil and Israel indicated, that a contribution the BNF by *Azospirillum* in graminoceous plants could cover up to 40% of the N requirements (Baldani & Baldani, 2005).

Diazotrophic bacteria have competitive advantages in a C-rich, N-poor environment. In soil-grown plants, the required microaerobic conditions for the nitrogenase activity have been presumably achieved by forming colonies in hypoxic microsites of the rhizosphere, at the rhizoplane or in the cortex (Gallori & Bazzicalupo, 1985). Contrarily to the symbiotic systems, the N_2 fixed by associative diazotrophs remains mainly in the bacterial cell and is released to the host mainly at a later stage of plant growth after bacterial death and mineralization of the biomass (Rao *et al.,* 1998). Because the BNF is energetically a very expensive process (Burris, 1991), diazotrophs, such as *Azospirillum* spp., might have developed an efficient mechanism of ammonia retention, becoming the major limiting factor in the supply of newly fixed N to the plant tissue (van Dommelen *et al.,* 1998; Christiansen-Weniger & van Veen 1991; Bashan & Holguin, 1997; Reinhold-Hurek & Hurek, 1998; Steenhoudt & Vanderleyden, 2000).

Non-symbiotic diazotrophs have preferences for certain plant hosts. After inoculation of field-grown wheat plants, homologous strains of *A. brasilense* (Sp245 and Sp107) were preferentially recovered from the interior of roots, while the heterologous strains (Sp7 and Cd) were mainly found on the root surface. Contrarily to the heterologous strains, wheat inoculaton experiments with endophytic *Azospirillum* strains (Sp245, Sp107) showed consistently an increase in total plant N contents (Baldani *et al.,* 1983). Similarly, de Oliveira *et al.* (*2*002) found greater affinity colonization by wheat homologous strains. Furthermore, it has been reported that the plant can actively be involved in the establishment of this association (Nogueira *et al*., 2001), which supports the existence of genotypic preferences to certain bacterial strains.

Inoculation with too high inoculant concentrations can inhibit root growth. For example, wheat plants showed an increased number and length of root hairs after inoculation with *A. brasilense*, at a concentration of 10^7 CFU mL^{-1}, while a 10-fold higher inoculum density led to a complete inhibition of root growth (Dobbelaere, 2002).

2.1.3.3 Environmental factors influencing non-symbiotic N_2 fixation

Field and laboratory studies on environmental factors influencing non-symbiotic N_2 fixation have been conducted in particular in the *Azospirilum brasilense*-wheat association (Bashan & Holguin, 1997; Kennedy *et al.,* 1997; Steenhoudt & Vanderleyden, 2000). Most of the positive effects have been reported in light soils under intermediate levels of water regime and NPK fertilization and with relatively low organic matter content (Okon & Labandera-González, 1994). Although less clear effects have been reported mostly in heavier soils and under high levels of fertilizer, it has also been observed that in heavy adequately fertilized soils, bacterial inoculation improved combined N uptake (Fages, 1994).

Nitrogen fertilization

The beneficial effect of inoculation with diazotrophic bacteria is most pronounced at intermediate levels of N fertilizer and declines at high levels of N fertilization (Dobbelaere *et al.,* 2001). Mineral N application not only inhibits nitrogenase activity, but also reduces the number of N_2-fixing bacteria in the rhizosphere. Martin *et al.,* (1989) showed in pot experiments that the number of non-symbiotic N_2-fixing bacteria decreased after soil or foliar N application, whereas the number of total bacteria increased. Likewise, the amount of cyanobacteria in paddy soils cultivated with rice correlate negatively with N fertilization (Fernández Valiente *et al*., 2000).

Plant growth stage

Both, the establishment and amplification of non-symbiotic N_2-fixing bacteria in the rhizosphere as well as the nitrogenase activity depend on the supply of carbon substrates from roots and this depends on the growth stage of the plant. The nitrogenase activity of bacteria associated to barley roots has been shown to be low during the first weeks of the host plant growth, while it increased to a maximum during the late reproductive stage (Vinther, 1982). In rice the highest nitrogenase activity was observed during flowering and was then subjected to diurnal fluctuations (Sano *et al.,* 1981). In maize, nitrogenase activity was particularly high in wet soils and under high light intensity (Dommergues *et al*.,

1973). Baldani and Baldani (2005) found the highest frequency of bacterial colonization even later, i.e. during grain filling, while Vinther (1982) noted a rapid decrease in the nitrogenase activity at the same stage. This indicates that statements on the influence of the plant growth stage on non-symbiotic N_2 fixation cannot be generalized.

Soil type

The soil pH might affect the adsorption capacitiy. *Azospirillum* strains isolated from roots or rhizosphere of wheat showed an optimum adsorption at pH 6.0. Strains isolated from other sources showed a maximum adsorption at pH 7.0 (de Oliveira Pinheiro *et al*., 2002). Furthermore, it has been reported that bacterial migration decreased with increasing soil weight (Bashan, 1986a).

Temperature

Temperature may also influence N_2 fixation. For example, Abrantes *et al.* (1976) have shown that an increase of the soil temperature by 4°C (24 to 28°C) led to an increase of the nitrogenase activity by 10-fold. Moreover, the decomposition of organic matter increases with temperature. It has been demonstrated by use of the $^{15}N_2$ method, that an increment of the soil temperature increases the N_2 fixation in rice in the presence of cellulose and rice straw (Kondo & Yasuda, 2003).

Light

A lack of sufficient light reduces the photosynthetic rate, and therefore delivery of assimilates to the roots and exudation, which is required as an energy source by diazotrophs in the rhizosphere (Cakmak *et al.,* 1998).

2.1.3.4 Methods to quantify biological N_2 fixation

Different methods are available to determine the absolute or relative amount of N_2 being fixed in a non-symbiotic association. While the Acetylene Reduction Assay (ARA) and the H_2-evolution determine the nitrogenase activity per se, ^{15}N isotope techniques estimate the relative amount of plant N that has been acquired from atmospheric N_2.

Acetylene reduction assay

Measuring the N_2 fixation rate can take advantage of the fact that the nitrogenase can reduce other substrates like acetylene. The ARA is the most widely used method because of its simplicity and low cost, but it is questionable because the product ethylene inhibits the activity of the nitrogenase, e.g. by 50% already after 30 min (Minchin *et al*., 1983). Moreover, it does not indicate if the fixed N is incorporated into the plant (Boddey *et al.,* 1995a). Therefore, it can be only used as an indirect method not providing absolute values.

H_2 sensor

In contrast to the ARA, the production of H_2 in the nitrogen fixation process correlates with the nitrogenase activity. Therefore, a flow-through H_2 analyser instrument allows in vivo real-time measurements (Hunt & Layzell, 1993). However, this technique is not suitable for large scale greenhouse experiments.

^{15}N Isotope Dilution Method

The most widespread methods for examining N_2 fixation in the field and in greenhouse experiments are the ^{15}N isotope dilution and ^{15}N natural abundance techniques (James, 2000). A great advantage with the use of ^{15}N isotope dilution technique to estimate N_2 fixed is its ability to provide an integrated estimate of N_2 fixation over a growing season or longer. This technique is also a very valuable technique for studies where soil mineral N levels are high (Miranda & Boddey, 1987).

To apply the ^{15}N isotope dilution technique, it is necessary to grow the "N_2-fixing" plant and a suitable non-N_2-fixing control plant (reference plant) in the same soil, where the mineral N is labelled with ^{15}N. It should be accepted that there is no isotope discrimination in the N uptake from the soil; usually ^{15}N. The extent to which this $^{15}N/^{14}N$ ratio is lowered in a N_2-fixing plant is a reflection of the magnitude of fixation, since 99.6% of atmospheric N_2 consist of ^{14}N isotopes (Boddey *et al*., 1995b).

Besides a homogenous spatial distribution of the ^{15}N labeled fertilizer in the soil, a homogenous temporal distribution is also required. To mitigate the differential temporal distribution it has also been proposed to use different methods, to use different reference plants in the same experiment, to plan more replicates, to use

higher ^{15}N labeling and to repeat independently the experiments (Boddey *et al*., 1995b; Hamilton *et al*., 1992).

^{15}N Natural Abundance

The natural abundance of ^{15}N in atmospheric N_2 is 0.3663% (Junk & Svec, 1958). However, small deviations from this value occur in nitrogen-containing compounds from a variety of other sources. Kinetic isotope effects almost always result in a ^{15}N enrichment of the substrate but depletion of the product because of the tendency of molecules bearing the lighter isotope to react somewhat faster than those which bear the heavier isotope. This results in a nitrogen isotope fractionation. In general, soil N is usually more abundant in ^{15}N than atmospheric N_2 (Shearer *et al.,* 1978). The use of the natural ^{15}N abundance for determining N_2 fixation requires that the $^{15}N/^{14}N$ ratio in the soil differs appreciably from that in the atmosphere, and it is based on the same principle as mentioned for the ^{15}N dilution method. Soils are naturally enriched in ^{15}N and the dilution of this by $^{14}N_2$ derived from the atmosphere has been used to estimate N_2 fixed in grain legumes (Kohl & Shearer, 1980). A reference crop with similar N uptake behaviour is needed to establish the change of the $^{15}N/^{14}N$ ratio from the soil to the crop. The $^{15}N/^{14}N$ ratio is site specific and must be empirically determined at each site of interest (Shearer & Kohl, 1986).

$^{15}N_2$ enrichment gas technique

Since N_2 is the substrate for N_2 fixation, incorporation of ^{15}N into plant tissues invariably provides the most direct and valid estimate of how much N_2 is fixed. However, this technique is not practicable in large-scale experiments. The use of ^{15}N-labelled N_2 is usually only feasible in short-term experiments under controlled conditions (de Polli *et al*., 1977).

2.2. Plant growth promoting effects of diazotrophs independent of N2 fixation

Apart from N_2 fixation, associative diazotrophs can exert their positive effects on plant growth directly or indirectly through different mechanisms or a combination of them (Glick *et al*., 1999).

2.2.1 Production of plant growth promoting substances

Some of the plant growth-promoting substances secreted by diazotrophs are phytohormones, enzymes, vitamins and recently nitric oxide has been reported to be produced by *A. brasilense* Sp245 and to act as a signal molecule in the hormonal cascade leading to root formation in tomato (Creus *et al.,* 2005).

2.2.1.1 Phytohormones

PGPR can produce morphological and physiological changes of the inoculated plants roots, which lead to an enhanced water and mineral uptake. The mechanism that has been most often invoked to explain these effects on plants is the production of phytohormones or analogous compounds. Their effect on plant growth is quite complex and depends on the plant hormonal status, the interaction with other phytohormones and the nutritional status of the plant (Mantelin & Touraine, 2004). Considering the numerous interactions that exist between the different hormone signalling pathways in plants, it is difficult to assess which of these pathways is the primary target of PGPR. More likely, rhizobacteria alter not just a single, but several hormonal pathways at a time (Mantelin & Touraine, 2004).

Stimulation of root growth by PGPR might be dependent on the availability of specific substrates as precursors of phytohormones (e.g. L-methionine for ethylene, L-tryptophan for IAA or adenine for cytokinines). These substrates might be found in plant root exudates, soil organic matter or in organic fertilizers (Marschner, 1995). An evaluation of bioactive molecules in different strains of *A. brasilense* has been recently reported (Perrig *et al*., 2007). These authors showed

different capability of different strains to produce five major phytohormones (IAA, zeatin, gibberellin, ethylene and ABA). This observation might have technological implications for inoculant formulation, since different strains may produce different concentrations of growth regulators under certain growth conditions to match the phytohormone requirement for optimal growth of certain crop plants.

Auxins

Indole acetic acid (IAA) is the most widely distributed, naturally occurring auxin. IAA stimulates apical dominance, root initiation and the differentiation of vascular tissues; it delays fruit ripening; and may inhibit leaf and fruit abscission. (Srivastava, 2002). Auxin further stimulates root hair (Ringli *et al.,* 2005) and lateral root formation (Hobbie, 1998). The production of auxin is widespread among the plant associated bacteria like *Azospirillum brasilense* (Dobbelaere, 2002), *Paenibacillus polymyxa* (Lebuhn *et al.,* 1997), *Azotobacter vinelandii* (Gonzalez-Lopez *et al.,* 1986) and *Gluconacetobacter* spp. (Pedraza *et al.,* 2004). Auxin biosynthesis by rhizobacteria has also been suggested as a tool for the screening of effective PGPR strains (Khalid *et al.,* 2004). Inoculation of wheat seeds with increasing concentrations of *A. brasilense* Sp245 cells had a pronounced effect on the development and morphology of roots, resulting in a decrease in total root length but an increase in root hair formation (Dobbelaere, 2002).

Gibberellins

Gibberellins are known to stimulate stem elongation, root growth and floral development and to break seed dormancy (Srivastava, 2002). Diazotrophs can also produce gibberellins (Gutierrez-Manero *et al.,* 2001) like *Azotobacter* spp. (Azcon & Barea, 1975), *Paenibacillus polymyxa* (Sattar & Gaur, 1987), *Glucoacetobacter diazotrophicus*, *Herbaspirillum seropedicae* (Bastian *et al*., 1998) and *Azospirillum* spp. (Cassan *et al.,* 2001; Piccoli *et al.,* 1999). Several observations suggest that these diazotrophs might promote growth in the host plants through the action of these hormones, e.g promotion of root growth in maize seedlings (Fulchieri *et al.,* 1993) and increase of height in dwarf maize (Lucangeli & Bottini, 1997). In addition, it has been demonstrated that the alleviation of water stress symptoms in wheat plants might be, at least partly, due to the bacterial

gibberellin production. Inoculation of wheat plants with *A. brasilense* significantly reverted part of the negative effects that salt or osmotic stresses produced on the relative elongation rate of shoots (Creus *et al.,* 1997). Similarly, maize plants under salt stress showed growth promotion when were sprayed with GA_3 or inoculated with *A. lipoferum* (Hamdia & El Komy, 1997).

Cytokinins

Cytokinins are compounds which promote cell division, the release of apical dominance and lateral root initiation (Srivastava, 2002). Cytokinins delay senescence (Gan & Amasino, 1997) and stimulates leaf expansion (Walch-Liu *et al.,* 2000); enhance stomata opening (Tanaka *et al.,* 2006) and stimulate tillering (Wang & Below, 1996). Some PGPR have been shown to produce cytokinins like *Azotobacter* spp. (Nieto & Frankenberger, 1991; Azcon & Barea, 1975), *Pantoea agglomerans* (Feng *et al.,* 2006), A*zospirillum brasilense* (Martinez-Morales *et al.,* 2003) and *Paenibacillus polymyxa* (Timmusk *et al.,* 1999). So far, no direct evidence for a role of cytokinin production in plant growth promotion by using mutant strains producing no or more cytokinins has been provided.

Ethylene

Ethylene is also known to influence many aspects of plant growth, like breaking seed dormancy, stimulating fruit ripening, senescence and epinasty of leaves and flowers, and forming root hairs and aerenchyma (Srivastava, 2002). There are numerous and contradictory reports on the effects of ethylene on root growth, which appear to depend on the applied concentrations (Marschner, 1995). Ribaudo *et al.* (2006) found recently that tomato plants were positively affected in growth after inoculation with *A. brasilense*, higher concentrations of ethylene and IAA. They concluded that ethylene is a central regulator involved in phytohormonal signalling pathways. Some PGPR may promote plant growth by lowering potentially deleterious levels of ethylene in plants. This is attributed to the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which can cleave the plant ethylene precursor ACC (Glick & Pasternak, 1998). However, the agronomic use of PGPR which can hydrolyse ACC may be limited to dicots, because monocots are less sensitive to ethylene and therefore less responsive to these bacteria (Holguin & Glick, 2001).

Abscisic acid

In general, abscisic acid (ABA) acts as a growth inhibitor, but these effects depend on the plant tissue, developmental stage and stress factors (Sharp & LeNoble, 2002). ABA protects against stresses, e.g. by stimulating the closure of stomata inducing seed dormancy or inhibiting shoot growth under water stress. ABA further induces abscission and senescence (Srivastava, 2002) and the transcription of proteinase inhibitors, which play a role in pathogen defense (Huang *et al.,* 2007). ABA has been detected in different diazotrophs like in the rice endophyte *Pantoea agglomerans* (Feng *et al.,* 2006). Its positive effect on shoot and root growth of maize seedlings grown under water stress, was to prevent an excess of ethylene (Sharp & LeNoble, 2002).

Recently, it has been proposed that hormones like auxin and ABA, together with nitrate, would act as long-distance signals responsible for the systemic control of lateral root development in *Arabidopsis*, which is a key determinant for a nutrient and water-use efficiency in crops (Casimiro *et al*., 2003).

2.2.1.2 Enzymes

Soil microorganisms are able to solubilize P by producing various organic acids or by releasing phosphatase enzymes (see chapter 2.2.3). Other enzymes produced by diazotrophs are cell wall-degrading enzymes, such as glucanase by *Azoarcus* sp. BH72 (Reinhold-Hurek *et al.,* 1993), *Burkholderia* sp. (Compant *et al.,* 2005b) and *Raoultella terrigena* (Jayamani, 2006); chitinase by *Bacillus polymyxa* (Mavingui & Heulin, 2007); or polygalacturonase by *Burkholderia* sp. (Compant *et al.,* 2005b). The capacity to degrade pectin is a feature of many plant-associated bacteria, especially of plant pathogens. *Azospirillum irakense* shows pectinase activity, which supports the infection process and allows better penetration of the next tissue (Bekri *et al.,* 1999). Also cellulase activities might participate in the initial colonization of wheat roots by *Azospirillum*. An inoculation with *Azospirillum* enhanced the cellulase activity in wheat root extracts, but this effect was directly dependent on the strain-plant combination (Mostajeran *et al*., 2007). Cellulose degradation has been reported to occur concomitantly with N_2 fixation in aerobic shipworms bacteria (Waterbury *et al.,* 1983).

2.2.1.3 Production of vitamins

Diazotrophs may enhance mycorrhizal development by releasing vitamins into the rhizosphere, since mycorrhizal fungi have been shown to be stimulated by certain vitamins (Björkman, 1970; Dobbelaere *et al.,* 2003). Production of water-soluble vitamins has been related to the ability of *Azotobacter* spp. to enhance crop yield, as exogenous application of vitamins may affect plant growth at different levels (Oertli, 1987). *Azotobacter* spp. produces different quantities of B-group vitamins under certain C/N ratios (Revillas *et al.,* 2000). *Azospirillum brasilense* produce thiamine, niacin riboflavin, biotin and pantothenic acid depending on the C source in artificial media (Rodelas *et al.,* 1993; Sierra *et al.,* 1999). However, studies with appropriate mutants lacking the ability for vitamin production are needed to confirm the significance of their contribution to plant growth stimulation.

2.2.2 Interaction with other microorganisms

2.2.2.1 Antagonism (Biocontrol)

Direct mechanisms of biocontrol mediated by PGPR include competition for an ecological niche, with effective root colonization, competition for nutrients and the production of inhibitory allelochemicals. Some of these allelochemicals are substances that detoxify pathogen virulence factors or that quench a pathogen quorum-sensing capacity by interfering with N-acyl-homoserine lactone-mediated communication; other allelochemicals are siderophores, antibiotics and a variety of enzymes like chitinases, proteases and glucanases (Compant *et al*., 2005a). Recently, it has been reported, that *Azoarcus* sp. strain BH72 uses a novel autoinducer molecule (HSF, hydrophilic signal factor) for the intercellular communication involved in quorum sensing (Böhm, 2006). Approximately twenty bacterial biocontrol based products have been marketed (for an inventory list see http://www.anbp.org/products_bcweeds.htm), but there still is a need to optimise the efficacy of these products.

An indirect plant growth promotion can be through the induction of systemic resistance (ISR) in host plants towards a broad spectrum of plant pathogens.

PGPR-triggered ISR fortifies plant cell wall stability and alters host physiology and metabolic responses, including an enhanced synthesis of plant defence chemicals after being challenged by pathogens and/or abiotic stress factors. Different traits to trigger ISR have been proposed, volatile organic compounds, flagellation and production of siderophores or lipopolysaccharides (Compant *et al*., 2005a). A description of the molecular basis of biocontrol processes has been provided by Bloemberg and Lugtenberg (2001).

Paenibacillus polymyxa is a potential commercially useful biocontrol agent because it has a broad host plant range, an ability to form endospores and produce different kinds of antibiotics. *Paenibacillus polymyxa* colonizes predominantly the root tip forming biofilms, and it may also be found in the intercellular spaces outside the vascular cylinder (Timmusk *et al.,* 2005). *Paenibacillus polymyxa* plant growth promotion may rely on indirect mechanisms, e.g. eliminating other pathogens since under gnotobiotic conditions no PGP was observed (Lindberg *et al*., 1985) or inducing drought tolerance as it has been shown for *Arabidopsis* (Timmusk & Wagner, 1999). Recently, a novel diazotrophic bacterium *Delftia tsuruhatensis* has been isolated from the rhizoplane of rice and it has been found to be an effective antagonist of three major rice pathogens, the rice bacterial blight (*Xanthomonas oryzae*), rice sheath blight (*Rhizoctonia solani*) and rice blast (*Pyricularia oryzae*) (Han *et al*., 2005) but the mechanisms of action are still unclear.

2.2.2.2 Synergism

The plant growth-promoting potential of some diazotrophs can be further improved through co-inoculation with other microorganisms for additive and synergistic effects. *Azospirillum brasilense* has been used as a phytostimulating inoculant when co-inoculated with *Rhizobium* increasing the nodulation of legumes. Stimulation of nodulation may be derived from an increase in the production of lateral roots, root hairs density and branching (Burdman *et al*., 1998b) or an increase of the secretion of flavonoids by plant roots (Burdman *et al.,* 1996). A positive interaction between arbuscular mycorrhiza infection in roots and *Azotobacter chroococcum* survival in the rhizosphere of wheat was also found (Behl *et al.,* 2003). In tomato the highest percentages of mycorrhizal colonization

were achieved by co-inoculating *Azospirillum brasilense* with AM. Furthermore, this co-inoculation leads to higher contents of N, P and K in tomato and in onion (Pulido *et al*., 2003). A combined inoculation of *Azospirillum brasilense* with *Trichoderma harzianum*, a biocontrol fungus which can solubilize inorganic P, did not show significant differences in growth of plants, unless the inocula concentration of both microorganisms was reduced. Many yield parameters in bean and wheat were improved, relative to a single inoculation with either microbial species, provided that rock phosphate was supplied at a low rate (Ögüt *et al.,* 2005).

2.2.3 Enhanced mobilization of nutrient elements

Although no experimental evidence for the enhancement of mineral uptake by diazotrophs has been provided, an accumulation of minerals in stems and leaves have been observed. This increase mineral uptake has been suggested to be an indirect mechanism, due to the improved root growth, due to e.g. the production of phytohormones. However, it has been reported that inoculation with *Azospirillum brasilense* alters the root membrane proton efflux of wheat roots resulting in a rhizosphere acidification, which facilitates the mobilization of minerals (Marschner, 1995). NO_3^- uptake by the roots of spring wheat was stimulated by *A. brasilense* strain (Saubidet *et al*., 2002); (Mantelin & Touraine, 2004). Increased root development and the stimulation of NO_3^- uptake in response to PGPR inoculation are probably independent of each other, suggesting an additive effect (Saubidet *et al*., 2002). Inoculation of corn (*Zea mays* L.) seeds with *A. brasilense* has been shown to enhance by 30 to 50% the uptake of NO_3^-, K^+ and H_2PO_4, however, the underlying mechanisms are unknown. Possible mechanisms have been proposed such as hormonal effects or pectinolytic enzyme productions (Lin *et al.,* 1983).

An explanation for a higher N accumulation, as observed after *Azospirillum* inoculation of wheat, could be the activity of the bacterial nitrate reductase. In contrast to a wild type strain, a nitrate reductase mutant of *A. brasilense* Sp245 produced only a very small inoculation response in wheat (Boddey *et al*., 1986a). It has been proposed that NO_3^- used by the PGPR decreases the NO_3^- concentrations at the root cell surface and this change may trigger lateral root development (Mantelin & Touraine, 2004).

To overcome the low solubility of iron in alkaline and calcareous soils, several PGPR like *Azotobacter vinelandii* (Tindale *et al*., 2000; Knosp *et al.,* 1984), *Azospirillum brasilense* (Bachhawat & Ghosh, 1987) and *A. lipoferum* (Saxena *et al.,* 1986) secrete siderophores as chelators for sparingly soluble Fe(III). It was suggested that plants can benefit indirectly from the microbialy enhanced Fe mobility in soils with low Fe availability. Siderophore cross-utilization among rhizospheric bacteria (Joshi *et al*., 2006) could cause an indirect plant growth promotion, enhancing other PGPR or delivering Fe to other soil bacteria with a high Fe requirement, like for instance *Nitrosomonas europae*, an important bacterium for the mineralization of organic matter (Wei *et al*., 2006). In contrast, no evidence exists for a direct utilization of bacterial Fe-siderophores by plants, although exchange chelation of Fe with phytosiderophores seems to be possible (Crowley & Kraemer, 2007).

Solubilization of P is important for plant growth because P is an essential nutritional element for plants but it is at the same time one of the least soluble nutrient ions in the environment. Soil microorganisms are able to solubilize P by producing various organic acids or by releasing acid and alkaline phosphatases (Rodriguez & Fraga, 1999). Strains of *Herbaspirillum lusitanum* have been shown to produce alkaline phosphatases (Valverde *et al.,* 2003). A wide range of microorganisms, including the diazotrophs *Paenibacillus polymyxa* (Hu *et al.,* 2006)*, Azospirillum brasilense, A. lipoferum* (Rodriguez *et al.,* 2004), *Azotobacter* (Garg *et al.,* 2001) and G*luconacetobacter* sp. (Loganathan & Nair 2003), are known to have the ability to solubilize inorganic P from insoluble sources (Chincholkar *et al*., 2001). It has been argued that an increased P uptake, as often observed in plants treated with P-solubilizing bacteria, is a side-effect of that interaction and actually reflects a better developed root system. Canola (*Brassica napus* L.) inoculated with several P-solubilizing rhizobacteria, e.g. *Xanthomonas maltophilia* or *Paenibacillus polymyxa*, significantly increased plant height and yield, but not P-uptake, indicating that P solubilization was not the main cause for plant growth promotion (de Freitas *et al.,* 1997).

2.2.4 Enhanced stress tolerance

In sorghum plants subjected to osmotic stress, leaf senescence was delayed in plants inoculated with *A. brasilense*, thus favouring dry matter accumulation and grain filling (Sarig *et al.,* 1990). Wheat seedlings inoculated with *A. brasilense and A. lipoferum* had a better water status under drought, osmotic or salt stress than non-inoculated control plants (Creus et al., 1998; Bacilio *et al*., 2004). Under drought stress conditions during anthesis, wheat plants inoculated with *A. brasilense* Sp245 exhibited smaller losses in grain yield than non-inoculated plants (Creus *et al*., 2004). The role of *A. brasilense* in the alleviation of salinity stress was examinated by Hamdia *et al.* (2004) comparing different salt-tolerant maize cultivars. Higher salt tolerance was associated with a higher K^+/Na^+ ratio in shoots. *Azospirillum brasilense* restricted Na^+ uptake but enhanced the uptake of K^+ and Ca^{2+}, leading to a higher salt tolerance in a salt-sensitive cultivar.

Arabidopsis plants inoculated with *Paenibacillus polymyxa* were more resistant than control plants to biotic and abiotic stress factors. The higher expression of stress-responsive genes indicated that *P. polymyxa* induced mild biotic stress triggering the expression of plant defense reactions (ISR) (Timmusk & Wagner, 1999).

In vitro inoculation of *Vitis vinifera* L. explants with the PGPR *Burkholderia phytofirmans* increased the cold tolerance of grapevine, resulting in better root growth and shoot biomass production. The inoculated plantlets had significantly increased levels of starch, proline and phenolics (Ait Barka *et al.,* 2006).

Apart from alleviating osmotic and cold stress in plants, inoculation with diazotrophs can also enhance oxidative stress tolerance. Reactive oxygen species (ROS) are generated by oxidative metabolism in most cells and in particular under stress situations. ROS are highly reactive and harmful to plant membranes or DNA. The inoculation of sugar beet with *Azotobacter chroococucum* increased oxidative stress defences in leaves, by inducing antioxidant enzymes (SOD, peroxidase and catalase) leading to a higher content of chlorophyll and carotenoids (Stajner *et al.,* 1997).

2.2.5 Associative interactions between plant roots and diazotrophic bacteria

Efficient colonisation of the root surface and a strong rhizosphere competence are pre-requisites for both, beneficial and pathogenic rhizosphere microorganisms when they interact with host plants (Fig. 2.2-1). It has been proposed that chemotaxis is strain specific and could be an adaptation of bacteria to the rhizosphere conditions created by the host plant (Reinhold *et al.,* 1985). Therefore, differential root exudation is likely to be an important factor for the initiation and success of microbial root colonization. This section describes the most important factors determining the bacterial-root colonization process.

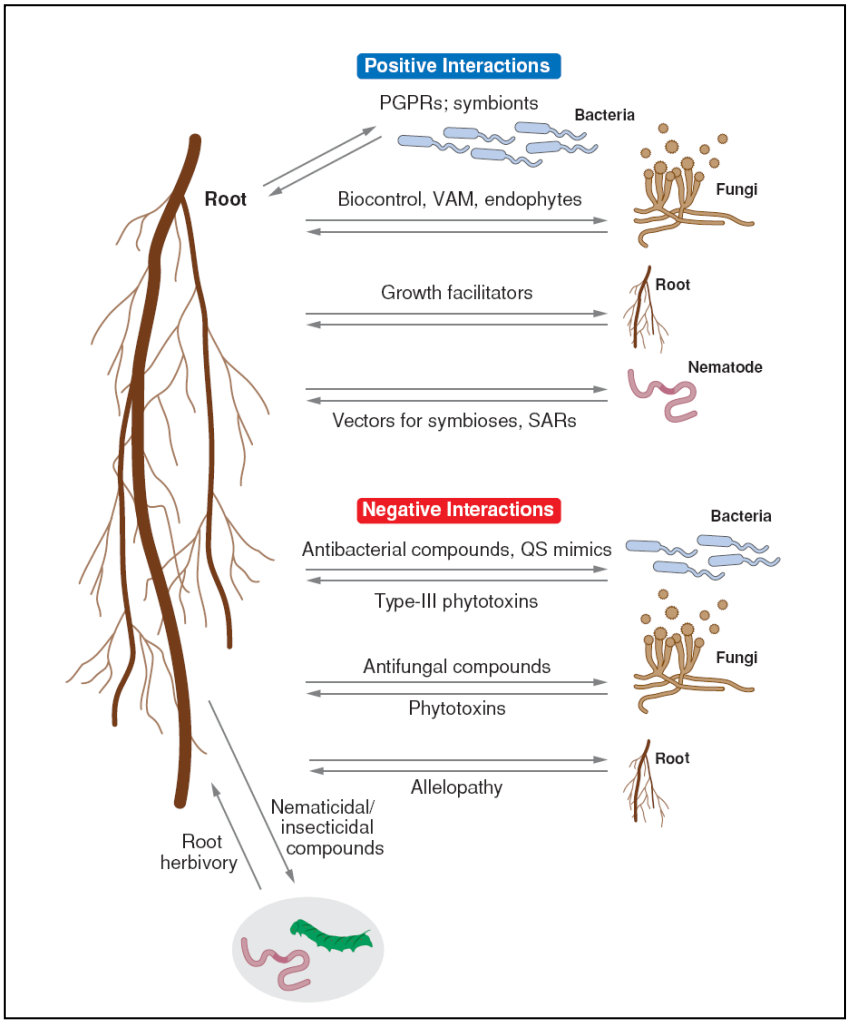

Figure 2.2-1 The role of root exudates in interactions between plants and rhizosphere organisms (After Bais *et al.,* 2006).

2.2.5.1 Plant signalling compounds recognized by microbes

Exudation can provide chemical benefits to the plants. Root exudates and sloughed-off cells are an important source of carbon and energy for microorganisms present in the rhizosphere, but they can also act as precursors for phytohormone production (see chapter 2.2.1.1) and as antimicrobial and antifungal substances (Walker *et al.,* 2003). Root exudates can also be signals for recognition, playing an important role in the colonization process through chemotaxis of soil microorganisms, such as *Bacillus subtilis* (Rudrappa *et al*., 2008).

It has been estimated that in the seedling stage, between 30-40% of photosynthetically fixed C is released by the roots (Whipps, 1990). Plant root exudates consist of a mixture of sugars, organic acids, phenolic compounds (ectoenzymes), phytosiderophores, vitamins, amino acids, inorganic ions, gases, enzymes and root border cells. The quantity and quality of root exudates depend on external factors, such as the environmental conditions, nutritional status of the plant, plant developmental stage and mechanical impedance of the soil (Marschner, 1995). In addition, the presence of microorganims can stimulate root exudation (Volpin *et al.,* 1996; Neumann & Römheld, 2007). Bioreporter studies have shown that plant exudates are not distributed evenly across the root surface; they are differently located depending on the plant species and on the nutritional or physiological status of the plant (Lugtenberg, 2007).

Root exudates include high (HMW) and low-molecular-weight (LMW) compounds. Mucilage, polysaccharides, fatty acids and ectoenzymes (phenolics) are components of the HMW fraction and organic acids, sugars, amino acids, phytohormones and phenolic compounds are components of the LMW fraction. The mechanisms of root exudation are either diffusion of LMW compounds, controlled release through ion channels or carriers, e.g. for carboxylates, and vesicle transport for HMW compounds (Bertin *et al.,* 2003) (Fig. 2.2-2). The exudation of compounds involves a certain cost for the plant, and must therefore provide a selective advantage, such as mobilizing mineral nutrients (e.g. phytosiderophores in graminaceous species mobilize iron from calcareous soils), detoxification of metabolic waste products or attraction and repelling of

microorganisms (Marschner, 1995). It should be mentioned that the collection of root exudates in distilled water can severely damage cell membranes, which in some cases can lead to the burst of root cells and the loss of cell contents into the external medium modifying the real organic acid profile and concentrations (Jones, 1998).

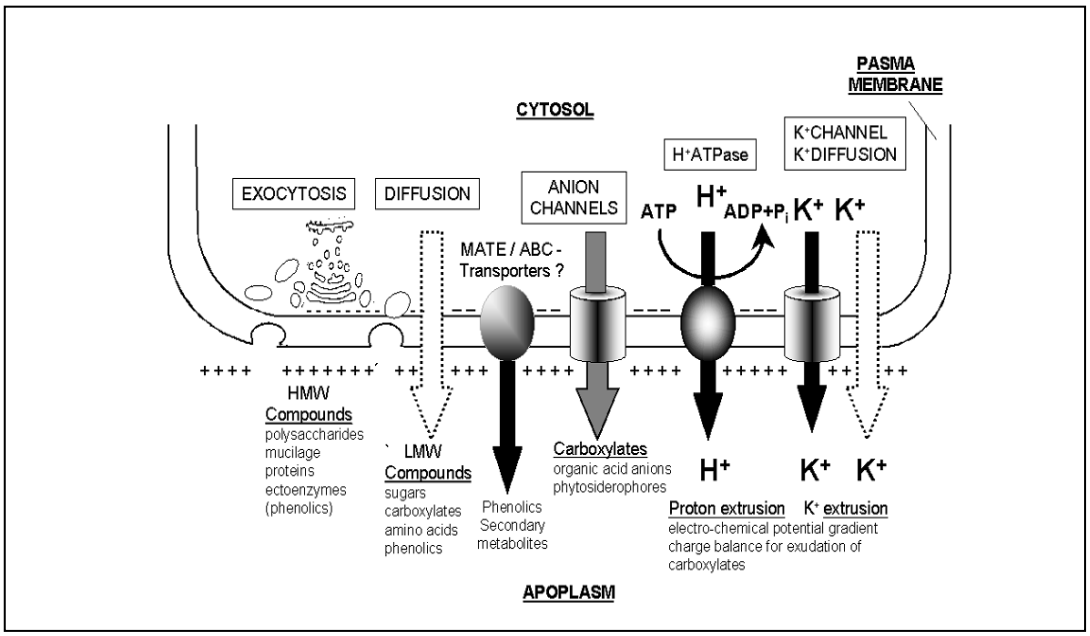

Figure 2.2-2 Mechanisms involved in the release of root exudates through the plant cell membrane (After Neumann & Römheld, 2007).

Root exudates of maize grown in nutrient solution may consist of up to 65% of sugars (arabinose, glucose, fructose, sucrose), up to 33% of organic acids (citric acid, lactic acid, succinic acid) and only up to 2% of amino acids (alanine, serine, glutamic acid) (Kraffczyk *et al*., 1984). The major organic acids reported in wheat root exudates have been identified as citric, malic, lactic, oxalic, and succinic acid (Rovira & McDougall, 1967), however, their composition depends on the wheat cultivar (Basu *et al*., 1994). In leguminous plants, root exudates consist of flavonoids, sugars, amino acids and organic acids, especially malonic acid (Burdman *et al.,* 1996; Rovira, 1956).

Plants produce more than 100,000 LMW natural products (Dixon, 2001), some of them may play important roles as chemical signals in the rhizosphere, for example phenolics with allelopathic activity exuded by wheat roots (Wu *et al*., 2000; Bertin

et al., 2003). When functioning as a signal for soil microorganisms only very low concentrations of secondary metabolites are required. The active components released for stimulating *nod*-gene expression in Rhizobia are flavonoids (Bauer & Caetano-Anolles, 1990). A different profile and a significant increase of the *nod*-gene inducers (flavonoids) in alfalfa root exudates were observed after *A. brasilense* inoculation, suggesting positive interactions between Rhizobia and associative Plant Growth Promoting Rhizobacteria (PGPR) (Volpin *et al.,* 1996). Recently, some biofertilizers containing flavonoids as active ingredients have been commercialised to increase beneficial microorganisms in the rhizosphere, for instance Exu-Root® (www.innovakglobal.com) and SoyaSignal®, which contains genistein and daidzein (Leibovitch *et al.,* 2001).

Organic acids in root exudates of tomato appeared to be important for the colonization by *Pseudomonas* (Lugtenberg *et al.,* 2001). Organic acids, like malic acid, can stimulate N_2 fixation by non-symbiotic bacteria (Wood *et al.,* 2001) or induce chemotaxis of different strains of *Azospirillum brasilense* (Sp7, Cd and Sp 59b) (Lopez-de-Victoria & Lovell, 1993). Chemotaxis towards malate has been proven to rely on stereospecific recognition of this organic acid (Rudrappa *et al*., 2008).

Another component in root exudates is the mucilage secreted by the root cap cells. Mucilage and the root cap cells seem to play a role in the establishment of specific plant root-microbial interactions. Mucilage contributes to the structural stability of soils by producing water-stable aggregates in the rhizosphere, reducing friction between root tip and soil and thereby facilitates ion uptake. Mucilage of maize has been shown to have a strong chemotactic action on strains of *Azospirillum lipoferum* isolated from the maize rhizoplane but not on strains isolated from the rice rhizoplane (Mandimba *et al.,* 1986). Furthermore, root cap cells seem to carry host-specific traits into the rhizosphere for the establishment of a characteristical bacterial flora, and to suppress certain soil-borne root pathogens (Hawes, 1990; Gochnauer *et al*., 1990).

Plant lectins may also play a role in the legume-Rhizobia symbiosis (Hirsch, 1999; Ridge *et al.,* 1998) and in wheat root colonization by *A. brasilense*. Wheat root

lectins, as sugar-binding proteins, are able to distinguish the cell surface sugar composition of the different rhizosphere bacteria, and by this way, they may support the selective binding of beneficial rhizobacteria to the roots. The presence of wheat germ agglutinin (WGA) has been reported to promote a two to four fold of *A. brasilense* proliferation (Tugarova *et al*., 2007) and to play a role in nitrogen fixation**.** The WGA-receptor complex of *A. lipoferum* generates a stimulus that leads to an enhanced transcription of two components of the regulatory cascade of nitrogen fixation (*glnB* and *nifA* genes) (Kárpáti *et al.,* 1999). Interestingly, Heinrich (1984) found that *A. lipoferum* showed a neutral chemotaxis towards WGA, but a positive chemotaxis to sugars, sugar alcohols and amino acids. On the other hand, it has been found that the extent of lectin-mediated attachment of *A. brasilense* is influenced by its physiological state and the C sources in the medium (Burdman *et al.,* 2000b; Laus *et al*., 2006).

Azospirillum strains have been reported to show a positive chemotaxis to sugars, to various organic acids such as succinic, malic and fumaric acid, amino acids (Okon *et al.,* 1980; Barak *et al*., 1982; Reinhold *et al.,* 1985), aromatic compounds (Lopez-de-Victoria & Lovell, 1993) and root mucilage (Mandimba *et al.,* 1986). However, *A. brasilense* Sp7 is not attracted by glucose and most amino acids (Alexandre *et al.,* 2000). Furthermore, this group demonstrated that the presence of poly-ß-hydroxybutyrate (PHB) in the cells prevents chemotaxis to any chemoattractant, although the cells were fully motile and able to respond to repellents.

Several plant-microbe interactions (pathogenic and beneficial) are mediated by Quorum Sensing (QS). Some evidence suggest that the cross talk between plants and bacteria may occur through compounds that are capable of interfering with QS processes in pathogenic bacteria. Compounds that interfere or mimic QS have been found in exudates of higher plants. For example, swarming in *Serratia liquefaciens* appeared to be specifically induced by *Pisum sativum* exudates, which induce the gene responsible for the synthesis of serrawettin, a surfactant required for surface swarming (Bais *et al.,* 2006). Also in the context of plant-microbe interactions, it has recently been shown that root secretions of L-malic acid are induced by a foliar pathogen and elevated levels of L-malic acid promote

binding and biofilm formation of beneficial rhizobacteria (*Bacillus subtilis* FB17) on *Arabidopsis* roots (Rudrappa *et al*., 2008).

2.2.5.2 Microbial signalling compounds recognized by the plants

Recognition of microbial signalling compounds is considered to be the initial key event in the response of plants to microbes. It can occur through physical interactions (flagella, pili, polysaccharides, chitin fragments and other adhesins) or through signalling by small molecules, such as biotin and amino acids. The type IV pili from bacteria are involved in their attachment to plants, e.g. by the associative N_2-fixing bacterium *Azoarcus* sp. (Dorr *et al.,* 1998), to fungal cell surfaces, to abiotic surfaces forming biofims (O'Toole & Kolter, 1998b) and they are also involved in adhesion among bacterial cells (Darzins & Russell, 1997). Some bacteria can secrete polysaccharides on their surface. These extracellular polymers comprise lypopolysaccharides (LPS), capsular polysaccharides (CPS), which form a cohesive layer linked to the cell surface, and exopolysaccharides (EPS), which form a slime layer loosely attached to the cell surface (Madigan *et al*., 1997). EPS can also be secreted into the environment and play an important role in bacterial colonization of plant surfaces. They modify the soil macroporosity, with an increase in N uptake from fertilizer, and they are involved in resistance to water stress (Alami *et al.,* 2000).

2.2.5.3 Bacterial motility

There are different types of surface motility, such as swimming, swarming, twitching, gliding and sliding. Various bacteria often produce polysaccharides to enhance flagella-dependent swarming motility, pili-dependent twitching motility, and gliding motility on surfaces (Harshey, 2003). Swimming and swarming are dependent on flagella (Harshey, 1994). *Azospirillum brasilense* migrates towards roots by swimming through the water space using the polar flagellum (Bashan *et al*., 1986a; Moens *et al.,* 1995). These modes of surface translocation play an important role in the colonization of natural environments by microorganisms (Harshey, 1994). Whereas swimming is an individual movement, swarming is the movement of a group of bacteria (Harshey, 2003). The most active swarming

occurs near the periphery of the colony, where the cells become multinucleate, elongate, synthesize large numbers of flagella, secrete surfactants and advance across the surface in coordinated packs (Harshey, 1994). Cells in the interior of the colony are less motile and appear to dedifferentiate to a vegetative morphology. Isolated swarmed cells generally do not move unless the agar concentration is low or the agar is supplemented with surfactants. In wet colonies, bacterial cells swarm normally, whereas on dry plates, flagellin assembly fails (Berg, 2005). Swimming motility in *Azospirillum* is thought to play a role in the movement of the bacteria toward the plant roots, whereas swarming across the surface of the roots may be important for long-term colonization. Twitching motility is primarily a social activity, involving cell-to-cell contact and can promote outward movement of colonies under nutrient-rich conditions, as well as bringing cells together to form fruiting bodies under conditions of nutrient depletion (Shi *et al.,* 1996). This motility form is also involved in the biofilm formation (O'Toole & Kolter, 1998a). Sliding is produced by proton motive forces of a growing colony in combination with reduced surface tension. There is a strong correlation between the production of surfactants such as lipopolysaccharides, and the sliding phenomenon (Brown & Hase, 2001; Matsuyama *et al.,* 1995; Recht *et al.,* 2000). Although it is a passive movement, this mode of translocation plays a significant role in bacterial surface colonization (Recht & Kolter, 2001).

2.2.5.4 Role of bacterial exopolysaccharides in root colonization

The attachment of *Azospirillum* to the plant root is essential for efficient association with the host plant. This attachment is a biphasic process (de Oliveira Pinheiro *et al*., 2002). In a first step, the bacteria are adsorbed to the roots as single cells in a rapid, weak and reversible way. This phase is mediated only by the polar flagellum and the *Azospirillum* Calcofluor-binding polysaccharides play no role in the adsorption phase (Vande Broek *et al.,* 1998). This phase is followed by the anchoring phase, which firmly and irreversibly binds the bacteria to the root. In this phase, the secreted EPS play a role in the bacterial aggregation and eventually formation of biofilms (Steenhoudt & Vanderleyden, 2000). It has been proposed that most bacteria are somehow autoaggregated, e.g. bound to the plant surface, caught under the mucigel layer or aggregated to other bacteria (Chin *et*

al., 1997). Once the bacteria are attached, a common feature is the expression of large quantities of EPS and the downregulation of the flagella biosynthesis (Harshey, 2003).

Moreover, EPS also protect the bacteria against environmental influences, such as desiccation, osmotic pressure, antibiotics and antagonists (Becker & Pühler, 1998; O'Toole *et al.,* 2000; Stoodley *et al.,* 2002; Davey & O'Toole, 2000) and they can also stabilize secreted enzymes. Furthermore, they can help to hold metals and nutrients near the cell because of their anionic nature (Michiels *et al.,* 1991; de Troch & Vanderleyden 1996; Skvortsov & Ignatov, 1998). Moreover, EPS together with LPS and cellulose, play an important role in the establishment of the symbiosis between rhizobia and legumes (del Gallo *et al*., 1989; Becker & Pühler, 1998).

The flocculation process (aggregation into macroscopic clumps) in *Azospirillum* is generally correlated with the production of EPS with a high C/N ratio and with the ability to develop into cyst-like forms as resistance against environmental stress conditions. Cyst-like forms contain PHB granules as carbon storage compound inside the cells. The PHB is surrounded by EPS (Katupitiya *et al*., 1995; Alexandre *et al.,* 2000). It might be expected that under a high C/N ratio, the EPS concentration is rising too, but this is not the case in all the aggregated strains of *Azospirillum*, indicating that not only the amount of EPS plays a role in aggregation, but also the composition of the EPSs (e.g. amount of arabinose) (Burdman *et al.,* 1998a; Burdman *et al.,* 2000a). The monomer structural diversity of EPS depends on the species and stress conditions and enables to function as informational molecules in cell-to-cell recognition (Okon, 2007).

2.3. Objective and research questions

The general objective of this thesis was first to establish plant growth-promoting effects in greenhouse pot trials, and second to understand the mechanisms underlying plant growth promotion by different rhizobacteria in association with cereal crop plants. Following the isolation of diazotrophic bacteria by an Austrian cooperation partner in the EU project “Micro-N-Fix”, the following strains were used in this study: *Paenibacillus polymyxa, Raoultella terrigena* and *Brevibacillus reuszeri*.

The chapter 4.1 therefore describes a series of inoculation experiments, in which several factors were investigated that may affect plant growth promotion by non-symbiotic diazotrophs.

The following chapters then describe investigations or mechanisms that may be involved in the establishment of diazotrophs in the rhizosphere or in the transfer of N from the N_2-fixing bacteria to the roots of host plants.
As an initial step in the association, a targeted movement of the bacteria to the root surface is required. Therefore, seed and root exudates were collected and investigated for a possible chemotactic action (chapters 4.2, 4.3). Then, another bacterial response to root exudates was investigated, which might be or is the production of expopolysaccharides (chapter 4.3).

Finally, the delivery of N from non-symbiotic N_2-fixing bacteria to host plants was attempted to be characterized by using transgenic tomato plants that were supposed to show a lower capacity for high-affinity NH_4^+ uptake. As these transgenic plants were not yet physiologically characterized, studies on their NH_4^+ uptake capacity in roots had to be conducted before looking at their performance in a bacterial association in more detail.

3. MATERIAL AND METHODS

3.1. Inoculation experiments in the greenhouse

3.1.1 Preparation of soil

Wheat was grown in pots filled with agricultural soils. The soils derived from either the Ap horizon of a Luvisol from the local agricultural area close to the University of Hohenheim (Filderlehm); or from the Ap horizon of a Luvisol/ Xerosol from the Negev region (Israel) (Table 3.1-1). To improve the soil structure, perlite was washed with distilled water, sterilized at 110°C during 24 h and mixed with either soil at 10% v/v.

In the first and second greenhouse trials the Filderlehm/ Perlite mixture was sterilized by γ-radiation (25 kGy).

Except for the last 2 greenhouse trials (to generate more stressful conditions to the plants), the soil was fertilized once at the beginning with P, K and Mg. Phosphorus was applied as $Ca(H_2PO_4)_2$ at 100 mg kg^{-1} soil, potassium as a K_2SO_4 at 150 mg kg^{-1} soil and magnesium as a $MgSO_4$ at 50 mg kg^{-1} soil. The different fertilizers were mixed with the soil substrate in a concrete mixer. Before filling the pots the soil was sieved through a 2 mm mesh.

In some experiments a range of different N-fertilizer doses (0 to 90 mg N kg^{-1} soil) was applied as a $Ca(NO_3)_2$. In some experiments a treatment with high doses of N fertilizer (170 or 200 mg N kg^{-1} soil), applied also as a $Ca(NO_3)_2$, was included to determine the maximal plant growth.

Soil was labelled with the stable isotope ^{15}N as $K^{15}NO_3$ (99.4 atom% ^{15}N at a purity of 99.5%; Chemotrade GmbH, Leipzig, Germany, charge KSH22168) in a range from 14% to 4% ^{15}N atom excess and mixed homogenously before filling the pots.

A N dose of 30 mg N kg^{-1} is comparable to an application of 117 kg N ha^{-1}, assuming a soil density of 1.3 and a soil depth of 0.3 m.

Plastic pots (1.4 L, 9,5 cm diameter, approx. 20 cm of height) were filled out with 1540 g dry soil up to a level of 2 cm from the top, with a bulk density of 1.1 g cm^{-3}.

3.1.2 Plant material and growth conditions

Seeds of the spring wheat cultivars *Triticum aestivum* L. cv. Paragon were obtained from Dalgety Arable Limited (Essex, UK) and *Triticum aestivum* L. cv. Atir from Agron Ltd. (Rehovot, Israel). Seeds of Atir were coated with a fungicide, which was tested to have no negative effects on all the bacteria tested (data not shown). Non-sterilized seeds from these cultivars were germinated directly in the pots. For the experiments I and II, seeds were imbibed overnight at 4°C between filter paper moistened with saturated $CaSO_4$ solution. Depending on the experiment, 6 or 8 seeds per pot were sown at a depth of 2 cm and thinned to the half after germination (Table 3.1-2). Inoculation with different bacterial strains was performed as indicated in Table 3.1-2. After germination the soil surface was covered with 150 g of coarse quartz sand to reduce evaporation.

Table 3.1-1 Analysis of investigated soils.

	Filderlehm soil	**Israeli soil**
Soil type	Silty-clay	Loamy-clay
Soil classification (FAO)	Luvisol	Luvisol, Xerosol
P (CAL-extract VDLUFA)	29 mg P kg^{-1}	127 mg P kg^{-1}
K (CAL-extract VDLUFA)	70 mg K kg^{-1}	174 mg K kg^{-1}
Mg ($CaCl_2$-extract VDLUFA)	80 mg Mg kg^{-1}	290 mg Mg kg^{-1}
N-total	0.11%	0.07%
$N\text{-}NO_3^-$ (CFA)	21 mg N kg^{-1}	2.7 mg N kg^{-1}
Organic matter (C)	0.97%	0.83%
pH ($CaCl_2$-suspension)	7.3	7.5

Source: Landesanstalt für Landwirtschaftliche Chemie, Hohenheim, 09/2002.

Experiments were generally conducted with 4 or 5 replicates and arranged in a completely randomized block design. Each block was rotated weekly. For the determination of the apparent nitrogen mineralisation from the Filderlehm soil 2 replicates were analyzed.

The plants were grown in the greenhouse for 4-5 months until complete maturation of the spikes, except for the experiment V in which the plants were harvested at the 6-leaf stage after 1.5 months.

The greenhouse experiments I-IV were conducted in a greenhouse with poorly controlled conditions. The displayed temperatures in the table 3.1-2 correspond to the means of the months with peak air temperatures in the greenhouse. The plants from the trial III were discarded because of poor growth, as a consequence of extremely hot temperatures in summer 2003.

The last 2 experiments (V and VI) took place in a S1 greenhouse under controlled environmental conditions (Fig. 3.1-1). The temperature was constant (18° or 19°C depending on the experiment) until the 6-leaf stage (EC29) and was then mounted gradually up to 21-23°C every week until harvest (EC92). The temperature at night was always 3°C lower. Natural light was supplemented with artificial illumination from 400 W High Pressure Sodium Lamps (Philips SON-T Agro 400). The relative humidity was adjusted to 50%.
Water losses were determined gravimetrically and every two days adjusted by adding distilled water to a soil water holding capacity (WHC) of 60, 48 or 40% depending on the experiment.

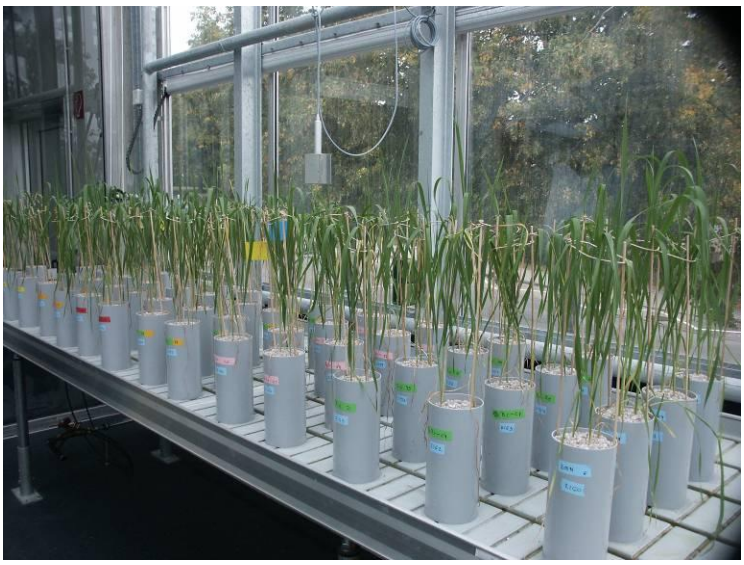

Figure 3.1-1 Arrangement of a pot experiment in the greenhouse under controlled growth conditions.

Table 3.1-2 Overview of the treatments in the 7 consecutive greenhouse experiments.

	Napp min.	**Trial I**	**Trial II**	**Trial IV**	**Trial V**	**Trial VI**
***Triticum aestivum* L. cv.**	Paragon	Paragon	Paragon	Paragon	Paragon	Atir Paragon
Soil type	Filderlehm +Perlite	Filderlehm +Perlite	Filderlehm +Perlite	Filderlehm +Perlite	Filderlehm +Perlite	Israeli soil
Soil sterilization	—	√	√	—	—	—
P, K, Mg fertilisation	√	√	√	√	—	—
N fertilization (mg N kg^{-1} soil)	0, 30, 90	30, 90	15+15, 60	30	30	0, 36, 72
WHC (%)	60	60	60	60	48	40
^{15}N enrichm. (at% excess)	—	14	10	10	4	4
Bacteria	—	Graminante® *A.brasilense*	Bactofil®A *A. brasilense*	*P. polymyxa* *A. brasilense*	*A.b nif, A.b nif^{+}, R.t*, *X.f, X.v*, *B.r, P.p*, *A.b*	*P. polymyxa*
Method of inoculation	—	Liquid at 3-leaf stage	Liquid at 1-leaf stage	Liquid Seed coating Capsules	Liquid at 1-leaf stage	Liquid at 1-leaf stage
Inoculum concentration (CFU mL^{-1})	—	10^3, 10^5	10^3, 10^5, 10^6	L 10^3, 10^6 S 10^3, 10^5 C 10^2	5.10^7	5.10^7
Growth period	Dec02-Apr03	Dec02-Apr03	Mar03-Jun03	Jul03-Nov03	Aug04-Sep04	Apr05-Jul05
Temperature (monthly extremes means)	14→ 20°C	14→ 20°C	18→ 27°C	26→ 18°C	18→ 21°C* *fixed temp.	19→ 23°C* *fixed temp.
Day / night light period	12/12→ 14/10 h	12/12→ 14/10 h	12/12→ 14/10 h	12/12→ 14/10 h	12/12→ 18/6 h	12/12→ 18/6 h
Supplemented light intensity (µmol m^{-2} s^{-1})	338	338	245	245	325	325
RH (%)	—	—	—	—	50%	50%
Plants pot^{-1}	4	4	4	3	3	3
Replicates	2	5	5	5	4	4

3.1.3 Preparation of bacterial inoculants and inoculation method

Non-symbiotic bacterial N_2-fixing strains from different European agricultural soils were isolated and pre-characterized in the framework of the EU project (www.micro-N-fix.org) by the partner Ecowork Laboratories Consulting GmbH (Vienna, Austria). The selection was based primarily on bacterial growth on nitrogen-free medium, the presence of a nitrogenase gene (*nifH*) as determined by PCR and nitrogenase activity as determined by the Acetylen Reduction Assay (ARA). In trial V, a nif^- mutant strain and one transgenic strain with a 4 to 5-fold enhanced N_2-fixation capacity were tested. The Table 3.1-3 presents an overview of the different bacterial strains used in all the experiments.

Frozen stocks of the bacteria were prepared in 25% glycerol and stored at –70°C. For preparing the inoculum one colony was added to 4 mL of Yeast Extract Peptone (YEP) medium (containing 10 g yeast extract, 10 g peptone, 5 g NaCl and 15 g agar per litre at pH 7.0) and grown overnight at 28°C and 190 rpm. One mL of this pre-culture was transferred to a larger volume of the same medium to a desired Optical Density (OD_{600}). In a preliminary experiment, the relation between the OD_{600} of a culture and the number of bacterial cells present in that culture was determined by plating out dilution series obtained from cultures sampled at different OD_{600}. Cells of the late exponential growth phase were harvested by centrifugation (4,000x g, 10 min., 10°C), washed with sterile phosphate buffer (containing 1.24 g K_2HPO_4, 0.39 g KH_2PO_4 and 8.80 g NaCl in 1 L of water and pH 7.0) and diluted with autoclaved distilled water to a density of about 10^7 CFU mL^{-1}. This density has been reported to be optimal for inoculation trials with *Azospirillum* spp. (Bashan, 1986b; Dobbelaere, 2002). As a control, the cell suspension was sterilized by autoclaving (120°C, 20 min, 1.2 bar) prior to inoculation.

Table 3.1-3 Description of the bacterial strains used for the inoculation of wheat plants in pot experiments.

Strains	Characteristica	Source or reference
Graminante®	*Azospirillum* sp. with Ca and Mg carbonate as a carrier	Commercial biofertilizer from Laboratorios Alquimia S.A. (Santa Fe, Argentina)
Bactofil® A	Mixture of *Azospirillum brasilense, Azobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens* and *Steptomyces albus*. Minimal amount 3.10^8 CFU mL^{-1}, pH 5.0-6.5	Commercial biofertilizer from Agrinova GmbH (Mühlheim, Germany)
Azospirillum brasilense Sp7	Wild type strain isolated from *Digitaria decumbens*; strain is motile by flagella.	(Tarrand *et al.*, 1978)
Paenibacillus polymyxa	Wild type strain; $Gram^+$; motile by flagella; endopore-forming strain; produces antibiotics (He *et al*., 2007)	Provided by Ecowork Lab. Consulting GmbH, (Vienna, Austria) within the EU-Micro-N-fix project
Raoultella terrigena TF1-08	Wild type strain; $Gram^-$; non motile (Drancourt *et al*., 2001); not endospore forming (Li *et al*., 2004)	Provided by Ecowork Lab. Consulting GmbH, (Vienna, Austria) within the EU-Micro-N-fix project
Xanthobacter flavus	Wild type strain; $Gram^+$; produces PHB; no motility; N_2-fixer under low O_2 pressure (Bergey *et al*., 1994)	Provided by Ecowork Lab. Consulting GmbH, (Vienna, Austria) within the EU-Micro-N-fix project
Xanthomonas vesicatoria	Wild type strain; $Gram^-$; produces no PHB; motile by flagella (Bergey *et al*., 1994)	Provided by Ecowork Lab. Consulting GmbH, (Vienna, Austria) within the EU-Micro-N-fix project
Brevibacillus reuszeri	Wild type strain; $Gram^+$; motile by flagella; endospore-forming strain (Bergey *et al*., 1994)	Provided by Ecowork Lab. Consulting GmbH, (Vienna, Austria) within the EU-Micro-N-fix project
Azospirillum brasilense Sp7 (nif^-)	Mutant defective in nitrogenase gene expression	(Jara *et al*., 1983)
Azospirillum brasilense ORF280 ::Tn5 (nif^+)	Trasposon-inserted strain with a 4-fold higher nitrogenase activity	(Revers *et al*., 2000)

Liquid inoculation was performed by pipetting one mL directly onto the soil surface close to the base of the shoots at the 1-leaf or 3-leaf stage, depending on the experiment (see Table 3.1-2), followed by watering with distilled water. To determine the exact concentration of bacteria present in each inoculum, dilutions series were plated out on YEP agar and the CFU were counted after incubation. In experiment IV, different inoculation methods were tested: liquid inoculation as described above, but directly applied to the seeds; seed coating with a bacterial suspension as performed by Dalgety Arable Limited (Essex, UK), with surface sterilized seeds as a control (3 min in ethanol, washed 3 times with sterilized water, 30 min with a solution with 3% hypochlorite and 1% SDS and rinsed with sterilized water); and encapsulated inoculum, where capsules were produced by ENITIIA (Nantes, France) and placed close to the seeds during sowing. As control, capsules were sterilized by autoclaving (120°C, 20 min, 1.2 bar). In the experiment I a commercial biofertilizer, Graminante® (Laboratorios Alquimia S.A., Santa Fe, Argentina), was applied at a rate of 13 g powder per pot. In the experiment II the biofertilizer Bactofil® A (Agrinova GmbH, Mühlheim, Germany) was applied at the 1-leaf stage and at the heading stage at 3 mL plant^{-1} (= 3x 10^{7} cells plant^{-1}) were applied as a liquid inoculum to the basis of each plant, according to the instructions of the manufacturer.

3.1.4 Analysis of growth and yield measures

Germination rate, evapotranspiration and Spectral Plant Analysis Diagnostic (SPAD) values were recorded. SPAD provided rapid and non-destructive relative indications of leaf chlorophyll contents to estimate the N content in the plant tissue (Scheppers *et al.*, 1998). SPAD values were recorded by a chlorophyll meter (Minolta, type SPAD 502, Ahrensburg, Germany) from the last totally expanded leaf and measured once in the middle of the leaf on 2 plants per pot.

At harvest, plants were cut off directly above the soil surface for analysis of fresh and dry matter of grains, shoots and roots, plant height (length of the whole plant up to the emerging spikes), number of grains per row of a spike, number of spikes, N concentration of grains, shoot and roots. $^{15}N/^{14}N$ ratio of grains, shoot and roots were also determined. Dry weight of plants was determined after drying at 55°C until constant weight. The dried plant material was homogenized to a fine powder

with a steel-grinding mill (model TS 100, Siebtechnik, Mülheim an der Ruhr, Germany).

Nitrogen concentrations and $^{15}N/^{14}N$ ratio were determined by an elemental analyser, model Eurovector EA, according to the Dumas method (HEKAtech GmbH, Wegberg, Germany) and by a mass spectrometer model Delta-Advantage (Thermo-Finnigan, Bremen, Germany), respectively. Analysis of the N concentrations in soils to investigate the soil N mineralisation rate was performed with an elemental analyser, model VarioMax CN (Elementar Analysensysteme GmbH, Hanau, Germany).

Data were analysed within the statistical software SigmaStat® for Windows Version 2.03 (SPSS Inc., Chicago, USA). Results of the different measurements were subjected to two-way analysis of the variance (ANOVA) and significance at the 5% level was tested by Tukey´s test (Fisher's LSD test in Appendix 4). Means are shown as bars or absolute values ± SD in all graphs and statistical differences are indicated by different letters.

3.2. Plant root exudates

3.2.1 Collection of seed and root exudates

Plant material

Seeds and seedlings of bean (*Phaseolus vulgaris* L.), spring wheat (*Triticum aestivum* L. cv. Paragon) and maize (*Zea mays* L. cv. Tassilo) were used for the collection of seed and root exudates.

Plant growth conditions

Seeds were germinated between filter papers saturated with $CaSO_4$. After germination, seedlings were transferred (10 plants per 2.5 L) to continuously aerated nutrient solution and grown under long day conditions. The pH was adjusted to pH 7 by adding approximately 2 g of $CaCO_3$ to the nutrient solution. The nutrient solution was replaced every 2 days. The compositions of the nutrient solutions for maize, wheat and bean are shown in Table 3.2-1.

Collection of exudates

Before collection of exudates, 4 d old seedlings were rinsed with saturated $CaSO_4$ for 30 min and seedlings older than 4 d were rinsed shortly with deionised water.

Table 3.2-1 Composition of the nutrient solutions used in hydroponic culture.

Elements	Bean (final conc.)	Maize / Wheat (final conc.)
K_2SO_4		0.7 mM
KCl		0.1 mM
$MgSO_4 . 7H_2O$	1.2 mM	0.5 mM
$CaCl_2. 2H_2O$	2 mM	
KH_2PO_4	1 mM	0.1 mM
H_3BO_3	25 µM	1 µM
$MnSO_4 . H_2O$	2.5 µM	0.5µM
$ZnSO_4 . 7H_2O$	0.25 µM	0.5 µM
$CuSO_4$	0.25 µM	0.2 µM
$Na_2MoO_4 . 2H_2O$	0.25 µM	
$(NH_4)_6Mo_7O_{24}$		0.01 µM
Fe-EDTA	50 µM	100 µM
For N starvation	1mM K_2SO_4	2 mM $CaCl_2$
For complete medium	1mM KNO_3 0.5 mM $(NH_4)_2SO_4$ 0.5 mM K_2SO_4	2 mM NH_4NO_3

For the collection of exudates the following seeds or seedlings were used: i) 50 seeds of bean or maize or 100 seeds of wheat; ii) 20 4 d old seedlings of bean or 25 4 d old seedlings of maize and wheat; iii) 10 seedlings of either plant species older than 4 d were used for exudate collection over a time period of 4 h (Fig. 3.2-1), starting 2-3 hours after onset of light. Seedlings were put into beakers containing 100 mL of aerated, deionised water. Collection times between 2 and 4 hours have been found to be experimentally the most suitable for analyzing organic acids, sugars, amino acids (Neumann & Römheld, 2007) as increasing the time of collection can lead to microbial degradation of exudates.

After the exudation period, the volume of the exudate solution was adjusted to 100 mL with deionised water. For motility assays the root washings were concentrated 20-fold by lyophilization. Exudate solutions were filter sterilized (0.2 µm FP 3/0.2 PA-S sterile filter, Schleicher & Schuell, Dassel, Germany) and adjusted to 100 µg C mL^{-1}. The total carbon concentration of the samples was analysed by an elemental analyser, model Eurovector EA, by the Dumas method (HEKAtech GmbH, Wegberg, Germany).

3.2.2 Characterization of exudates by HPLC analysis

For HPLC analysis the crude exudates were filtered (589[2] White Ribbon ∅ 55 mm, Schleicher & Schuell, Dassel, Germany) and stored at –70°C until further analysis. Ten mL of exudates were vacuum concentrated overnight in a plastic tube using a Speed vac concentrator (Savant, GMI, Richmond, CA, USA). The pellet was resuspended with 500 µl 18 mM KH_2PO_4 solution (pH 2.15 used as HPLC eluent) and centrifuged 5 min at max. 14,000x g. The supernatant was used for reversed phase HPLC analysis with suppressed ionisation. Separation of the organic acids was performed on a reversed-phase C-18 column (GROM-SIL 120 ODS-5 ST, particle size 5 mm; length 250 mm, ID 4.6 mm) with a Hypersil ODS guard column (length 20 mm, ID 4.6 mm; GROM, Herrenberg, Germany). A sample volume of 20 µL was injected into the isocratic flow of the eluent (18 mM KH_2PO_4, pH 2.25, 35°C, flow rate 0.5 mL min^{-1}) and detected spectrophotometrically at 215 nm with a UV detector. Identification and quantification of the organic acids were carried out by comparing retention time, peak area and absorption spectra with those of known standards.

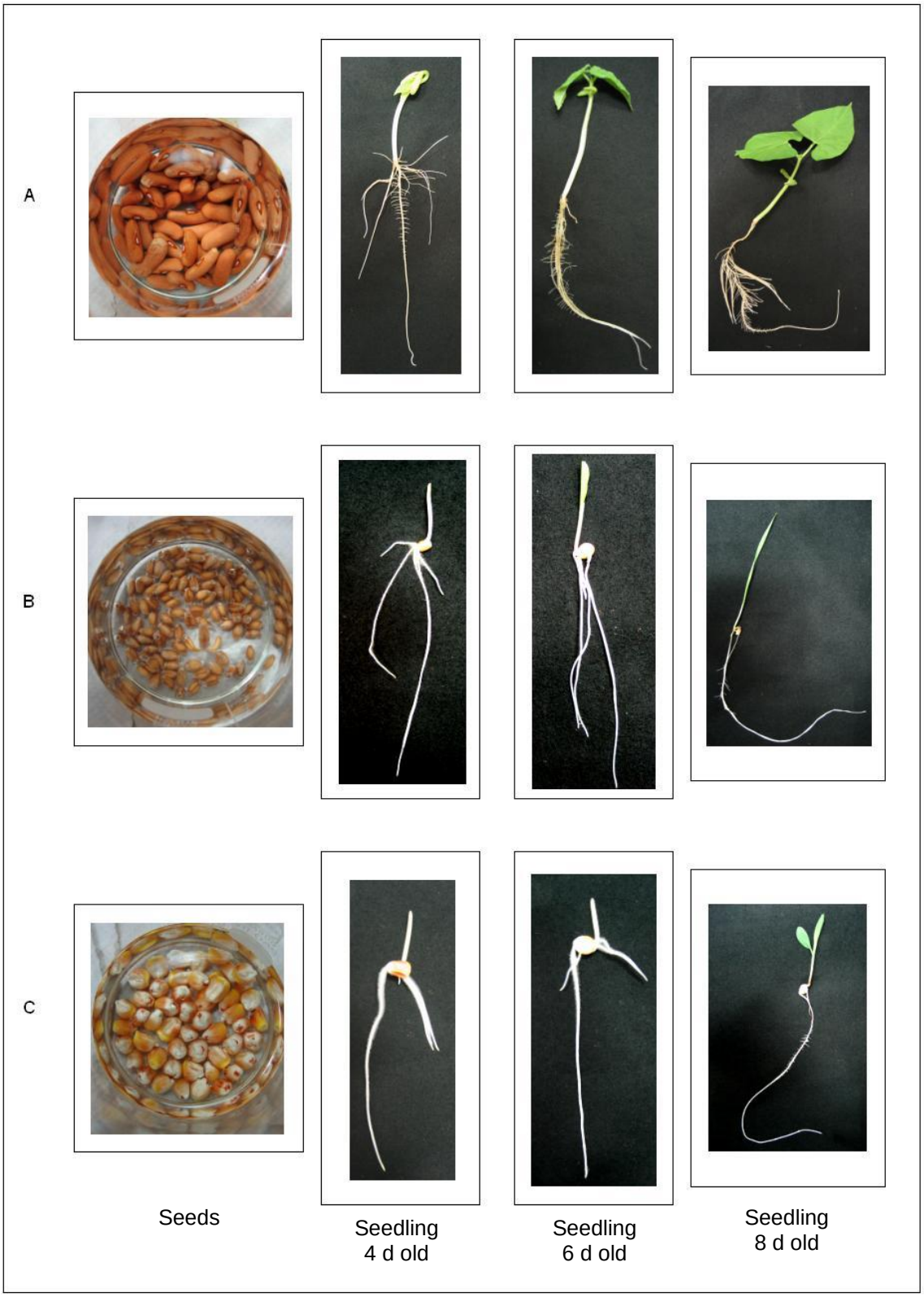

Figure 3.2-1 Developmental stages of bean (A), wheat (B) and maize (C) seedlings at the different exudate collection dates.

3.3. Chemotaxis assays

3.3.1 Bacteria strains and media

It has been reported, that the combination of a relatively low nutrient media solidified by a suitable quality of agar is important for the bacterial motility. In preliminary experiments in this study, different media were tested, such us 10-fold diluted YEM medium, water with agar and R2A-agar medium (data not shown). The latter was selected as medium for the motility assays because it allowed motility for *B. reuszeri* and other N_2-fixing bacteria. Motility plates contained 20 mL of 5-fold diluted R2A-agar medium (CM0906 with pH 7.2, Oxoid Ltd., Basingstoke, UK) resulting in a semisolid medium (0.33% agar).

3.3.2 Motility assay

Stock cultures of the strains (*A. brasilense* Sp7, *Paenibacillus polymyxa*, *Brevibacillus reuszeri* and *Xanthomonas vesicatoria*), already described in Table 3.1-3, were maintained at –70°C in 25% (v/v) glycerol. Bacterial pre-cultures (4 mL) were grown overnight at 28°C at 190 rpm in Yeast Extract Peptone (YEP) media (containing 10 g yeast extract, 10 g peptone, 5 g NaCl and 15 g agar per liter, pH 7). One mL of this pre-culture was further cultured to an Optical Density (OD) corresponding to the late exponential phase, as determined by the growth curve of the respective bacteria strain. In a preliminary experiment the relation between the OD of a culture and the number of bacterial cells present in that culture was determined by plating dilution series obtained from culture sampled at different OD_{600}. Cells of the late exponential growth phase were harvested by centrifugation (1,700x g, 15 min at 8°C), washed with sterile phosphate buffer (containing 1.24 g K_2HPO_4, 0.39 g KH_2PO_4 and 8.80 g NaCl in 1 L of water and pH 7) and centrifuged again. The pellet was resuspended in PBS to a viscous solution (10 mL of original culture resuspend to 200 µL).

Filter paper discs (5 mm diameter, water uptake capacity= 15 µL $disc^{-1}$, Macherey-Nagel, Düren, Germany), previously washed with methanol and distilled water, autoclaved and dried, were soaked with filter-sterilized exudate solutions or

standard solutions (adjusted to 100 µg C mL^{-1}) and placed 2 cm left and right of the inoculation spot (Fig. 3.2-2). Each filter paper disc contained 1.5 µg C. It has been shown that an outward migration of the spotted bacterial cells occurs after a few hours after inoculation on appropriate medium. This corresponds to the time when the colony has reached the required cell density for migration (Toguchi *et al*., 2000). Similarly, Brown and Hase (2001) showed that dilutions of the pellet significantly reduced the mobility of the cells. In the system used, 20 µL of a 50-fold concentrated bacterial suspension were carefully spotted onto the centre of the motility plates (Ø 90 mm) and incubated in a humidified chamber on a horizontal surface at 28°C for 8 h in the darkness.

A first motility response of bacterial cells resulted in the formation of a ring after about 15 min (data not shown). A second motility response in the form of an outer ring was formed after 8 h. The outer border of this ring was then re-drawn onto the bottom of the plate by a pen, following the approach of Grimm and Harwood (1997). The area of this ring was quantified. This approach is preferential to the measurement of the diameter because the bacterial rings do not have a regular shape. This ring was scanned by an EPSON Scan version 2.20A (Seiko Epson Corporation, 2002) and the area was quantified using Quantity-One-software version 4.02 (BioRad, Munich, Germany).

It should be note that the order of placing exudates or bacteria might affect the results. In this study, it has been observed that the spotted bacteria migrated outward at a higher velocity when the exudates were supplied before relative to the situation when the bacteria were spotted first.

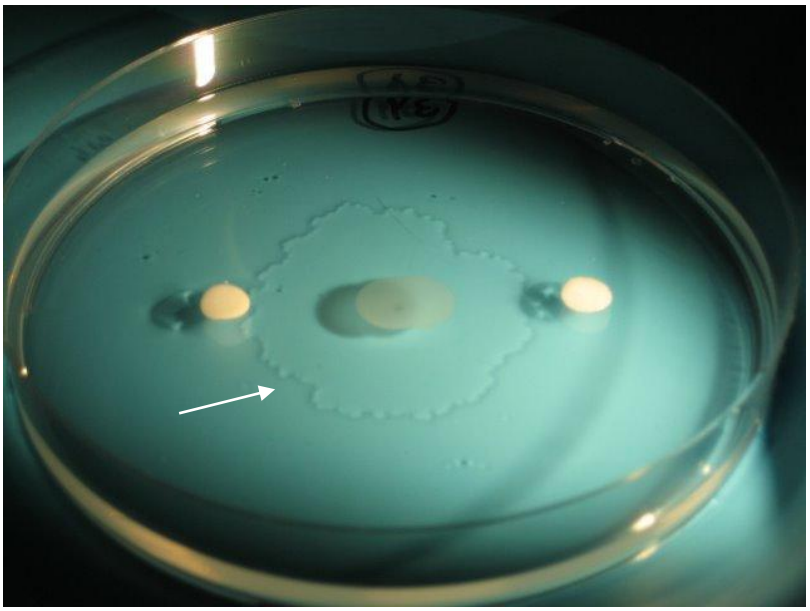

Figure 3.2-2 Motility ring formed by *Brevibacillus reuszeri* in semisolid agar medium after 8h of incubation in the presence of exudate-soaked filter paper discs.

Different filter sterilized organic acids were tested: L(-)-malic acid (Sigma, St. Louis, USA), t-aconitic acid (Serva, Heidelberg, Germany), malonic acid (Merck). D(+)-glucose monohydrat (Applichem, Darmstadt) was also tested, as *Brevibacillus spp.* can utilize glucose as sole carbon source (Petrie *et al.*, 2003). All organic acid solutions were adjusted to pH 7. Deionised and filter sterilized water was used as a control.

Data were analyzed using the statistical software SigmaStat® for Windows Version 2.03 (SPSS Inc., Chicago, USA). Results of the different measurements were subjected to analysis of the variance (ANOVA), and significance at the 5% level was tested by Fisher's LSD test. Every experiment consisted of 5 replicates. The experimental protocol was repeated and improved several times. It should be noted that, despite a large quantitative variability, all data from preliminary and presented experiments show the same trend. The displayed results correspond to the assay with the smallest standard deviations.

3.3.3 Detection of bacterial exopolysaccharides (EPS) by Calcofluor staining

A stock solution (3.5 mg mL^{-1}) of Calcofluor White (Fluorescent Brightener 28, Sigma-Aldrich, St. Louis, USA) was prepared with deionised water and stored at –20°C in the dark. Calcofluor white was freshly added in a 1:100 dilution to the semisolid medium before pouring the agar. Fluorescence of Calcofluor-binding compounds of *B. reuszeri* was photographed under UV light (excitation wavelength 365 nm) after incubation during 4 d with the exudates in the dark.

3.4. Hydroponic culture of tomato

3.4.1 Hydroponic culture conditions

Plants were grown hydroponically under non-sterile conditions for 4 weeks in a climate chamber under the following conditions: 24°C during the light period (7:00 to 23:00) and 22°C during the dark period (23:00 to 7:00), 280 µmol m^{-2} s^{-1} light intensity and 70% relative humidity. Seeds were germinated in the climate chamber on washed and autoclaved fine sand moistured with saturated $CaSO_4$ solution. Two weeks after sowing, the hypocotyls were covered by Saran foil, wrapped in foam and transferred into 3 L pots (10 plants per pot) with full nutrient solution adjusted to pH 5.8-6.0 with KOH (Table 3.4-1). The nutrient solution was changed every 3 days. After one week, plants were transferred to 5 L pots (5 plants per pot) with full nutrient solution buffered to pH 7.0 with approximately 2 g of calcium carbonate. The nutrient solution was renewed twice in the first week and every 2 days until harvest. Nitrogen was supplied as 2mM NH_4NO_3. Nitrogen starvation was induced using N-deficient nutrient solution for 3 days. Ammonium was re-supplied to the nutrient solution after 3 days of N starvation either for 2 hours (with 200 µM NH_4^+) or for 12 hours (with 2 mM NH_4^+) (Table 3.4-1).

To prevent nutrient depletion, during the 12 hours of re-supply, 100 L of 200 µM NH_4^+ solution was prepared and continuously circulated between a tank and the pots. The 200 µM NH_4^+ solution for the 2 hour-pulses was 10% enriched by ^{15}N and composed of 10 µM 95% ^{15}N-$(NH_4)_2SO_4$ and 90 µM $(NH_4)_2SO_4$. Plants of the same age were harvested at the same time of day, usually 6 h after onset of light.

Table 3.4-1 Composition of the nutrient solution for tomato plants.

Elements	Final concentration
$MgSO_4 .7H_2O$	1.2 mM
$CaCl_2. 2H_2O$	2 mM
KH_2PO_4	1 mM
H_3BO_3	25 µM
$MnSO_4 .H_2O$	2.5 µM
$ZnSO_4 .7H_2O$	0.25 µM
$CuSO_4$	0.25 µM
$Na_2MoO_4 .2H_2O$	0.25 µM
Fe-EDTA	50 µM
For complete medium	1mM KNO_3 0.5 mM $(NH_4)_2SO_4$ 0.5 mM K_2SO_4
For N starvation	1mM K_2SO_4
For 2 mM NH_4 re-supply: For 200 µM NH_4 re-supply:	 1 mM $(NH_4)_2SO_4$ 1 mM K_2SO_4 0.1 mM $(NH_4)_2SO_4$ 1 mM K_2SO_4

3.4.2 Physiological analysis

Plants grown under different N regimes were harvested at the same time of the day in the middle of the light period, with the exception of plants subjected to 6 days N starvation or 6 days N starvation with daily NH_4^+ pulses. These plants were harvested 3 days later at the same time of the day. At harvest, roots were washed in 1 mM $CaSO_4$ solution for 1 min. Roots of 4 single plants were cut, briefly dried on paper and their fresh weight was determined. The shoots were separated into 2 parts: the 3 last fully-expanded leaves (young leaves) and the remaining leaves (old leaves). All samples were weighed, frozen immediately in liquid N_2 and stored at -70°C before being grinded and lyophilized. After grinding of the frozen

samples, approximately 100 mg were taken for RNA extraction and 100 mg for ^{15}N analysis (see chapter 3.4.3).

For chlorophyll analysis, a piece of 40 mg from the first fully expanded leaf and from the third oldest leaf were taken, cut into small pieces and incubated with 4 mL N,N-dimethylformamide in the dark at 4°C for 24-48 hours. The extinction at 647 and 664 nm was measured using a photometer and chlorophyll concentrations were calculated according to Chlorophyll (a+b) = 7.04 $Ext._{664}$ + 20.27 $Ext._{647}$.

For ammonium determination, 50 mg of lyophilized sample were incubated with 1 mL of 10 mM formic acid. The sample was vortexed for 20 seconds and centrifuged at 2000 g in a pre-cooled centrifuge for 10 min. The supernatant was collected and kept on ice. The free ammonium concentration was quantified by fluorescence spectroscopy at neutral pH, using on-line derivatization of ammonium by o-phthalaldehyde (Husted *et al*., 2000). Twenty microliters of the formic acid extract were mixed with cold OPA buffer solution (3 mM o-phtalaldehyde, 10mM ß-mercaptoethanol and 100 mM potassium phosphate buffer, pH 6.8), incubated at 80°C for 15 min and immediately cooled down on ice. The fluorescence was measured at 470 nm with an excitation at 410 nm using a fluorescence spectrophotometer (F2000 Hitachi, Tokyo, Japan). Ammonium contents were directly calculated using a calibration curve from 0 to 2000 µM NH_4^+.

Three samples were analyzed for glutamine, glutamate, threonine, serine and aspartate concentration. For this purpose, samples were separated by HPLC on a cation-exchange column and derivatized before photometric detection (Pilot *et al*., 2004). These analyses were performed at the ZMBP (University of Tübingen, Germany).

3.4.3 ^{15}N-ammonium uptake studies

The roots of 4 to 5 week-old plants were rinsed in 1 mM $CaSO_4$ solution for exactly 1 min, followed by incubation in 5 L pots for 10 min in nutrient solution containing 200 µM ^{15}N-labelled ammonium (95 atom% ^{15}N) as sole N source, and finally washed in the same washing solution for 1 min. For each treatment and each genotype, roots and shoots (3 youngest leaves and rest of shoot) of 4 single plants were separated, weighed, frozen immediately in liquid N_2 and stored at -70°C. Frozen samples were grinded and 100 mg of the powder were dried at 65°C. About 1.5-2.5 mg powder of each sample was used for ^{15}N determination by isotope ratio mass spectrometry (MAT-DELTA^{plus}, Thermo Finnigan, Dreieich, Germany).

3.5. Plant molecular biology

3.5.1 Transformation of tomato plants (*Lycopersicon esculentum* L.)

For antisense repression, tomato plants (*Lycopersicon esculentum* L. variety Moneymaker) were transformed by the leaf disk transformation method using the pBR vector containing an antisense fragment of the *LeAMT1;1* or *LeAMT1;2* ORF lacking approximately 200 bp at the 3'-end. The selected homozygous lines of pre-tested antisense lines were 117, 123 and 204.2 for *LeAMT1;1* and 120.3, 258 and 264 for *LeAMT1;2*.

The strain of *Agrobacterium tumefaciens* GV2260 carrying a resistance gene for rifampycin and ampicilin in the genome and kanamycin in the Ti-plasmid (pBINAR) was used for transformation of tomato plants. Cotyledons of approx. 2 week-old plants were dipped in a solution containing transformed *Agrobacterium* cells which were previously cultivated in selective medium, washed and resuspended to obtain an OD_{600}= 0.5.

Seeds harvested from regenerated and selected tomato plants were sterilized by agitation at RT in sterilization solution (70% ethanol for 3 min, 1.5% sodium hypochlorite with Tween 20 for 10 min and 3 times washed with distilled water). T0

seeds were selected on plates containing half MS medium (2.2 g L^{-1} MS, Murashige and Skook medium, Duchefa Biochemie, Haarlem, The Netherlands), 10 g L^{-1} sucrose, 6 g L^{-1} agar Bitek (DIFCO, Detroit, USA) containing kanamycin (35 mg L^{-1}), ticarcillin disodium (100 mg L^{-1}) and Combactam® (100 mg L^{-1}). Resistant plants were transferred to soil and grown in the greenhouse until maturation. The T2 seeds were selected on half-strength MS medium and homozygous lines were selected.

3.5.2 RNA gel blot analysis

For RNA extraction, the grinded powder samples were processed using TRIzol® Reagent following the instructions of the manufacturer (Invitrogen GmbH, Karlsruhe, Germany). Samples were kept in liquid N_2 and centrifuges were pre-cooled. Immediately after addition of 1 mL of TRIzol® to 100-200 mg of sample, the samples were vortexed, incubated at RT for 5 min and 0.2 mL of chloroform was added. The samples were shaken vigorously for 15 s by hand and incubated again at RT for 2 min before being centrifuged at 12,000x g for 15 min at 4°C. The supernatant was collected and RNA was precipitated after addition of 0.5 mL isopropanol and incubation at RT for 10 min. After centrifugation at 12,000x g for 10 min at 4°C, the pellet was washed with 1 mL 75% ethanol for RNA (in DEPC-treated water), resuspended in 25 µL TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA in DEPC-treated water), incubated for 10 min at 60°C and stored at –20°C. The RNA concentration of the samples was determined by measuring absorption at 260 nm of a 1:500 dilution in water according to [RNA] = 0.04 x OD_{260} x dilution factor.

Northern blot analyses were conducted running 10 µg of total RNA in a 1.2% (w/v) agarose gel containing 1x MOPS buffer (80 mM MOPS, 20 mM Na-acetate DEPC-treated, 0.4 mM EDTA pH 8.0 DEPC-treated) and 6.5% formaldehyde in DEPC-treated water. The RNA samples were preincubated in denaturation buffer (0.5x MOPS buffer, 18% formaldehyde, 18.5% formamide, 1x blue buffer) at 65°C for 15 min and separated by electrophoresis on agarose gels. Equal RNA loading was verified on an UV transiluminator (Herolab GmbH Laborgeräte, Wiesloch,

Germany). The gel was washed 10-20 min in distilled water and capillary-blotted onto Hybond-N^+ nylon membrane (Amersham Little Chalfont, UK) by 10x SSC buffer (1.5 M NaCl, 0.15 M tri-sodium citrate pH 7.0) overnight. The membrane was washed with 2x SSC, 0.1% SDS for 5 min and with 2x SSC for 5 min, dried and fixed at 80°C for 2 h. The blot was pre-hybridized at 42°C overnight in a pre-hybridization buffer (50% formamide, 5x SSC, 1% w/v sarkosyl, 10% Dextran sulfate) containing 0.125 mg mL^{-1} tRNA (in RNAse-free TE buffer).

The probes used to specifically detect *LeAMT1;1* and *LeAMT1;2* sense mRNA were generated from a ~500 bp fragment composed of 200 bp of the respective ORF 3'-end which was not present in the antisense constructs and the 3'-UTR of the respective cDNA. To generate the LeAMT1;1 probe, a 500bp cDNA was cut by *Sma*I and *Hind*III from pBS harboring the *LeAMT1;1* ORF and 3'-end UTR. For *LeAMT1;2*, a ~450 bp fragment was excised by *EcoR*V from pBS containing the *LeAMT1;2* ORF and 3' UTR. The labelling of the probe was carried out using Megaprime DNA labelling system (Amersham Biosciences Europe, Freiburg, Germany), using 20-30 ng template DNA, 5 µL random primers, 10 µL of labelling buffer, 5µL of ^{32}P-radiolabelled dCTP (50 µCi) and 2 µL of Klenow fragment and incubating the mixture at RT for 1-2 hours. The reaction was stopped by adding 5 µL of 0.5 M EDTA pH 8.0. The radioactive probe was purified using a Sephadex G50 column (Amersham Biosciences Europe GmbH, Freiburg, Germany), denaturated at 95°C for 10 min and added to the hybridization solution for incubation overnight at 42°C. Membranes were washed at 42°C twice in 2x SSC with 0.1% w/v SDS buffer for 20 min, once in 0.2x SSC with 0.1% (w/v) SDS buffer for 20 min and finally 0.1x SSC with 0.1% SDS buffer for 20 min. Radioactivity on the membrane was detected on X-ray films (Hyperfilm MP, Amersham Biosciences GmbH, Freiburg, Germany) exposed to the membrane at -70°C. Ethidium bromide-stained gels were used as RNA loading control.

4. RESULTS

4.1. Influence of different factors on growth of wheat plants inoculated with diazotrophic bacteria

In all the greenhouse experiments the height of plants, number of spikes, number of grains per ear, DW, N concentrations and $^{15}N/^{14}N$ ratios in grains, shoots and roots were determined at harvest. At tillering and at the shooting stage evapotranspiration rates and SPAD values were also recorded. In order to test the response of plant growth to the inoculation with certain diazotrophs, a growth system was established, in which different factors in the association between plants and N_2-fixing bacteria were systematically tested. Some of the growth factors were also tested in combination, e.g. soil type with bacterial strain or plant genotype under different water regimes. An overview on all tested variables on growth and N measures is provided in the Table 3.1-2. This table describe the chronological sequence of performed experiments, whereas the following description of their outcome has been summarized and ordered along N fertilization treatments, soil properties, plant genotype and bacterial inoculation.

4.1.1 Nitrogen fertilization

As a prerequisite to adjust N fertilization in a suboptimal to optimal range, the amount of N delivered by mineralization of the soil organic matter was estimated. For this purpose nitrate was determined in the solution of Filderlehm soil after incubation in non-planted (Appendix 1) or planted pots (Appendix 2). With regard to N input, BNF was neglected, because leguminous plants were not in the soil, which could participate in BNF to a considerable extent; N derived from the air, e.g. NH_3, was also neglected. With regard to N output, N losses like via N_2, NH_3 and NO_2 were considered to be 0, because conditions for denitrification were unfavorable and soil pH (pH 7.3) was not sufficiently basic to promote NH_3 emissions to a considerable extent (Appendixes 1 and 2).

To find an adequate N supply at which the inoculated bacteria could promote plant growth, a wide range of N fertilization levels was tested 0, 15, 30, 36, 72, 60 and 90 mg N kg^{-1} soil. The lowest N level, which still allowed plant growth until

maturity, was selected. When the plants were cultivated at 15 mg N kg^{-1} soil the leaves were chlorotic already at the jointing stage (EC32) (Fig. 4.1-1). To allow the plant to reach the maturity stage other 15 mg N kg^{-1} soil were added. Therefore, 30 mg N kg^{-1} soil was considered to represent the minimal level of N to fertilize in the established soil system.

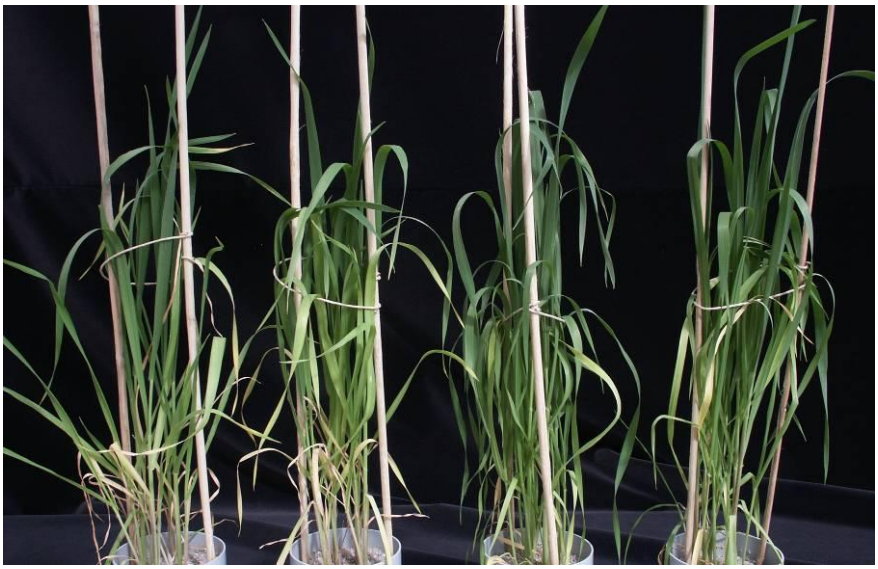

Figure 4.1-1 Chlorotic symptoms in wheat plants (cv. Paragon) at jointing stage (EC 32) inoculated with 10^5 CFU $seed^{-1}$ of *A. brasilense* and grown under 2 different N regimes
Left: 15 mg N kg^{-1} soil (control/ inoculated). Right: 60 mg N kg^{-1} soil (control/ inoculated).

Comparing different N fertilization levels among different greenhouse trials, plants cultivated at a higher N level (60, 72 and 90 mg N kg^{-1} soil) showed as expected increased height (Figs. 4.1-2; 4.1-3), higher SPAD values (Fig. 4.1-4), higher grain and shoot DW (Figs. 4.1-5; 4.1-6 A,B; 4.1-7 A,B), higher number of spikes (Fig. 4.1-8), more number of grains per ear (Figs. 4.1-9; 4.1-10), higher N concentration (Fig. 4.1-11) and $^{15}N/^{14}N$ ratio in grains (Fig. 4.1-12) than plants cultivated at a lower N level (30 and 36 mg N kg^{-1} soil). With respect to the $^{15}N/^{14}N$ ratios, the higher the N fertilization, the lower was the dilution of the ^{15}N-labelled N fertilizer; this has been conformed also in a subsequent experiment (Fig. 4.1-13). Likewise, plants cultivated at 36 mg N kg^{-1} soil showed higher SPAD values (Fig. 4.1-14) and higher grain and shoot DW than plants without N supply (Fig. 4.1-7). Contrarily, the N concentration in grains and roots was higher when no N was added (Fig. 4.1-15). This is probably the consequence of a concentration of nutrients in the plant tissues due to extremely reduced growth activity of N-deficient plants.

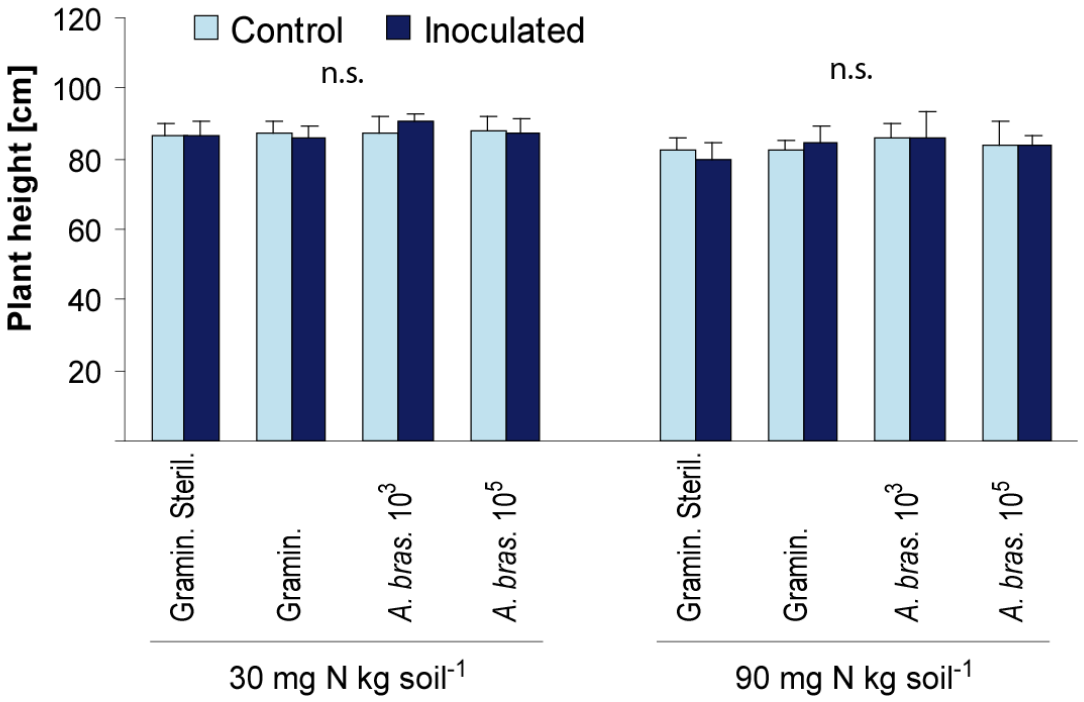

Figure 4.1-2 Plant height of wheat plants at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants (cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means (inside each N level) at $p \leq 0.05$ according to Tukey test, n.s.= not significant.

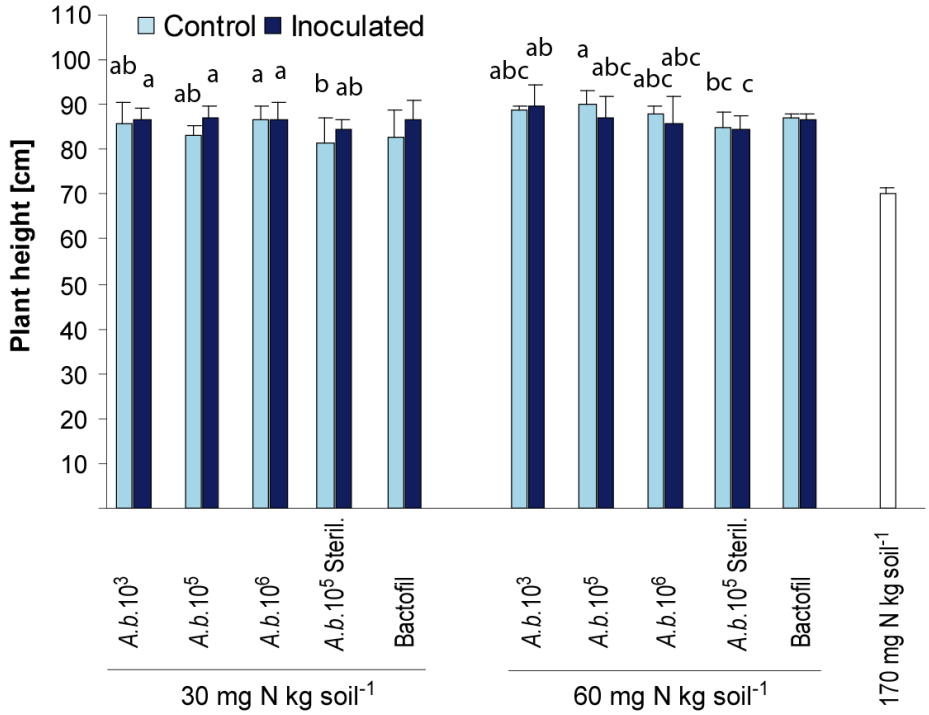

Figure 4.1-3 Plant height of wheat plants at harvest (EC 92) in dependence of biofertilizer and nitrogen application

A.b. 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A.b.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}, *A.b.* 10^6= *A. brasilense* in a concentration of 10^6 CFU mL^{-1}, *A.b.* 10^5 Steril.= *A. brasilense* in a concentration of 10^5 CFU mL^{-1} applied to sterilized soil, Bactofil= commercial biofertilizer. Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated via liquid inoculum and watered to 60% WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test. The treatments with 170 mg N kg^{-1} soil and Bactofil® are shown as reference and were not considered for the statistical analysis, n= 3.

In general, no significant effects of inoculated bacteria were observed on plant growth or on N_2 fixation. When no N fertilization was added and at low N levels (30, 36 mg N kg^{-1} soil) no effects from the inoculation could be observed in any of the performed trials. At 60 mg N kg^{-1} soil an inhibition effect on grain DW was observed when plants where grown in sterilized soil and inoculated with 10^5 CFU mL^{-1} of *A. brasilense* (Fig. 4.1-6 A). At 90 mg N kg^{-1} soil in sterilized soil, application of the biofertilizer Graminante® increased the number of spikes compared to the non-inoculated plants (Fig. 4.1-16).

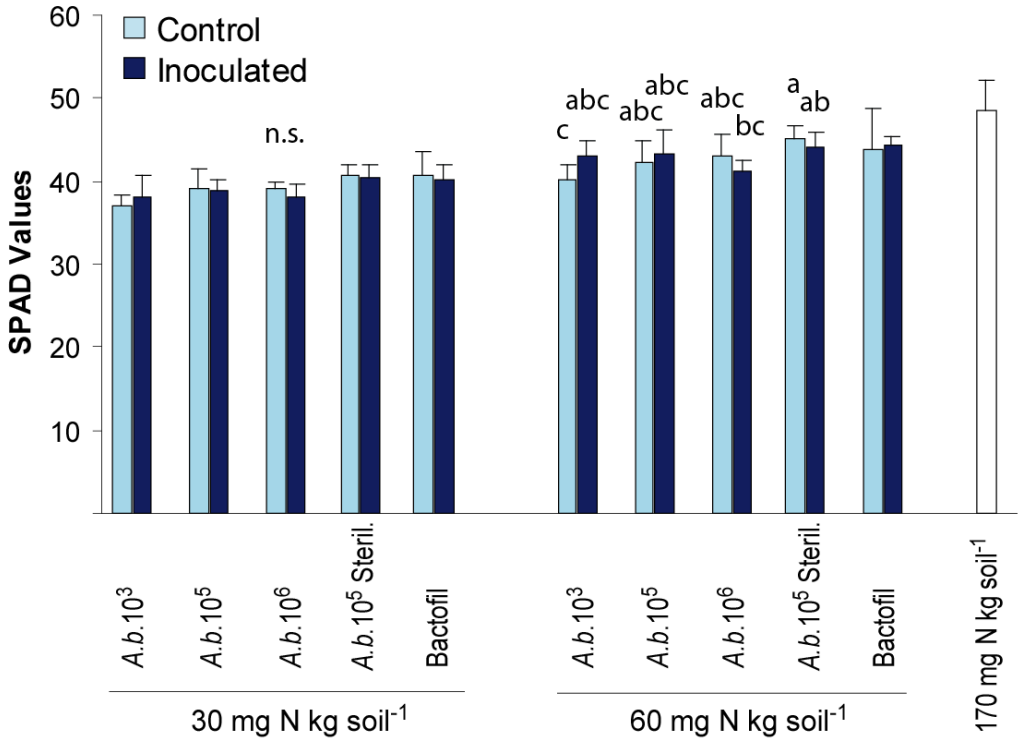

Figure 4.1-4 Relative chlorophyll densities (SPAD values) of wheat leaves at shooting stage (EC31-32) in dependence of biofertilizer and nitrogen application

A.b. 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A.b.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}, *A.b.* 10^6= *A. brasilense* in a concentration of 10^6 CFU mL^{-1}, *A.b.* 10^5 Steril.= *A. brasilense* in a concentration of 10^5 CFU mL^{-1} applied to sterilized soil, Bactofil= commercial biofertilizer. Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated via liquid inoculum and watered to 60% WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant. The treatments with 170 mg N kg^{-1} soil and Bactofil® are shown as reference and were not considered for the statistical analysis, n= 3.

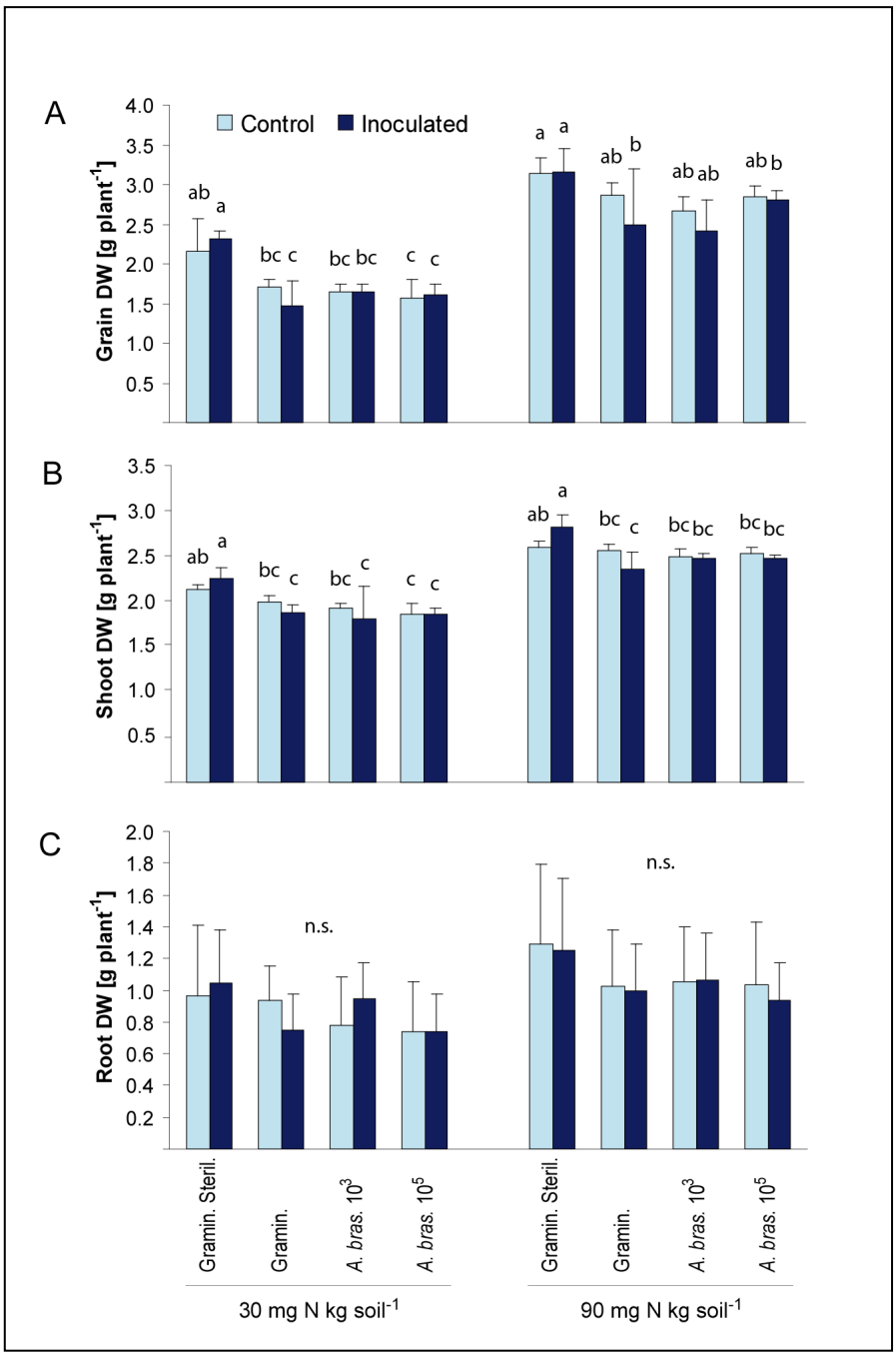

Figure 4.1-5 Grain (A), shoot (B) and root (C) dry weight of wheat plants at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants (cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s.= not significant.

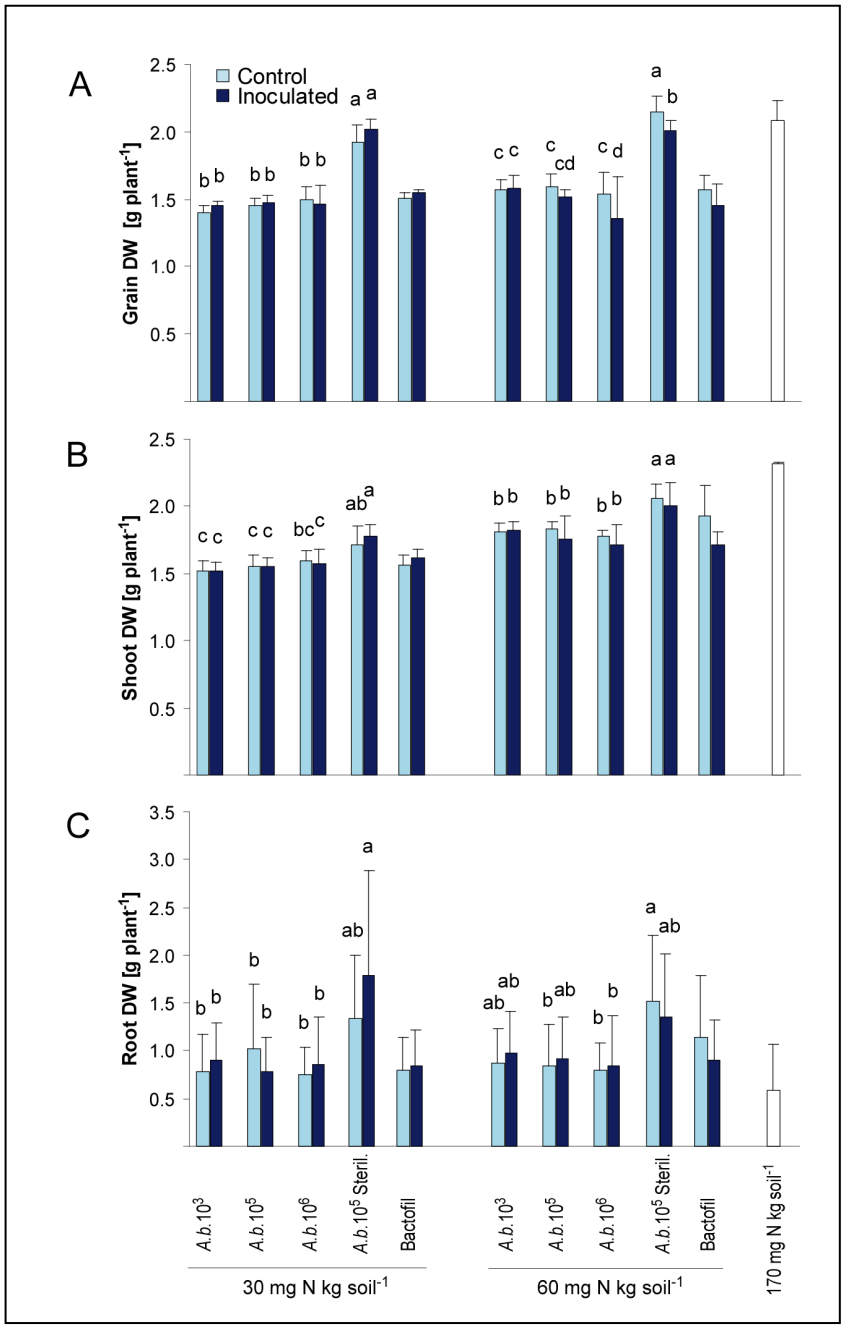

Figure 4.1-6 Grain (A), shoot (B) and root (C) dry weight of wheat plants at harvest (EC 92) in dependence of biofertilizer and nitrogen application

A.b. 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A.b.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}, *A.b.* 10^6= *A. brasilense* in a concentration of 10^6 CFU mL^{-1}, *A.b.* 10^5 Steril.= *A. brasilense* in a concentration of 10^5 CFU mL^{-1} applied to sterilized soil, Bactofil= commercial biofertilizer. Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated via liquid inoculum and watered to 60% WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test. The treatments with 170 mg N kg^{-1} soil and Bactofil® are shown as reference and were not considered for the statistical analysis, n= 3.

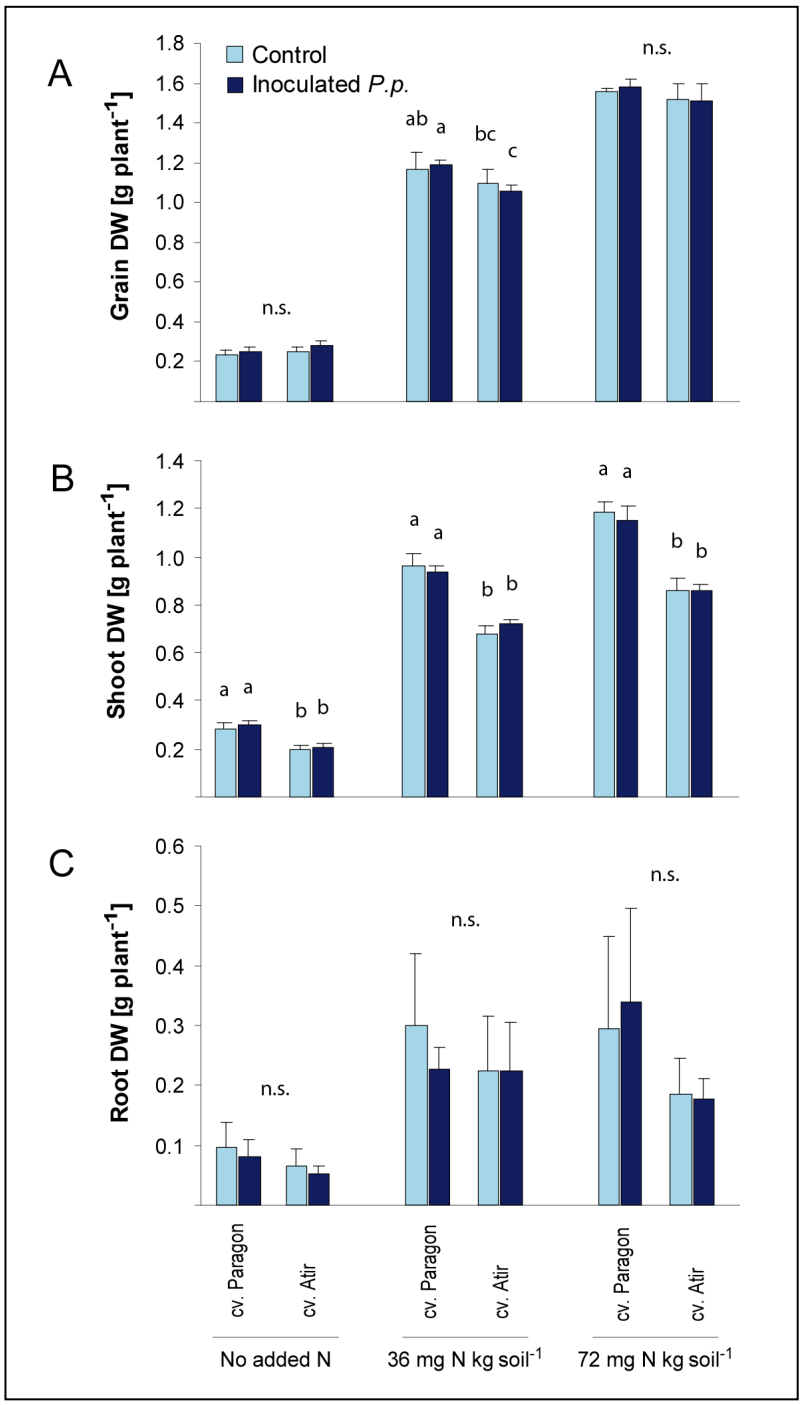

Figure 4.1-7 Grain (A), shoot (B) and root (C) dry weight of wheat plants at maturity in dependence of the wheat cultivar, the inoculation with *Paenibacillus polymyxa* (*P.p.*) and nitrogen supply

Wheat plants (cv. Paragon and cv. Atir) were inoculated at the 1-leaf stage with liquid inoculum (5.10^7 CFU $seed^{-1}$) and watered to 40% WHC. Autoclaved inoculum was used as a control. A Luvisol (from Israel) was used as substrate and supplied with 0, 36 or 72 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 4. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s.= not significant.

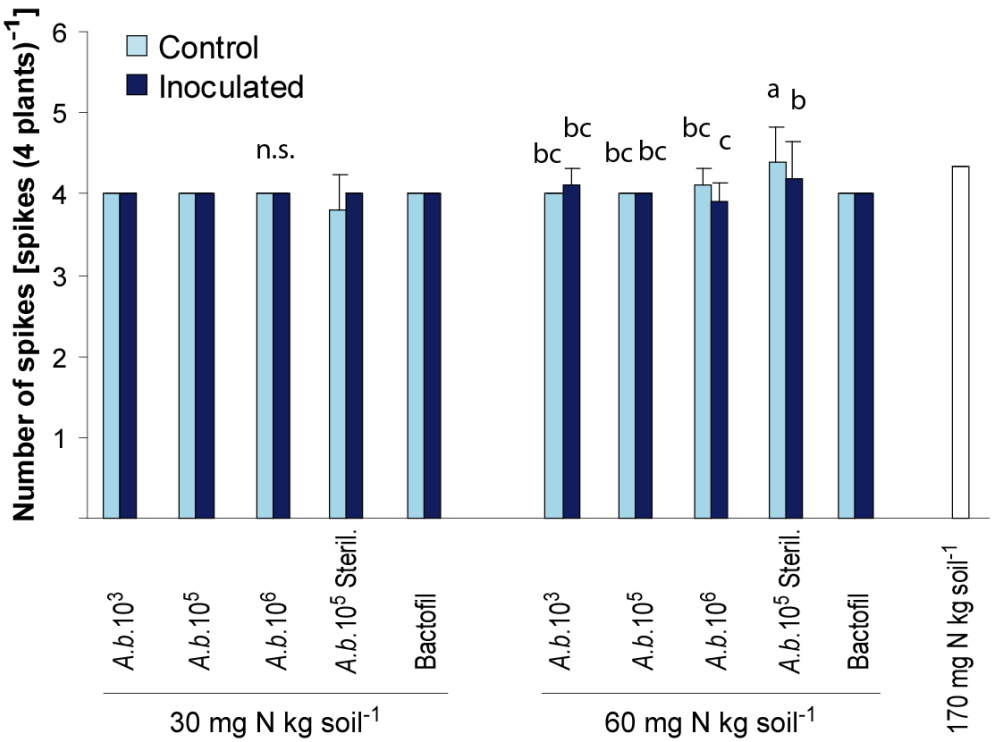

Figure 4.1-8 Number of spikes of wheat plants at harvest (EC 92) in dependence of biofertilizer and nitrogen application

A.b. 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A.b.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}, *A.b.* 10^6= *A. brasilense* in a concentration of 10^6 CFU mL^{-1}, *A.b.* 10^5 Steril.= *A. brasilense* in a concentration of 10^5 CFU mL^{-1} applied to sterilized soil, Bactofil= commercial biofertilizer. Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated via liquid inoculum and watered to 60% WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Spikes of all 4 plants per pot were added together. Bars indicate mean values± SD, in some treatments SD was too low to be visualized, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant. The treatments with 170 mg N kg^{-1} soil and Bactofil® are shown as reference and were not considered for the statistical analysis, n= 3.

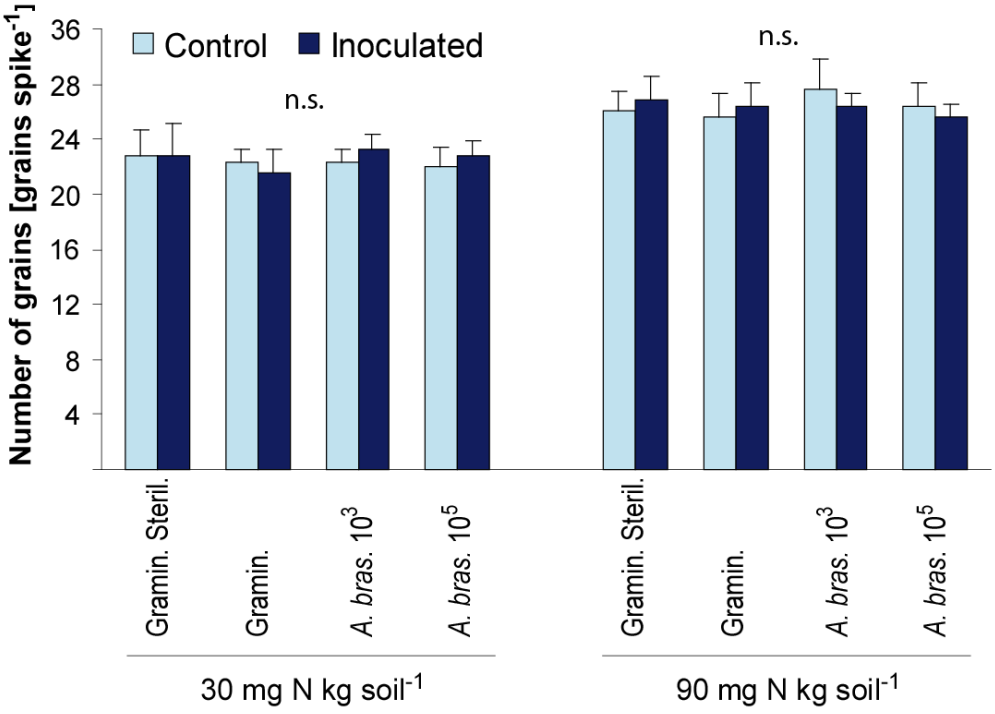

Figure 4.1-9 Number of grains per ear of wheat plants at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants (cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means (inside each N level) at $p \leq 0.05$ according to Tukey test, n.s.= not significant.

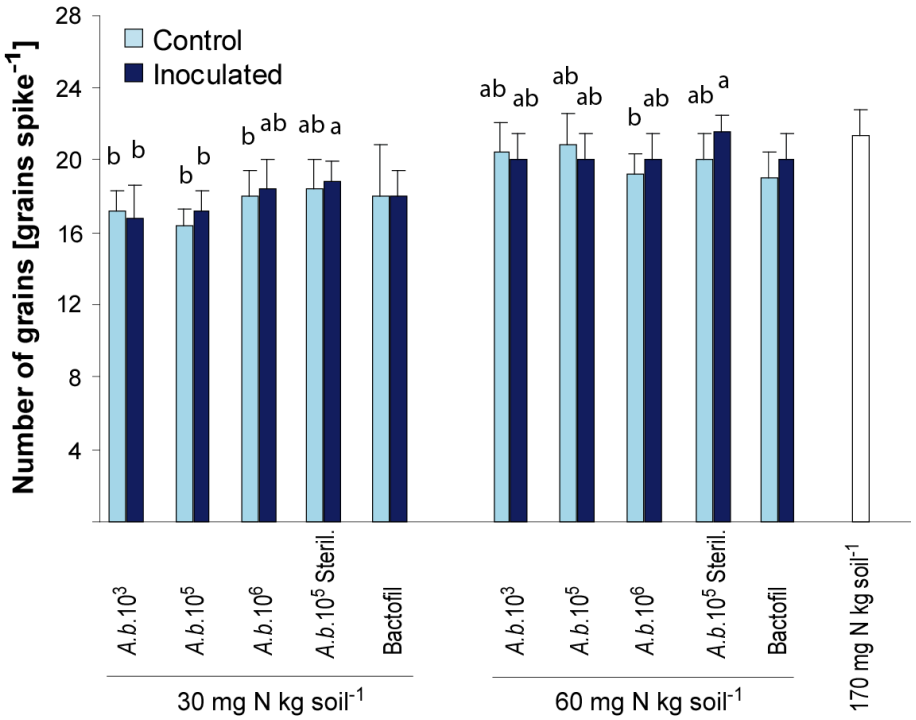

Figure 4.1-10 Number of grains per ear of wheat plants at harvest (EC 92) in dependence of biofertilizer and nitrogen application

A.b. 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A.b.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}, *A.b.* 10^6= *A. brasilense* in a concentration of 10^6 CFU mL^{-1}, *A.b.* 10^5 Steril.= *A. brasilense* in a concentration of 10^5 CFU mL^{-1} applied to sterilized soil, Bactofil= commercial biofertilizer. Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated via liquid inoculum and watered to 60% WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test. The treatments with 170 mg N kg^{-1} soil and Bactofil® are shown as reference and were not considered for the statistical analysis, n= 3.

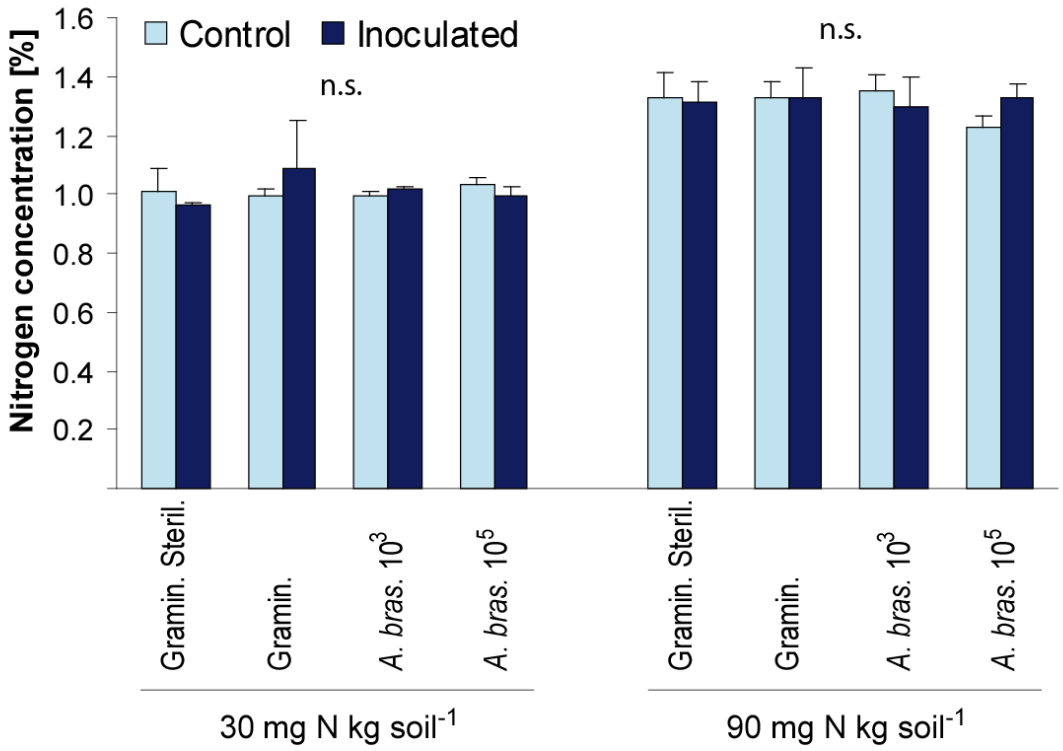

Figure 4.1-11 Nitrogen concentration of wheat grains at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants (cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s.= not significant.

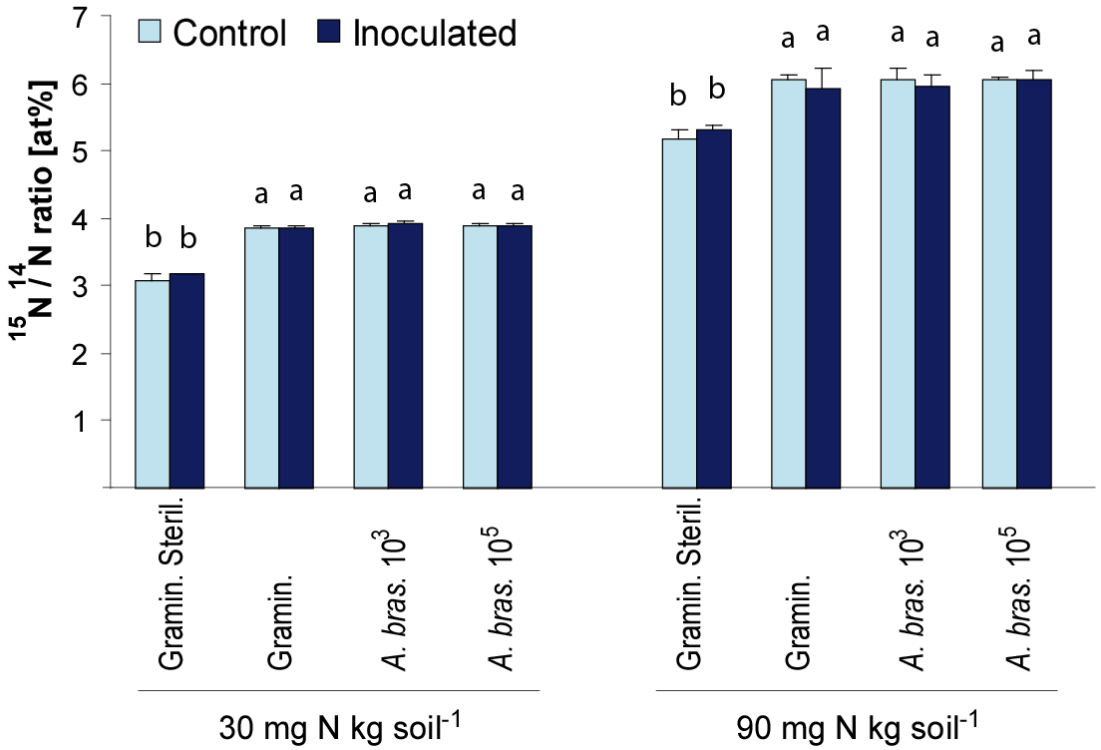

Figure 4.1-12 $^{15}N/^{14}N$ ratio of wheat grains at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants (cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture labelled with 14% ^{15}N was used as substrate. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test.

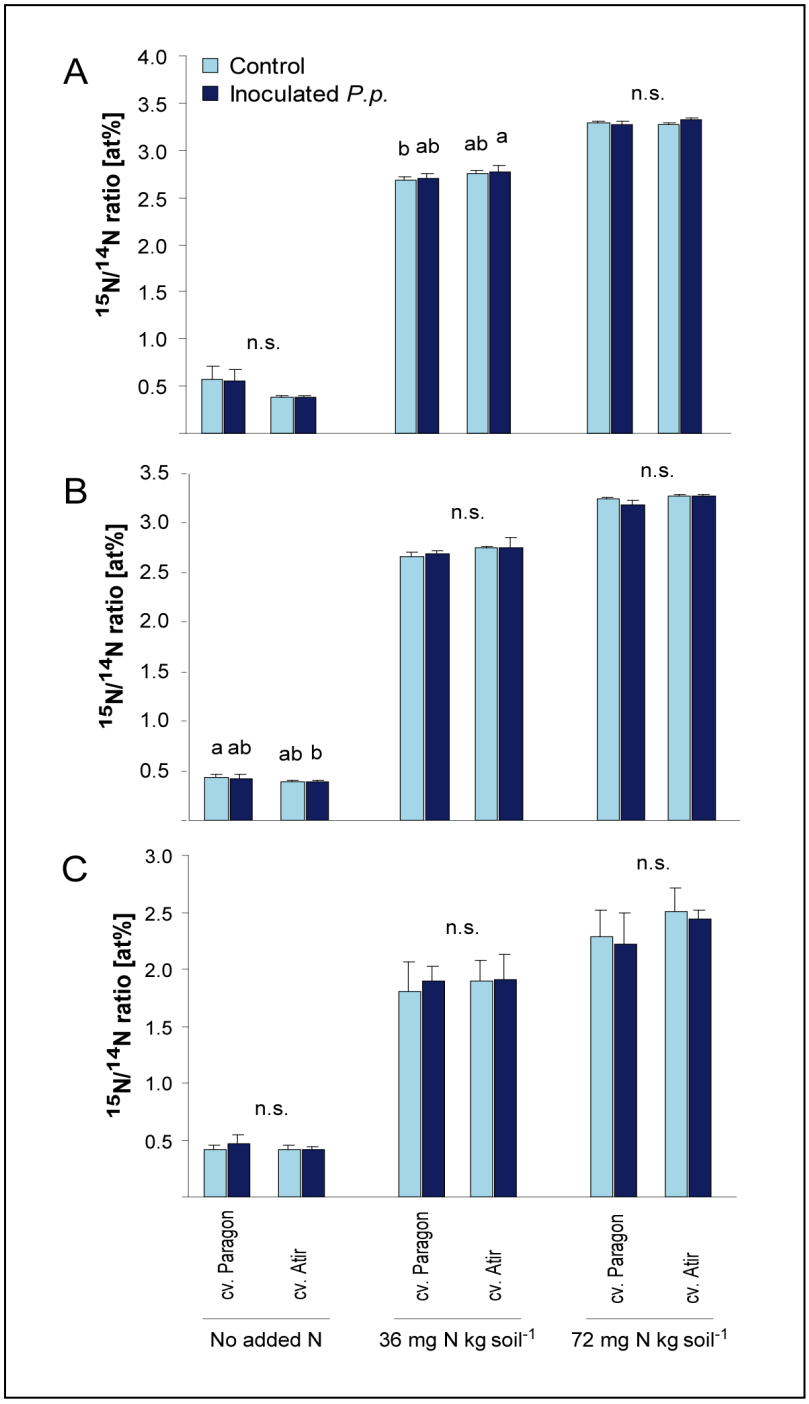

Figure 4.1-13 $^{15}N/^{14}N$ ratio in grain (A), shoot (B) and root (C) of wheat plants at maturity in dependence of the wheat cultivar, the inoculation with *Paenibacillus polymyxa* (*P.p.*) and nitrogen supply

Wheat plants (cv. Paragon and cv. Atir) were inoculated at the 1-leaf stage with liquid inoculum (5.10^7 CFU $seed^{-1}$) and watered to 40% WHC. Autoclaved inoculum was used as a control. A Luvisol (from Israel), labelled with 4%^{15}N, was used as substrate and supplied with 0, 36 or 72 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 4. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s.= not significant.

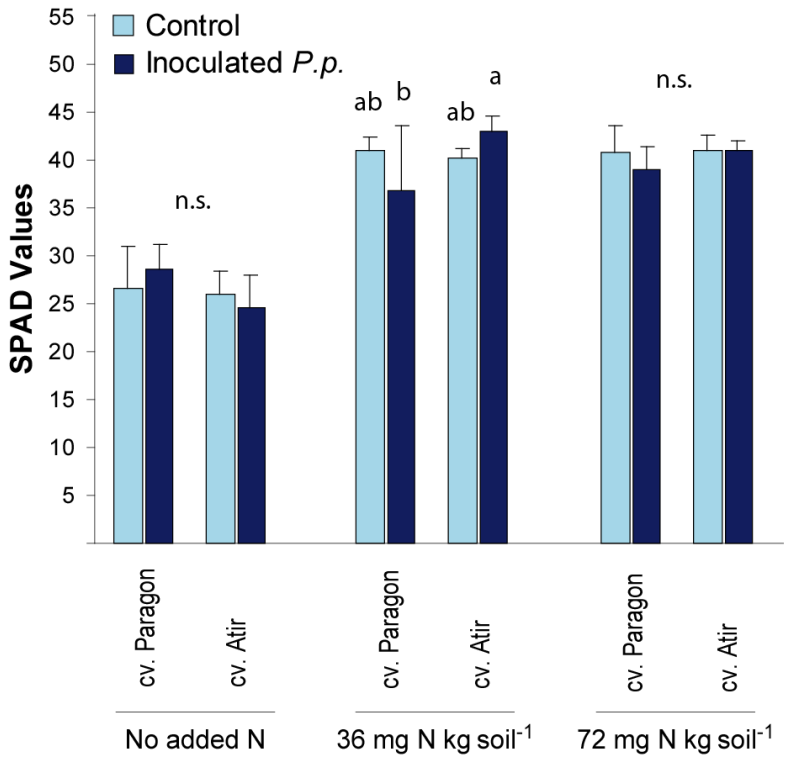

Figure 4.1-14 Relative chlorophyll densities (SPAD values) of wheat plants at tillering stage (EC25) in dependence of the wheat cultivar, the inoculation with *Paenibacillus polymyxa* (*P.p.*) and nitrogen supply

Wheat plants (cv. Paragon and cv. Atir) were inoculated at the 1-leaf stage with liquid inoculum (5.10^7 CFU $seed^{-1}$) and watered to 40% WHC. Autoclaved inoculum was used as a control. A Luvisol (from Israel) was used as substrate and supplied with 0, 36 or 72 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 4. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s. = not significant.

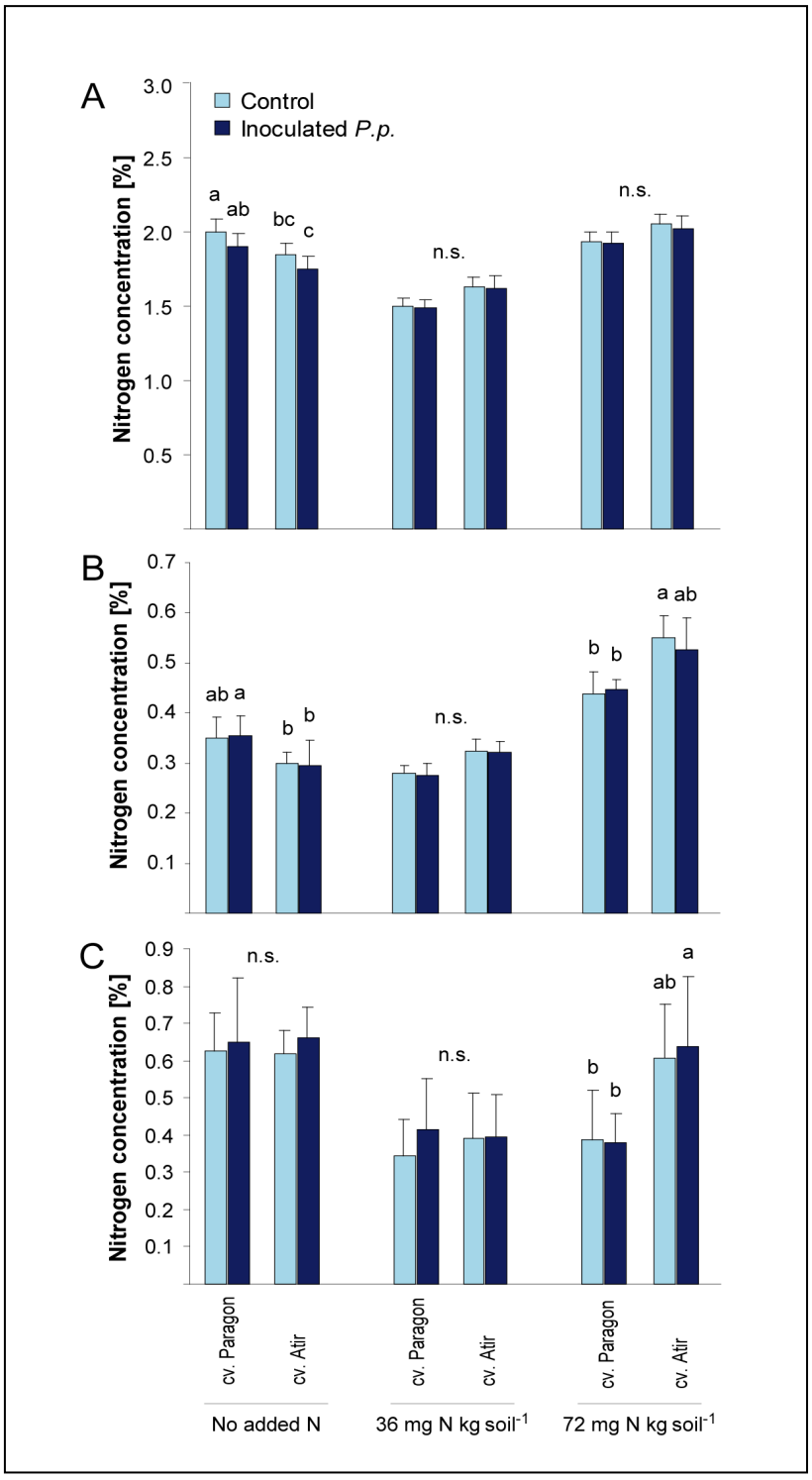

Figure 4.1-15 Nitrogen concentration of grain (A), shoot (B) and root (C) of wheat plants at maturity in dependence of the wheat cultivar, the inoculation with *Paenibacillus polymyxa* (*P.p.*) and nitrogen supply

Wheat plants (cv. Paragon and cv. Atir) were inoculated at the 1-leaf stage with liquid inoculum (5.10^7 CFU $seed^{-1}$) and watered to 40% WHC. Autoclaved inoculum was used as a control. A Luvisol (from Israel) was used as substrate and supplied with 0, 36 or 72 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 4. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s. = not significant.

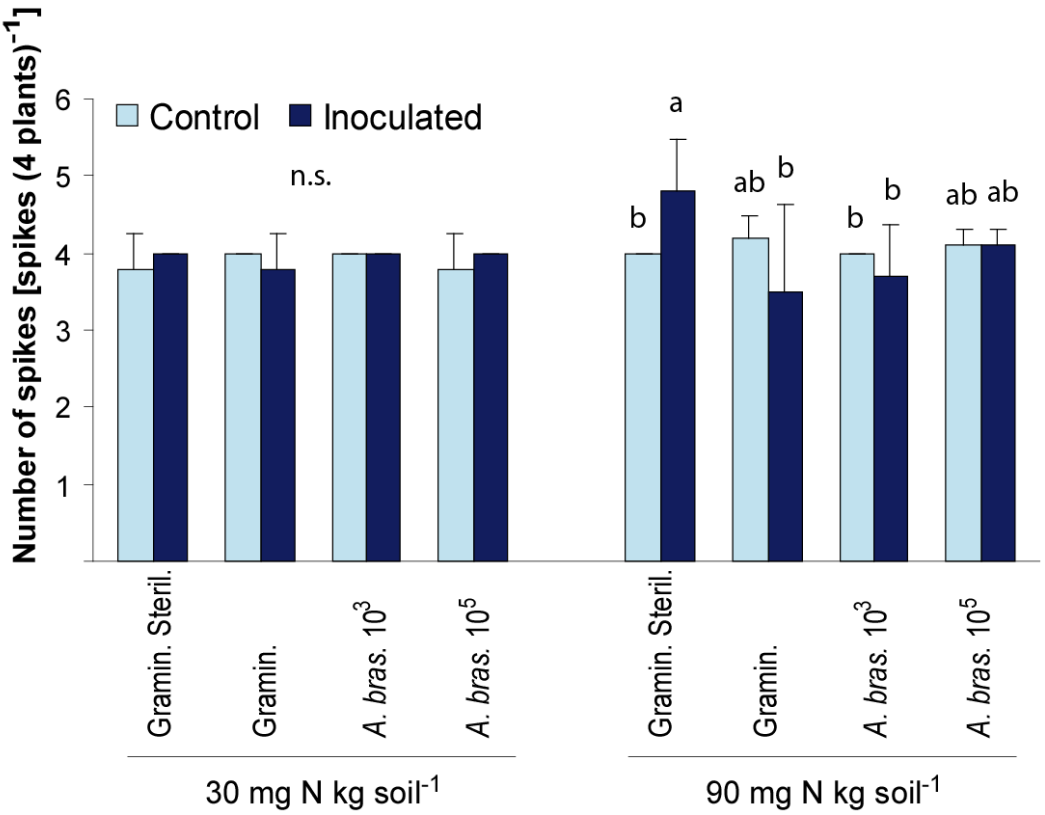

Figure 4.1-16 Number of spikes of wheat plants at maturity in dependence of bacterial inoculation and nitrogen fertilization

Gramin.= Graminante® (commercial biofertilizer), Gramin. Steril.= Graminante® in sterilized soil, *A. bras.* 10^3= *Azospirillum brasilense* in a concentration of 10^3 CFU mL^{-1}, *A. bras.* 10^5= *A. brasilense* in a concentration of 10^5 CFU mL^{-1}. Wheat plants **(**cv. Paragon) at the 3-leaf stage were inoculated via liquid inoculum and watered to 60% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate. Spikes of all 4 plants per pot were added together. Bars indicate mean values± SD, in some treatments SD was too low to be visualized, n= 5. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test, n.s.= not significant.

4.1.2 Soil type

The experiments were performed in two agricultural soils, a Luvisol from Germany (Filderlehm) containing relatively low concentrations of P, K and Mg but a high concentration of N and on the other hand, a Luvisol from Israel, containing higher concentrations of P, K and Mg, but a lower concentration of N. In both soils no significant effects of inoculated bacteria or biofertilizer application on plant growth or yield formation could be observed. Comparative evaluation of the plant growth and nutritional status in posterior experiments at the Institute on these two soils simultaneouly gave also no significant differences (Weishaar, 2007).
Sterilized Filderlehm soil was used to investigate a possible influence of native soil microorganisms on the investigated diazotrophs and on plant growth. In sterilized soil, a significantly positive effect of the inoculation with Graminante® at the highest level of N fertilization could be observed regarding the number of spikes (Fig. 4.1-16). On the other hand, a significant inhibition effect on the grain DW by the inoculation of *A. brasilense* at higher inoculation densities (10^5, 10^6 CFU mL^{-1}) was observed also in sterilized soil. However, this negative effect was not observed at lower inoculum densities or at a lower N fertilization level (Fig. 4.1-6 A).

4.1.3 Plant genotype

The wheat cultivar Atir showed positive response to inoculation with *Paenibacillus polymyxa* in field trials conducted by an Israeli cooperation partner in the EU project “Micro-N-Fix”. Therefore, cv. Atir was directly compared to cv. Paragon in a separate greenhouse trial (Figs. 4.1-7 to 4.1-13). At any of the N levels tested, no significant effects of an inoculation with *P. polymyxa* were observed in any cultivar. Only inoculated plants of the Israeli cv. Atir showed higher SPAD values than non-inoculated plants when cultivated at 36 mg N kg^{-1} soil (Fig. 4.1-14; statistical comparison via t-test). The plants from the cv. Paragon were higher (Fig. 4.1-17), had higher grain and shoot DW (Fig. 4.1-7 A,B) and when no N was added, had higher N concentrations in grains and shoot compared to the plants of the cv. Atir (Fig. 4.1-15 A,B). On the other hand, the cv. Atir showed higher N concentration in

shoot and roots compared to the cv. Paragon at the highest N fertilization level (Fig. 4.1-15 B,C).

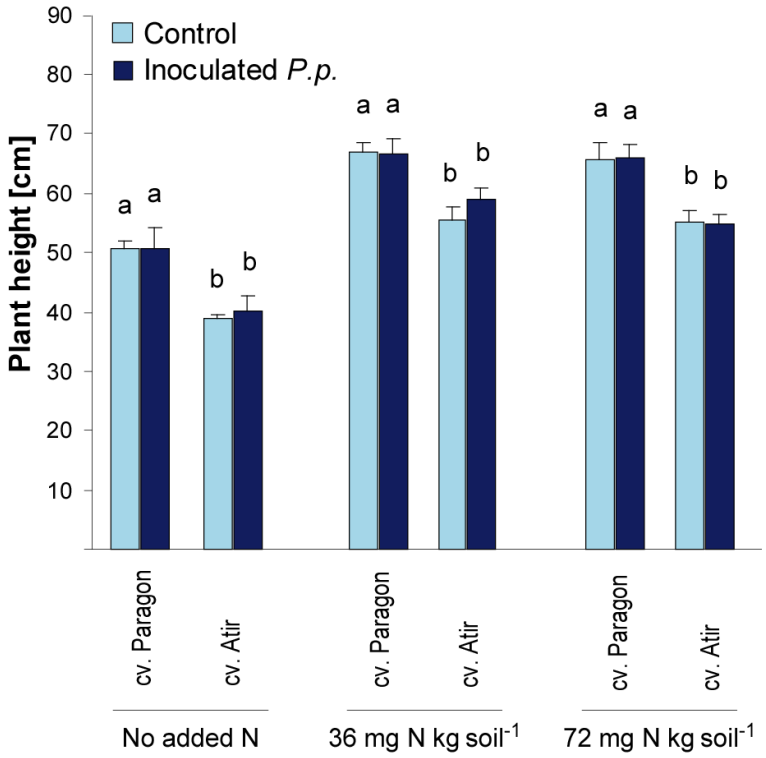

Figure 4.1-17 Plant height of wheat plants at maturity in dependence of the wheat cultivar, the inoculation with *Paenibacillus polymyxa* (*P.p.*) and nitrogen supply

Wheat plants (cv. Paragon and cv. Atir) were inoculated at the 1-leaf stage with liquid inoculum (5.10^7 CFU $seed^{-1}$) and watered to 40% WHC. Autoclaved inoculum was used as a control. A Luvisol (from Israel) was used as substrate and supplied with 0, 36 or 72 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 4. Different letters represent significant differences of the means (inside each N level) at p≤0.05 according to Tukey test.

4.1.4 Bacteria strain

Particularly in greenhouse trial V, different diazotrophic strains were compared for their possible effect on plant growth promotion or N_2 fixation (Table 3.1-3). In the previous experiments *Azospirillum brasilense* Sp7 was the main inoculant along with two commercial biofertilizers containing *Azospirillum*. In the following experiments plants were inoculated with *Paenibacillus polymyxa*, trying to reproduce a growth-stimulating effect of this strain on wheat growth in a field trial by one of the partners in the EU project “Micro-N-fix” (Figs. 4.1-18 to 4.1-23). This strain was compared to *Raoultella terrigena*, *Xanthobacter flavus*, *Xanthomonas vesicatoria*, *Brevibacillus reuszeri* and two mutants of *A. brasilense*, one defective in nitrogenase activity and another with enhanced nitrogenase activity (Figs. 4.1-25 to 4.1-26).

Taking together the results of all pot experiments, no significant differences were found neither in shoot and root DW (Fig. 4.1-25), nor in N concentrations (Fig. 4.1-26), or $^{15}N/^{14}N$ ratios (Fig. 4.1-24) by any of the tested strains. Only two biofertilizers gave significant positive results. Wheat plants from the cv. Atir inoculated with *P. polymyxa,* at 36 mg N kg^{-1} soil, showed higher SPAD values when assayed by t-test than non-inoculated plants (Fig. 4.1-14), and plants of cv. Paragon inoculated with Graminante® led to a higher number of spikes compared to the control at the highest N supply in sterilized soil (Fig. 4.1-16).

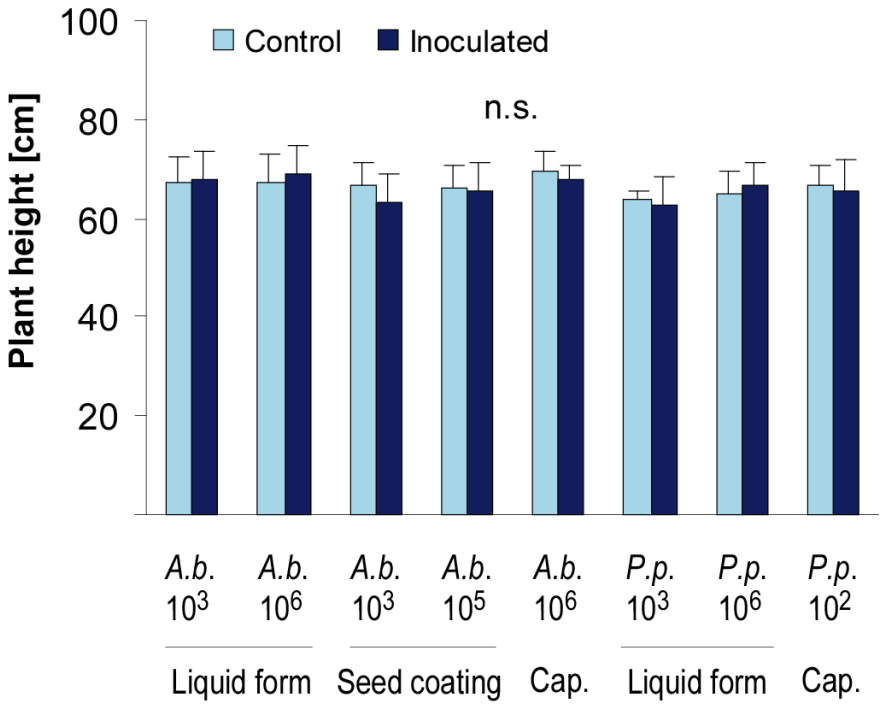

Figure 4.1-18 Plant height of wheat plants at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

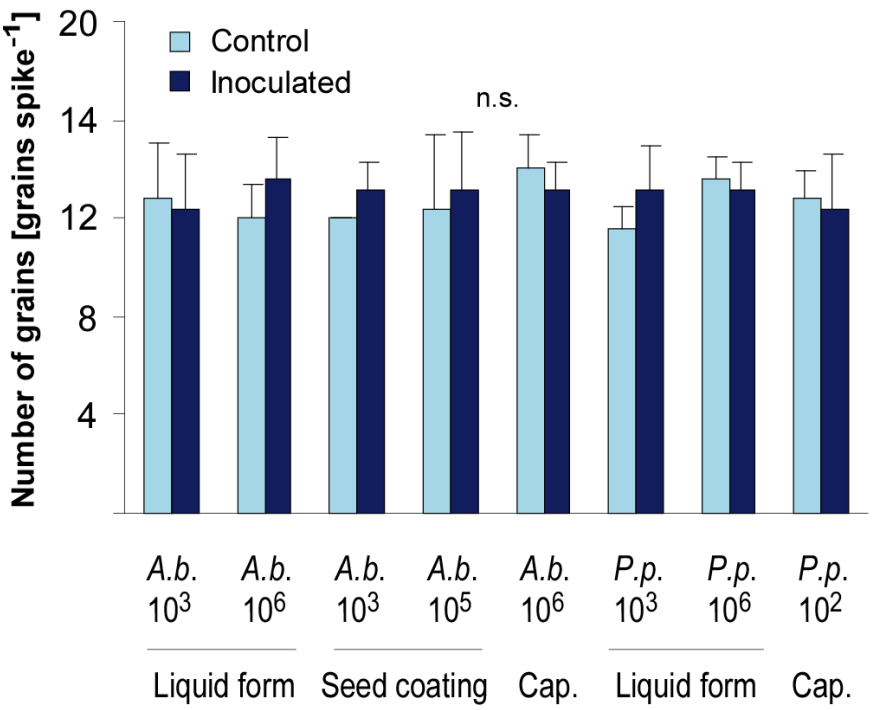

Figure 4.1-19 Number of grains per spike of wheat plants at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s. = not significant.

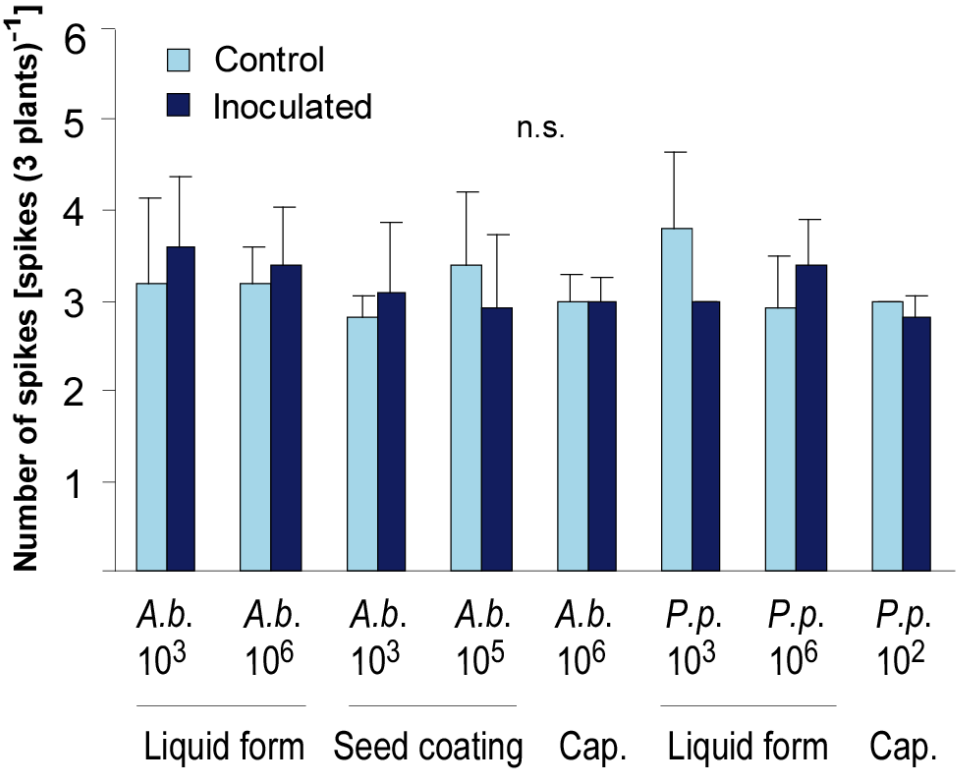

Figure 4.1-20 Number of spikes of wheat plants at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil. Spikes of all 3 plants per pot were added together. Bars indicate mean values± SD, in some treatments SD was too low to be visualized, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

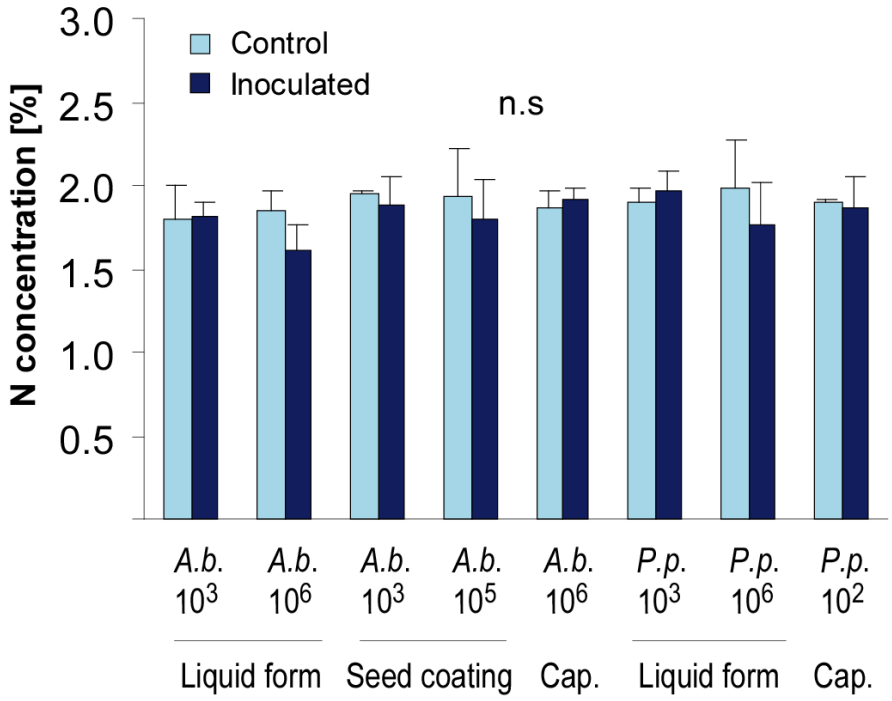

Figure 4.1-21 Nitrogen concentration in grains of wheat plants at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

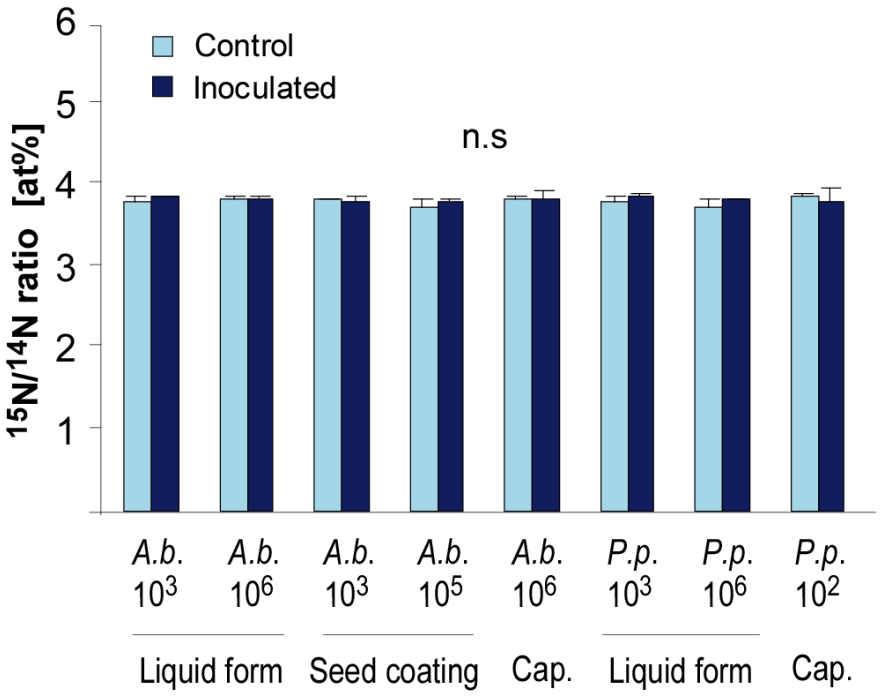

Figure 4.1-22 $^{15}N/^{14}N$ ratio in wheat grains at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil and labelled with 10% ^{15}N. Bars indicate mean values± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

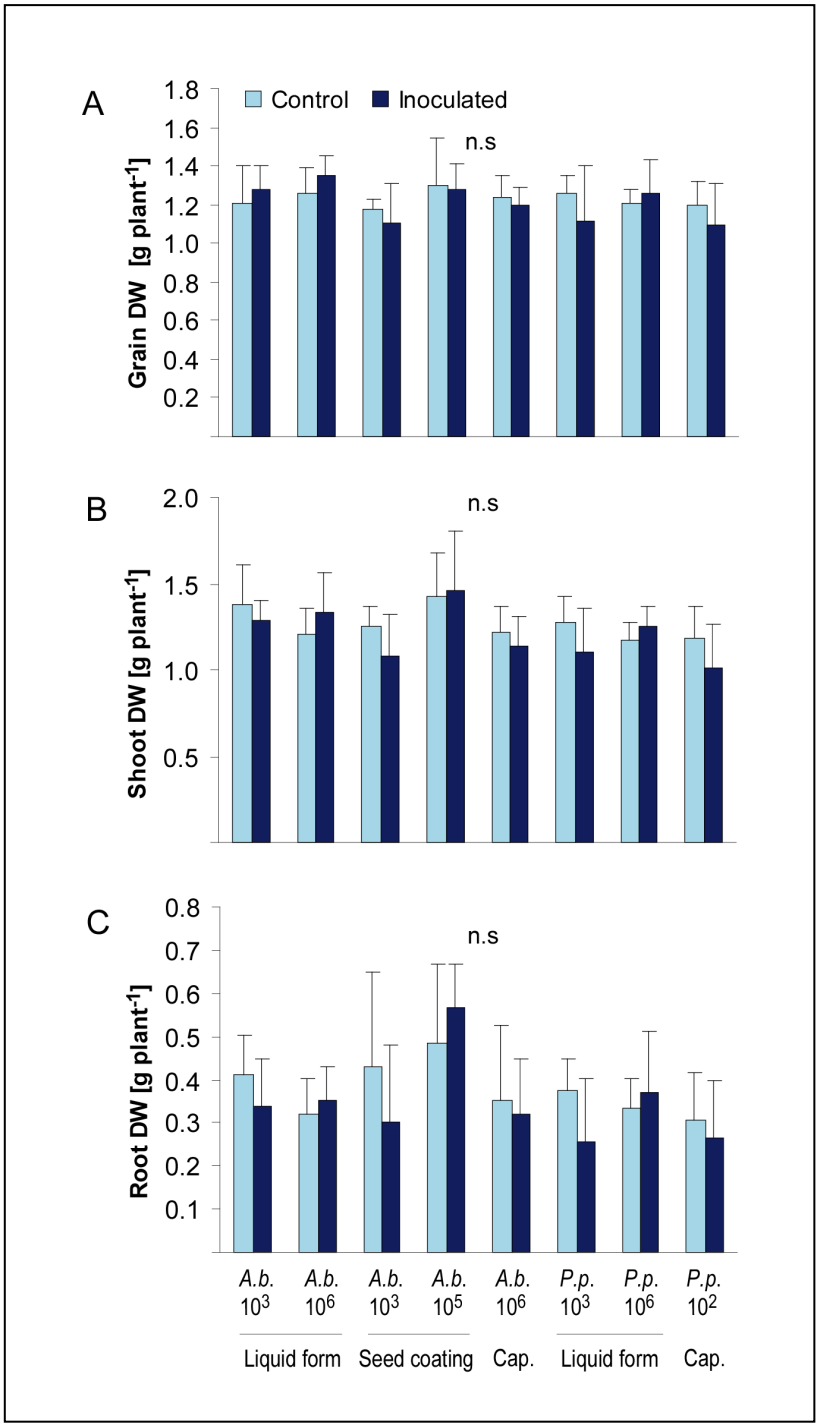

Figure 4.1-23 Grain (A), shoot (B) and root (C) dry weight of wheat plants at maturity in dependence of bacterial inoculation and the inoculation method

Wheat plants (cv. Paragon) were inoculated either with *A.b. (Azospirillum brasilense)* or *P.p. (Paenibacillus polymyxa)* via application of a liquid inoculum (10^3 or 10^6 CFU seed^{-1}), seed coating (10^3 or 10^5 CFU seed^{-1}) or application of capsules (10^2 or 10^6 CFU seed^{-1}). Autoclaved inoculum was used as a control. Watering: 60% WHC. A Luvisol (Filderlehm)/ Perlite mixture was used as substrate and supplied with 30 mg N kg^{-1} soil. Bars indicate mean values ± SD, n= 5. Different letters represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

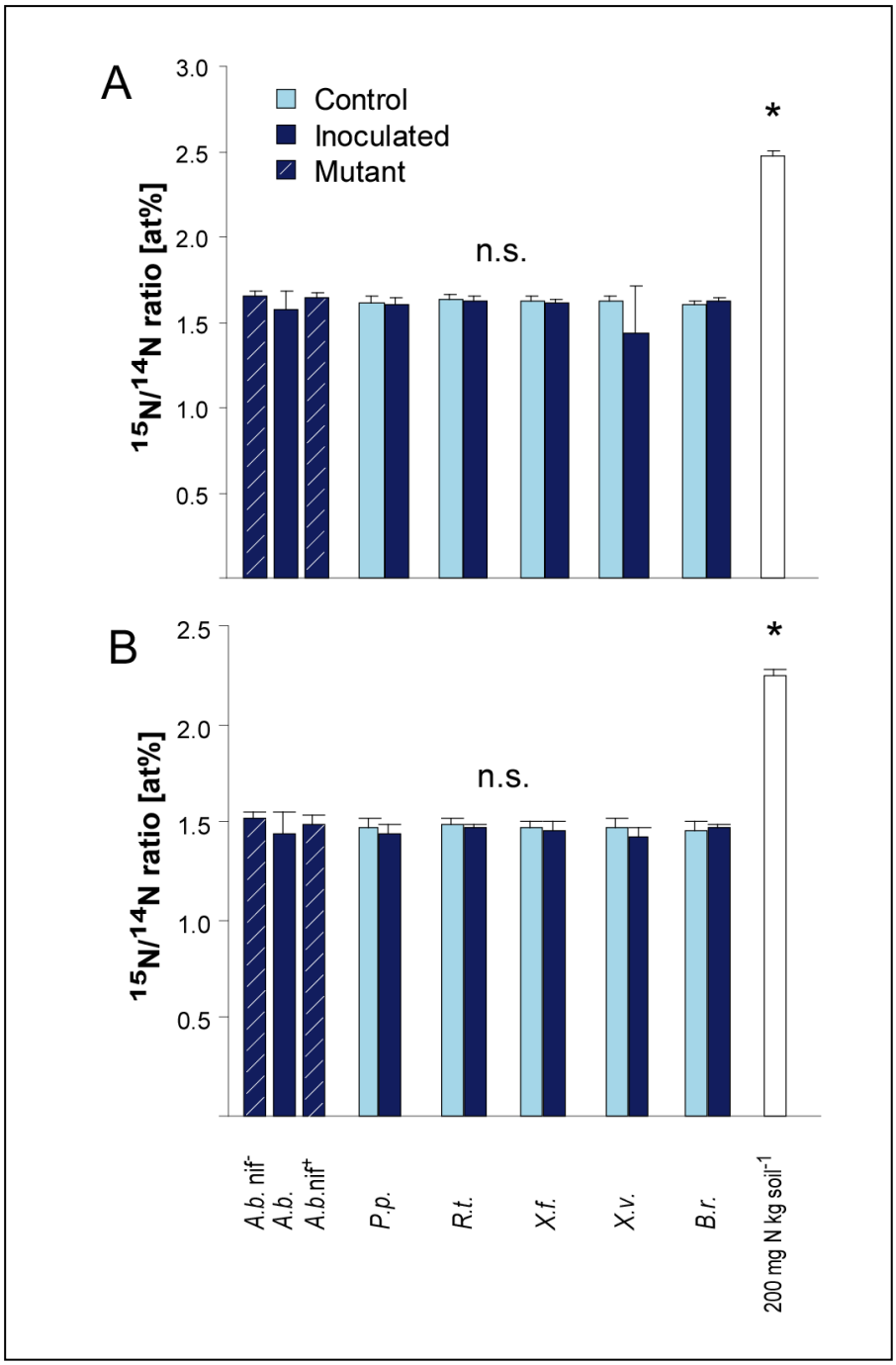

Figure 4.1-24 $^{15}N/^{14}N$ ratio in shoot (A) and root (B) of wheat plants harvested at the 6-leaf stage (EC 29) and inoculated with different N_2-fixing bacteria

Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated either with *A.b.* (*Azospirillum brasilense*), or *A.b. nif* (mutant of *A.b*. defective in nitrogenase activity), *A.b. nif*$^+$ (mutant of *A.b.* with enhanced nitrogenase activity), *P.p.* (*Paenibacillus polymyxa*), *R.t.* (*Raoultella terrigena*), *X.f.* (*Xanthobacter flavus*), *X.v.* (*Xanthomonas vesicatoria*) or *B.r.* (*Brevibacillus reuszeri*) by liquid inoculation (5.10^7 CFU seedling^{-1}) and watered to 48% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture supplemented with 30 mg N kg^{-1} soil, labelled with 4% ^{15}N, was used as substrate. A treatment with 200 mg N kg^{-1} soil was included as additional control. Bars indicate mean values± SD, n= 4. An asterisk represent significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant

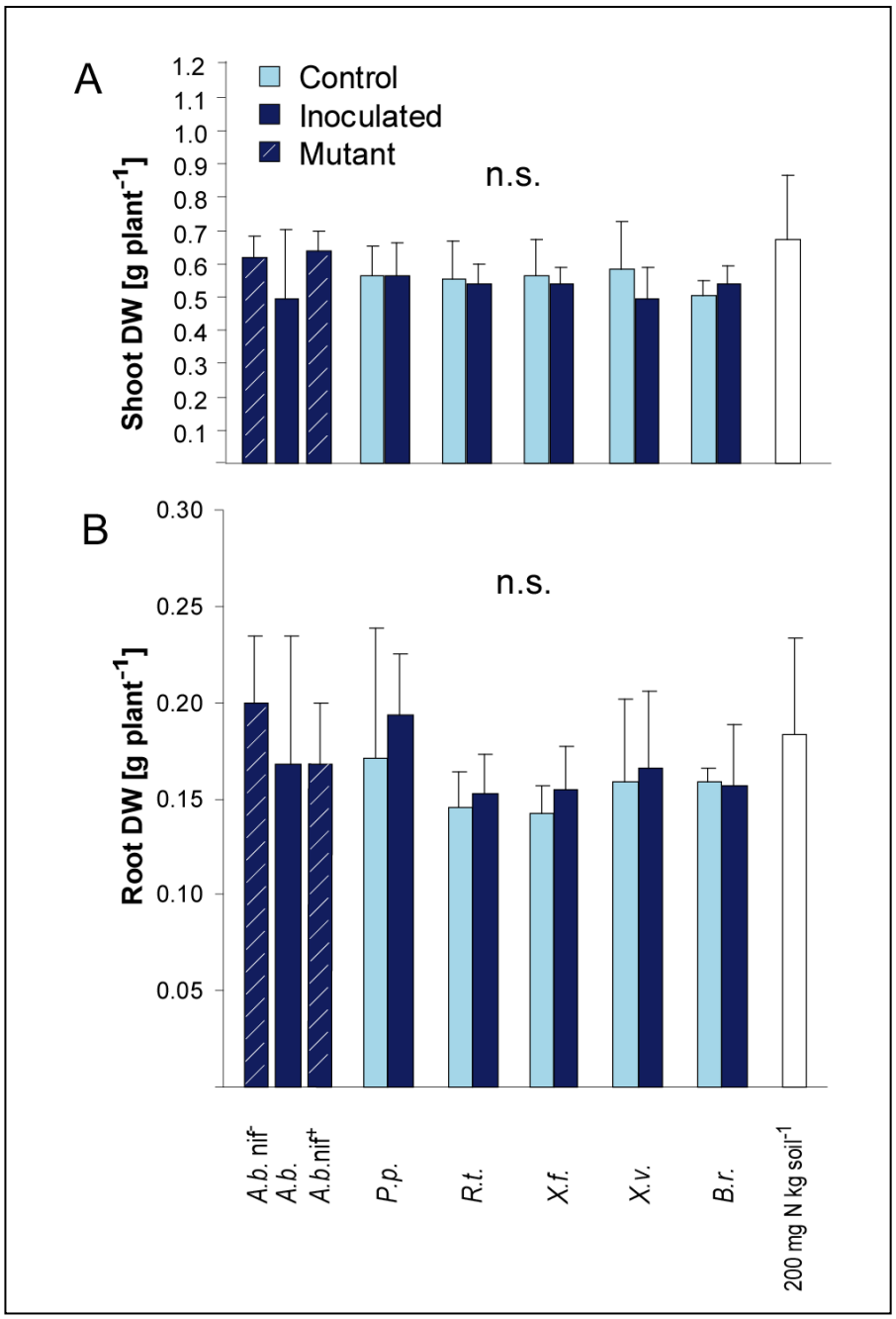

Figure 4.1-25 Shoot (A) and root (B) dry weight of wheat plants harvested at the 6-leaf stage (EC 29) and inoculated with different N_2-fixing bacteria

Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated either with *A.b.* (*Azospirillum brasilense*), or *A.b. nif* (mutant of *A.b*. defective in nitrogenase activity), *A.b. nif*$^+$ (mutant of *A.b.* with enhanced nitrogenase activity), *P.p.* (*Paenibacillus polymyxa*), *R.t.* (*Raoultella terrigena*), *X.f.* (*Xanthobacter flavus*), *X.v.* (*Xanthomonas vesicatoria*) or *B.r.* (*Brevibacillus reuszeri*) by liquid inoculation (5.10^7 CFU seedling^{-1}) and watered to 48% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture supplemented with 30 mg N kg^{-1} soil was used as substrate. A treatment with an application of 200 mg N kg^{-1} soil was included as additional control. Bars indicate mean values± SD, n= 4. Not significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

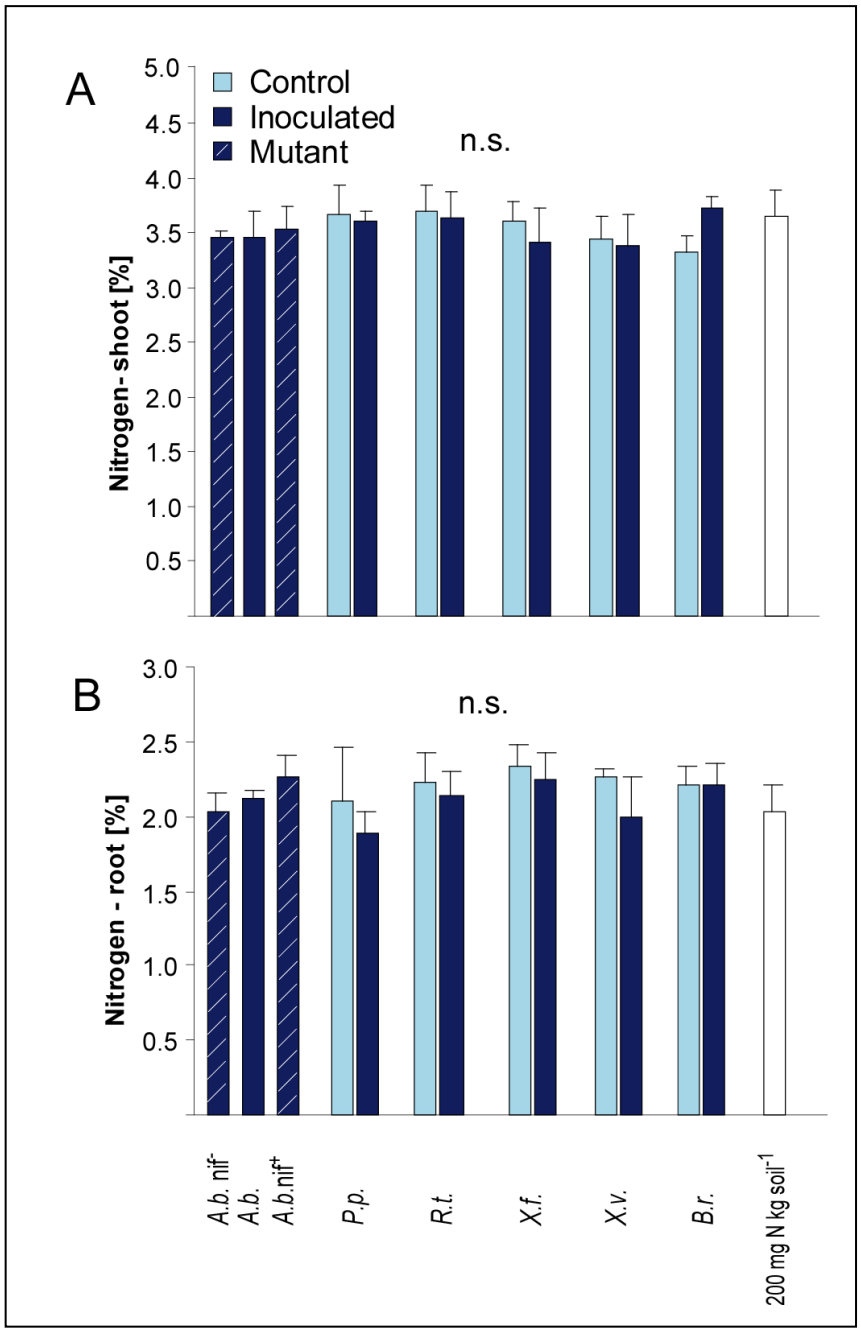

Figure 4.1-26 Nitrogen concentration in shoot (A) and root (B) of wheat plants harvested at the 6-leaf stage (EC 29) and inoculated with different N_2-fixing bacteria

Wheat plants (cv. Paragon) at the 1-leaf stage were inoculated either with *A.b.* (*Azospirillum brasilense*), or *A.b. nif* (mutant of *A.b*. defective in nitrogenase activity), *A.b. nif*$^+$ (mutant of *A.b.* with enhanced nitrogenase activity), *P.p.* (*Paenibacillus polymyxa*), *R.t.* (*Raoultella terrigena*), *X.f.* (*Xanthobacter flavus*), *X.v.* (*Xanthomonas vesicatoria*) or *B.r.* (*Brevibacillus reuszeri*) by liquid inoculation (5.10^7 CFU seedling^{-1}) and watered to 48% of WHC. Autoclaved inoculum was used as a control. A Luvisol (Filderlehm)/ Perlite mixture supplemented with 30 mg N kg^{-1} soil was used as substrate. A treatment with an application of 200 mg N kg^{-1} soil was included as additional control. Bars indicate mean values± SD, n= 4. Not significant differences of the means at p≤0.05 according to Tukey test, n.s.= not significant.

4.1.5 Concentration of inoculum

Aiming at finding bacterial inoculation densities that promote plant growth, a series of different inoculum concentrations were tested (10^2, 10^3, 10^5, 10^6 and 10^7 CFU mL^{-1}). Except for the last two experiments, different concentrations of bacteria were tested at the same time (see Table 3.1-2).

As a general trend, plant growth tended to be inhibited by high densities of the bacterial inoculum, although the observed effects were not statistically significant in all cases. Moreover, the expression of inhibitory effects of high bacterial inoculum densities was also influenced by the N fertilization level: with high N supply, significantly lower grain DW was produced at high inoculum density (10^6 CFU mL^{-1}) of *A. brasilense* in sterilized soil, but this negative effect was not observed at a lower N supply (Fig. 4.1-6 A). On the other hand, at highest N supply in sterilized soil, the inoculation of wheat plants with the biofertilizer Graminante® according to the product recommendations increased the number of spikes (Fig. 4.1-16).

4.1.6 Inoculum delivery mode

Applying liquid inoculum to the plants was the standard procedure used in most experiments. However, in greenhouse trial IV different inoculation methods were compared: application of liquid inoculum, versus seed coating and versus application of encapsulated bacteria at sowing (Figs. 4.1-18 to 4.1-23). None of the inoculation methods, however, allowed determining significant effects on plant growth or N uptake.

4.2. Seed / root exudates analysis

A major aim of this work was to investigate whether root and seed exudates contain compounds that exert a chemotactical attraction to associative diazotrophic bacteria. With respect to the large number of exuded compounds, the analysis was focused on organic acids. Different organic acids were analysed by HPLC such as malic, lactic, citric and succinic acid. Furthermore, fumaric and trans-aconitic acid were analysed only in wheat and maize, respectively. The profiles of these organic acids differed in dependence of plant species, plant age and N nutritional status (Fig. 4.2-1).

Carboxylate profiles in wheat, maize and bean exudates

Seed exudates of wheat and maize were dominated by malic, citric and succinic acid. Additionally, lactic acid was found in significant amounts in maize and traces of fumaric acid in wheat. Root exudates of maize and wheat contained malic, citric and succinic acid and also surprisingly high amounts of lactic acid. In maize additionally trans-aconitic acid was detectable. Data for root exudates of bean (*Phaseolus vulgaris* L.) seedlings were provided by Haase *et al.* (2008) comprising malic, citric, malonic and fumaric acid.

Influence of plant age on exudates composition

Lactic acid was the most abundant organic acid determined by HPLC in germinating seeds of maize and wheat and declined with increasing age of the seedlings. Finally, root exudates of wheat seedlings mainly contained malic and succinic acid, while in maize succinic, malic, trans-aconitic and citric acid were most abundant and detected in similar concentrations.

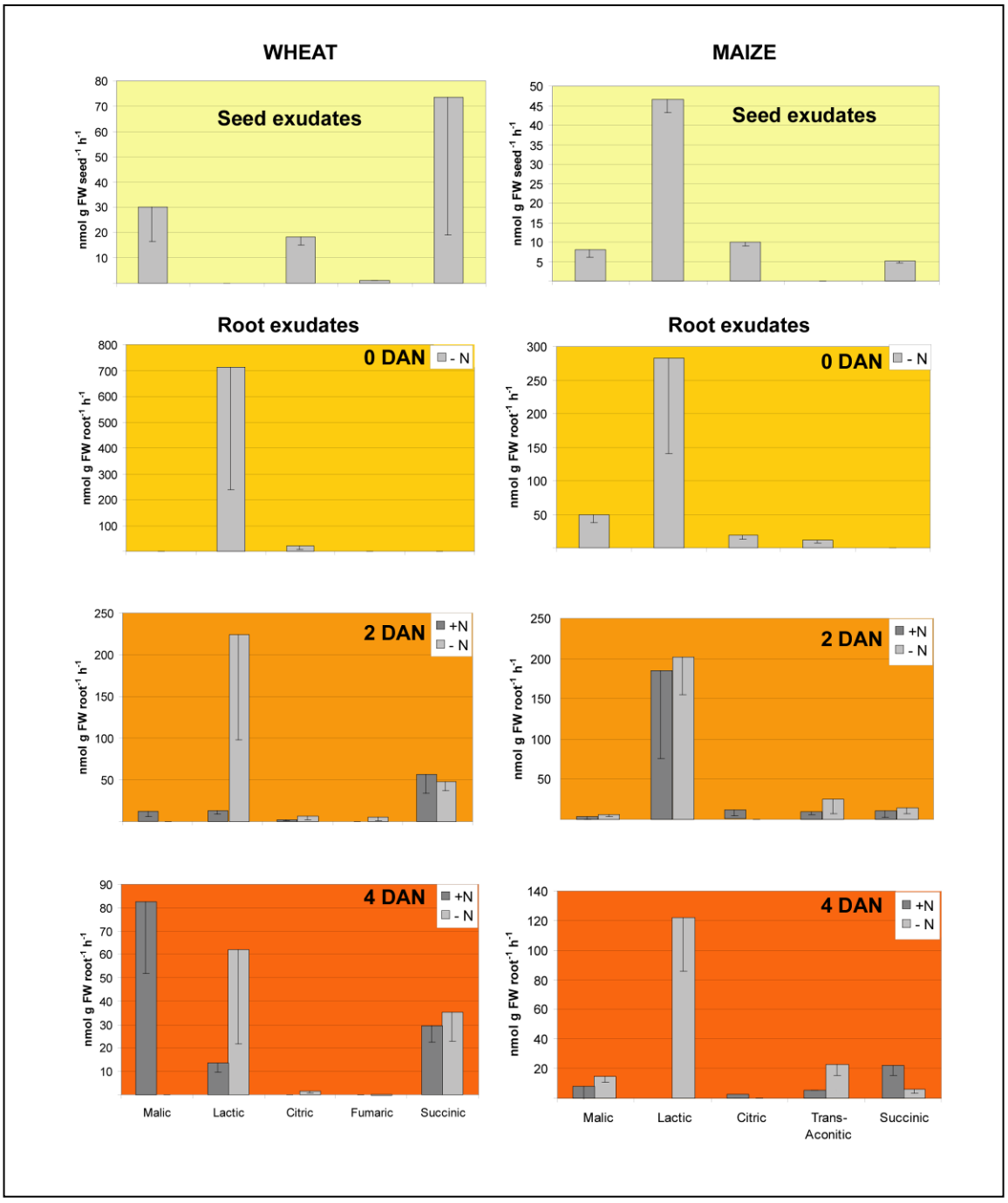

Figure 4.2-1: Organic acid composition in dependence of plant species, developmental stage and plant nutritional status

Exudates were collected under non-sterile conditions for a period of 4 h. –N= no nitrogen added, +N= 2 mM NH_4NO_3 added to the nutrient solution. DAN= days after N treatment. Bars indicate means ± SE, n= 3.

Influence of the N nutritional status on exudate composition

Nitrogen deficiency drastically modified root exudate profiles in all investigated plant species. In wheat and maize, the most prominent effect was an induction of lactic acid exudation after removal of N supply. Malic acid exudation was completely suppressed by N deficiency in wheat, while succinic acid exudation remained unaffected. In maize, apart from lactic acid exudation also the release of trans-aconitic and malic acid was stimulated by N deficiency while exudation of succinic and citric acid declined. In bean seedlings particularly the release of malonic and malic acid was stimulated by N limitation (Haase, 2008).

4.3. Assays for bacterial motility and EPS production

Another aim of this work was to develop a motility assay for testing the attraction of rhizosphere bacteria by the characterized seed and root exudates (chapter 4.2). The results from experiments dealing with bacterial motility on semisolid media strongly depended on the experimental conditions and the inoculation method. Therefore, part of this study was dealing with the set-up of a repeatable assay for assaying different substrates, such as organic acids, water and seed or root exudates.

The motility of the diazotrophs to a given substrate was determined in a motility assay based on the distance of spreading of the cells from the inoculation spot and the formation of a well-defined motility ring, the area of which was quantitatively assessed. In addition, the spreading of a bacterial colony was monitored via staining of bacterial EPS.

Data suggesting the involvement of EPS in aggregation and root atachment of diazotrophs have been published. There are different quantitative and qualitative methods for the analysis of EPS (Mironescu, 2003). A qualitative method is the staining with Calcofluor White, which is a dye that specifically binds ß-1,4- and ß-1,3-linked glucans and emits fluorescence under UV-light (Wood & Fulcher, 1978). This method has been successfully used in the literature (Michiels *et al.*, 1990; de Troch *et al.*, 1992) and it has been chosen for the staining of the EPS in this study. Extracellular polysaccharides are found in nature in two forms: attached to the

synthesising microbial cell as a discrete structure, called capsule polysaccharides (CPS), or as soluble mucilage excreted by cells into the environment (EPS). In this thesis, both forms have been summarized as EPS.

After testing different non-rich media, 5-fold diluted Oxoid R2A agar medium (0.33% agar) gave the best results. This semisolid medium allows bacterial motility. It was found that testing only one substance per Petri dish was most appropriate to avoid interferences and to have clearer and significant results. The best and fastest results of motility were observed when the bacterial culture was 50-fold concentrated and only 20 µL were spotted on the agar surface after the exudates had been placed. During all the steps of the protocol (pouring the plates, pipetting of bacteria and incubation) Petri dishes were placed on a completely horizontal surface. To make sure that all the plates were under a similar relative humidity, a chamber containing almost saturated humidity was put over the Petri dishes (Fig. 4.3-1).

In a preliminary experiment, different diazotrophs were tested for their migration towards wheat, maize and tomato exudates. The bacteria tested were *A. brasilense* Sp7, *Paenibacillus polymyxa, Xanthomonas* cf. *vesicatoria* and *Brevibacillus reuszeri*. *Xanthomonas vesicatoria* and *B. reuszeri* were found to migrate preferentially in the direction of wheat and maize exudates, especially when derived from N-deficient plants (data not shown). However, the highest motility was found for *B. reuszeri* and *P. polymyxa*, which were selected for subsequent experiments. In a second preliminary experiment, only exudates from N-deficient plants were used. It was observed that *P. polymyxa* was repulsed by bean exudates, but attracted by exudates from wheat seedlings 2 and 4 d after N starvation. *B. reuszeri* was attracted by exudates from wheat seedlings 4 d after N starvation.

Furthermore, a quantitative fluorimetric method was employed for the staining of EPS. However, during the tested inoculation periods (0-180 min) no differences were observed in Calcofluor-dependent fluorescence irrespective of the presence of bacteria, probably because the experimental incubation times were too short for

EPS production. More than 3d were needed to see fluorescence in the semisolid assay, thus this quantitative fluorimetric needs further optimization.

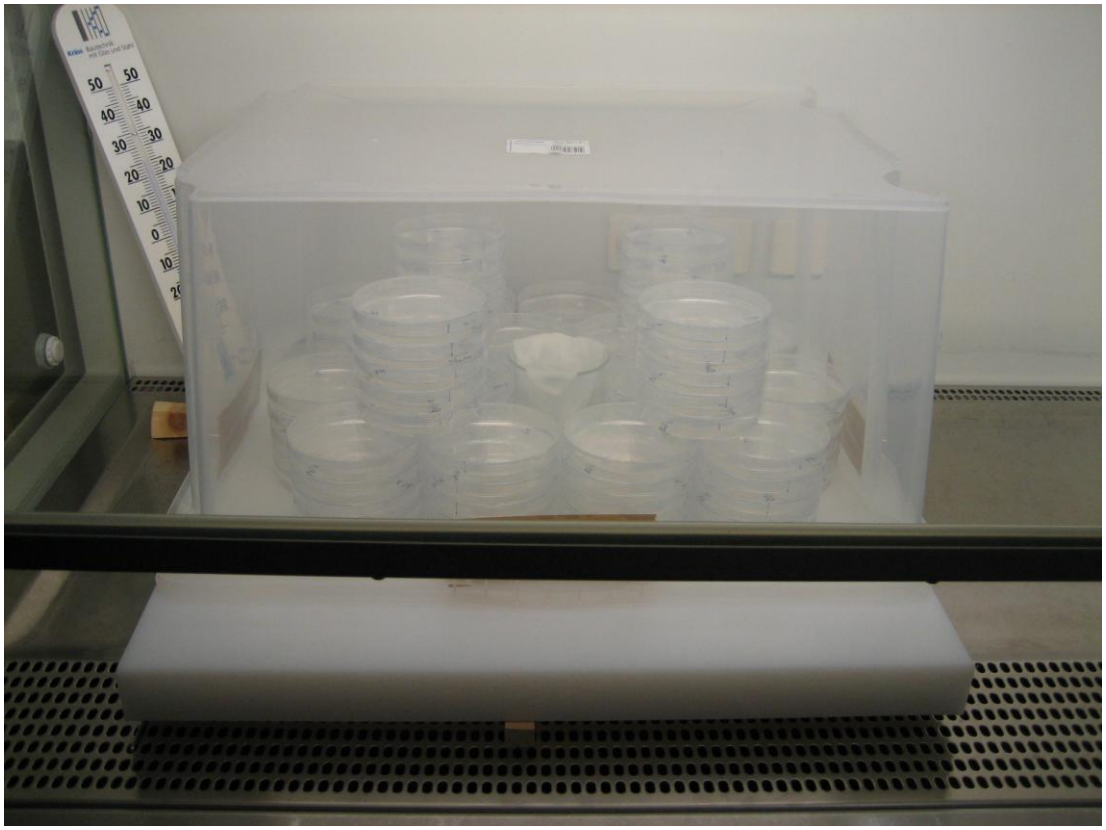

Figure 4.3-1 Chamber with saturated humidity for incubation of Petri dishes during the motility assay.

4.3.1 Motility in the presence of several pure substances

In the motility assay, different organic acids were tested, together with water and glucose. After 8 h of incubation, the diazotroph *Brevibacillus reuszeri* showed a significant higher motility in the presence of organic acids, such as malic, malonic and trans-aconitic acid than in the presence of water (Fig. 4.3-2 A). Glucose did not promote a significantly higher motility than water. These results correlated positively with the spreading of the colony when stained with Calcofluor and assessed after 4 d of incubation (Fig. 4.3-2 B).

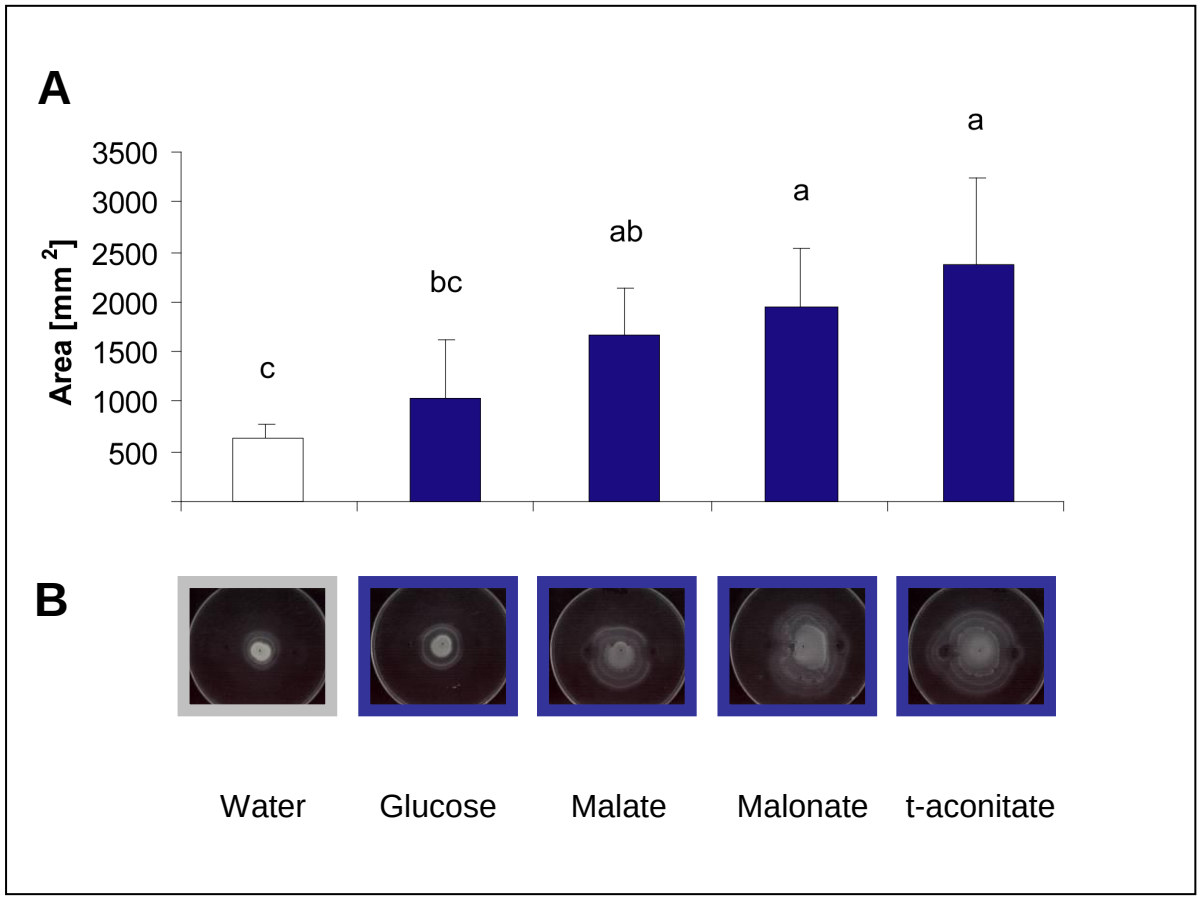

Figure 4.3-2 Motility of *Brevibacillus reuszeri* in the presence of water, glucose and different organic acids. A) Area (mm^2) of the motility ring formed after 8 h of growth around the spotted bacteria and B) fluorescence of bacterial colonies after 4 d of Calcofluor-staining

Filter paper discs (5 mm diameter) containing 1.5 µg C $disc^{-1}$ were placed at 2 cm distance left and right of the central inoculation spot. Bacterial colonies were incubated on semisolid agar medium (5-fold diluted R2A agar medium, Oxoid). Bars indicate means ±SD, n= 5. Different letters represent significant differences of the means at p≤0.05 by Fisher's LSD test.

4.3.2 Motility in the presence of seed / root exudates

Brevibacillus reuszeri was also tested in the presence of seed and root exudates collected from different plant species, different plant growth stages and under different N regimes. The results displayed in Figs. 4.3-3 and 4.3-4 are directly comparable because they were generated in the same experiment.

The presence of exudates of bean (*Phaseolus vulgaris* L.) increased the area of *B. reuszeri* macrocolonies relative to water. The exudates from seeds and the youngest seedlings (4 d old) provoked significantly higher motilities than the

exudates from 6 and 8 d old seedlings. No differences were observed between different N regimes at any of the different plant growth stages tested (Fig. 4.3-3 A).

Likewise, all the exudates of spring wheat (*Triticum aestivum* L. cv. Paragon) induced significantly higher motility of *B. reuszeri* than water. When the bacterial motility assay was performed in the presence of root exudates collected from seedlings grown under N-deficient conditions, the motility tended to be higher than from seedlings grown under adequate N nutrition (Fig. 4.3-3 B).

B. reuszeri was also more motile in the presence of exudates of maize (*Zea mays* L. cv. Tassilo) compared to water. Similarly to the results of wheat exudates, the presence of exudates from seedlings grown under N-deficient conditions tended to induce a higher motility than the exudates from seedlings grown under N-sufficient conditions. A significant difference between different N regimes was only seen 2 d after start of the N treatments and disappearing 4 d after onset of N deficiency (Fig. 4.3-3 C).

This differential behaviour of *B. reuszeri* in the presence of water or exudates was also observed by estimating the macrocolony diameter after Calcofluor-staining (Fig. 4.3-4). The macrocolony diameter was smallest in the presence of water. Bacterial colonies were more extended and showed higher fluorescence intensities in the presence of exudates from older seedlings of wheat and maize (Fig. 4.3-4 B, C). An influence of the N treatment appeared only in exudates from 4 d old seedlings of wheat and maize, which tended to provoke higher fluorescence. However, due to the formation of multiple rings differing in intensity, this assay was difficult to interpret.

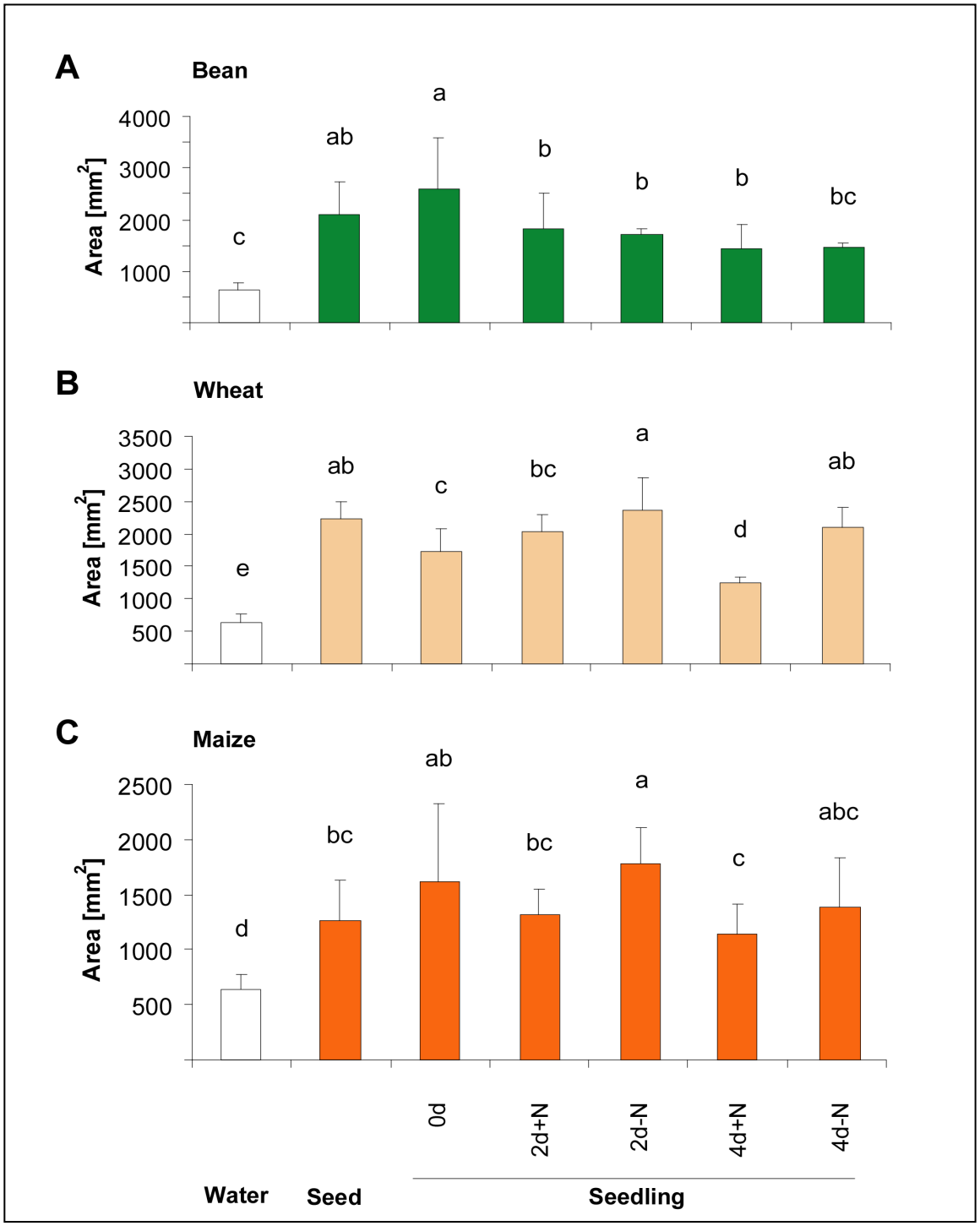

Figure 4.3-3 Motility of *Brevibacillus reuszeri* in the presence of water or exudates from seeds or seedlings of bean (A), spring wheat (B) or maize (C)

Area (mm^2) of the bacterial ring formed after 8 h of growth around the spotted bacteria in the presence of water or exudates on semisolid agar medium (5-fold diluted R2A-agar medium, Oxoid). Exudates were collected from roots of bean, wheat or maize seedlings that were pre-cultured in N-sufficient (+N) or N-deficient (-N) nutrient solution for 0, 2 and 4 days. Filter paper discs (5 mm diameter) containing exudates (1.5 µg C $disc^{-1}$) were placed at 2 cm distance left and right of the inoculation spot. Bars indicate means± SD, n= 5. Different letters represent significant differences of the means within each plant species at p≤0.05 by Fisher's LSD test.

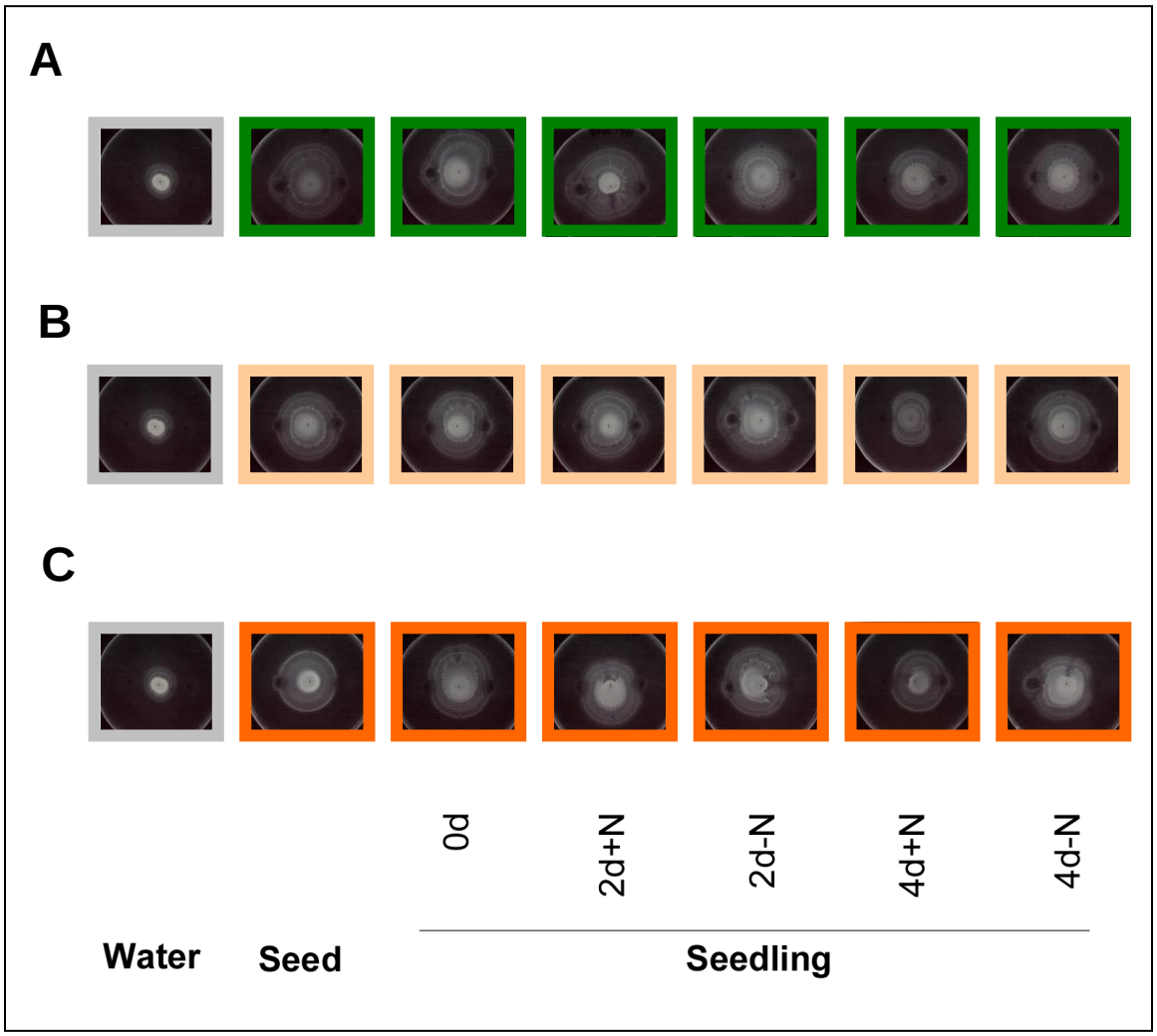

Figure 4.3-4 Fluorescence of bacterial colonies of *Brevibacillus reuszeri* after 4 d Calcofluor-staining in the presence of water or exudates from seeds or seedlings of bean (A), spring wheat (B) and maize (C)

Exudates were collected from roots of bean, wheat or maize seedlings that were pre-cultured in N-sufficient (+N) or N-deficient (-N) nutrient solution for 0, 2 and 4 days. Filter paper discs (5 mm diameter) were soaked in different exudates (1.5 µg C $disc^{-1}$) and placed at 2 cm distance left and right of the central inoculation spot. Bacterial colonies were incubated on semisolid agar medium (5-fold diluted R2A-agar medium, Oxoid), n= 5.

4.3.3 Bacterial macrocolony patterns

Formation of bacterial growth patterns by motile bacteria can be due to intercellular signalling (amino acids, proteins, pili), motility and chemotaxis (for a review see Shapiro, 1995). Differences in the growth pattern of *E. coli* are shown

for example in http://www.rowland.harvard.edu/labs/bacteria/index.html. The average size of macrocolonies may depend on the strain, the substrate, the concentration and the quality of agar, as well as the time and method of incubation (Brown & Hase, 2001). Some stress factors or environmental signals can lead to geometrically regular macrocolony morphologies, distinct patterns of cellular morphology, physiology and differential gene expression (Shapiro, 1995).

In this study, different bacterial colony patterns could be observed. While in the presence of water, the macrocolonies were symmetrical and not branched, in the presence of organic acids (Fig. 4.3-2) and seed or root exudates (Fig. 4.3-4) almost all formed rings had branched edges. In preliminary experiments, clear differences in the colony growth patterns could be observed in the presence of exudates from plants grown under different N regimes (data not shown). When *B. reuszeri* was incubated with water or exudates from N-starved plants, the motility ring formed showed a more regular shape than in the presence of root exudates from N-sufficient plants (Fig. 4.3-5). It has been reported that several bacterial species adjust to nutritional deprivation and to changes in agar concentrations by spreading in a more branched manner, frequently showing fractal patterns (Rauprich *et al.*, 1996; Shapiro, 1995). For example, bacteria such as *Bacillus subtilis* and *Paenibacillus* strains, produce distinct colony morphologies depending on the environment (wetness of the agar and surfactant production) and become more compact and symmetrical at low nutrient supply (Ben-Jacob *et al.*, 1998; Mendelson & Salhi, 1996).

Costerton *et al.* (1987) suggested that the study of the bacterial macrocolony patterns on motility plates can expand our understanding of the biological capabilities which bacteria employ in their natural environments, where biofilms and microcolonies are the predominant modes of existence. The Fig. 4.3-5 only provides an example supporting this suggestion without providing more details, but bacterial growth patterns should be further investigated for their potential to indicate the physiological status of bacteria.

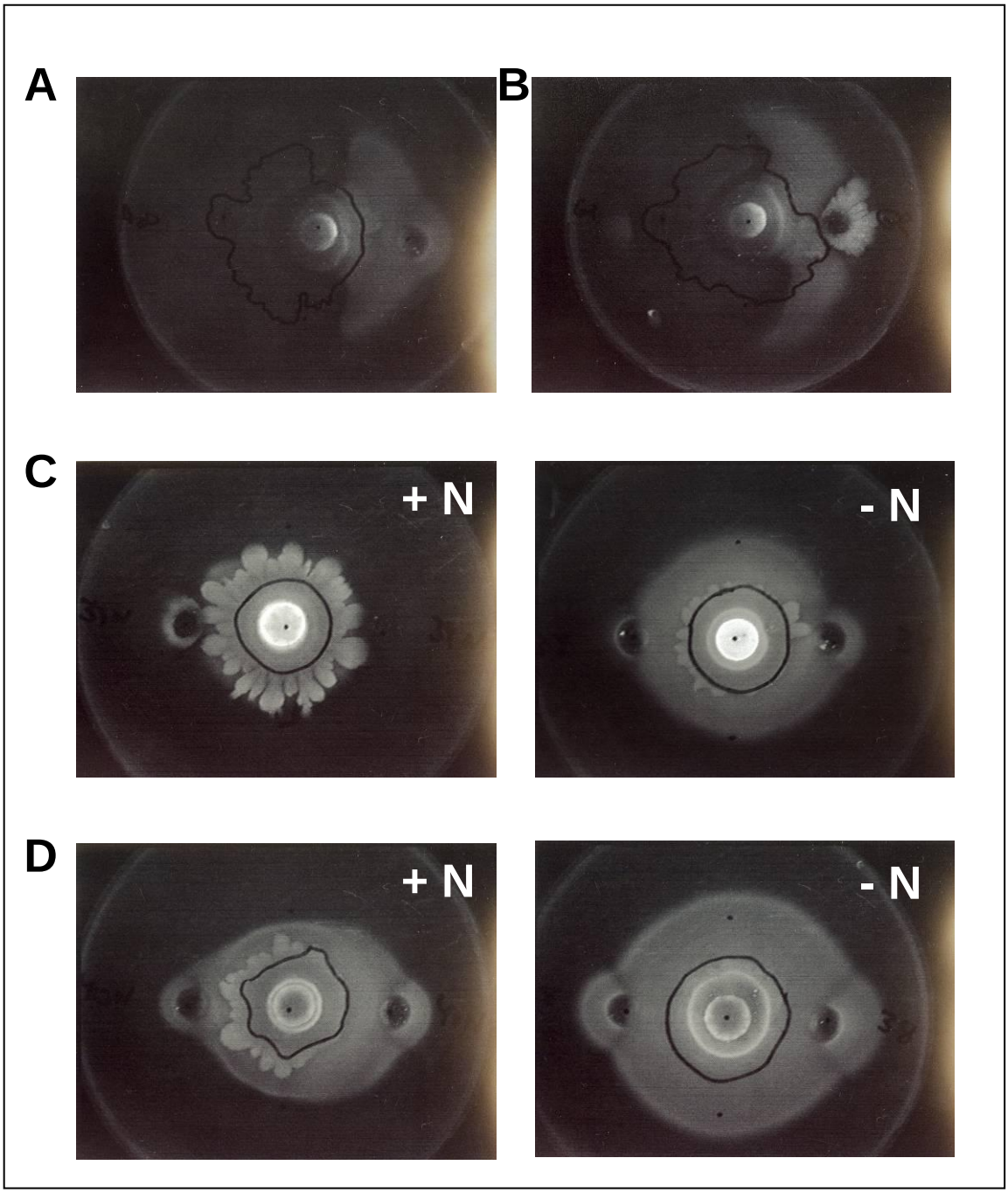

Figure 4.3-5 Differential bacterial colony patterns of *Brevibacillus reuszeri* after 4 d Calcofluor-staining in the presence of water (A), glucose (B) or exudates from root wheat seedlings pre-cultured in N-sufficient (+N) or N-deficient (-N) nutrient solution for 2 (C) and 4 days (D)

Filter paper discs (5 mm diameter) were soaked in different exudates (1.5 µg C $disc^{-1}$) and placed at 2 cm distance left and right of the central inoculation spot. Bacterial colonies were incubated on semisolid agar medium (5-fold diluted R2A-agar medium, Oxoid), n= 5.

4.4. Physiological characterization of LeAMT1;1 and LeAMT1;2 in tomato

Out of the three tomato AMT genes, two of them have been shown to be also expressed in roots, *LeAMT1;1* and *LeAMT1;2* (von Wirén *et al*., 2000b). In order to investigate a possible contribution of either LeAMT1 transporters to N transfer from associative diazotrophic bacteria, transgenic tomato plants with altered expression of these two genes have been generated and characterized. The transgenic tomato lines were generated by *Agrobacterium*-mediated transformation of cotyledons with gene fragments of *LeAMT1;1* or of *LeAMT1;2* in antisense orientation. Transgenic plants were selected on medium containing kanamycin, selfed and 3 independent, homozygous T2 lines of each gene construct were selected for further analyses. They were hydroponically grown along with tomato WT plants under non-sterile conditions and under different N-regimes. When grown in nutrient solution with adequate N supply, all tomato antisense lines grew similar to WT plants and showed no visual symptoms (data not shown).

All the experiments performed for the characterization of the tomato root ammonium transporters (chapter 4.4) were conducted in cooperation with and at equal contribution of Lucile Graff.

4.4.1 Characterization of the contribution of *LeAMT1;1* to ammonium uptake and the N nutritional status of tomato plants

Ammonium uptake capacity

LeAMT1;1 mRNA levels as well as the ammonium uptake capacity were studied in antisense lines (117, 123 and 204.2) and compared to wild-type (WT) plants. For this purpose, hydroponically-grown tomato plants were pre-cultured under adequate N conditions, then N-starved for 3 days followed by ammonium re-supply of 2 mM ammonium for 2 or 12 hours. For each treatment, 4 plants of WT and of the 3 transgenic *LeAMT1;1* lines were independently harvested and analysed.

The regulation of mRNA levels of *LeAMT1;1* in WT and antisense lines was then examined by northern blot analysis (Fig. 4.4-1). In WT roots, transcript levels of *LeAMT1;1* were strongly induced after 3 days of N starvation and remained at a high level after 2 or 12 h ammonium re-supply. This regulation was in agreement with the one described previously by von Wirén *et al.* (2000b). In the antisense lines, *LeAMT1;1* mRNA expression was reduced in plants of the lines 117 and 123, but not in the plants of line 204.2. The extent of *LeAMT1;1* repression increased in the line 204.2 ‹ 117 ‹ 123.

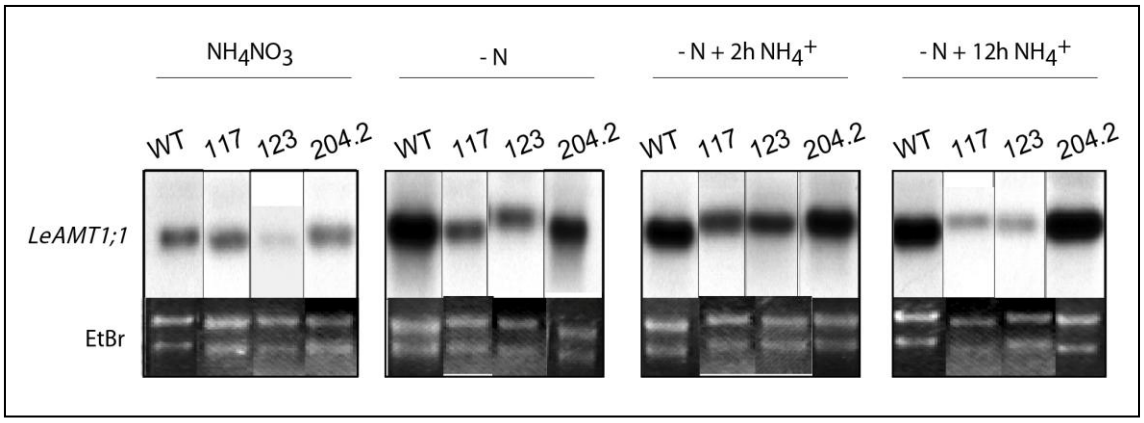

Figure 4.4-1 Nitrogen-dependent expression of *LeAMT1;1* in roots of wild-type tomato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2)

RNA gel-blot analysis performed on root of 4 week-old plants hydroponically pre-cultured at 2 mM NH_4NO_3, N-starved for 3 d (-N) or starved for 3 d and re-supplied with 2 mM ammonium for 2 h (-N +2h NH_4^+) or 12 h (-N +12h NH_4^+). Ethidium bromide-stained rRNA (EtBr) served as loading control.

In order to investigate the contribution of LeAMT1;1 to ammonium uptake by tomato plants, *LeAMT1;1* antisense lines and WT plants were subjected to short-term ammonium influx studies using 200 µM of ^{15}N-labeled ammonium as a measure for high-affinity uptake (Fig. 4.4-2). Depending on the N supply, WT plants showed different ammonium uptake rates. Under N-sufficient conditions, they showed a relatively low ammonium uptake rate, which was two-fold increased under N starvation. Under ammonium re-supply a further increase was observed. Ammonium uptake rates in the antisense lines did not differ significantly from that in the WT. However, plants of the lines 117 and 123 showed a reduction of

ammonium uptake already after N starvation and an even stronger reduction after ammonium re-supply. No difference in ammonium uptake rates in any of the treatments was observed between the plants of the line 204.2 and the WT.

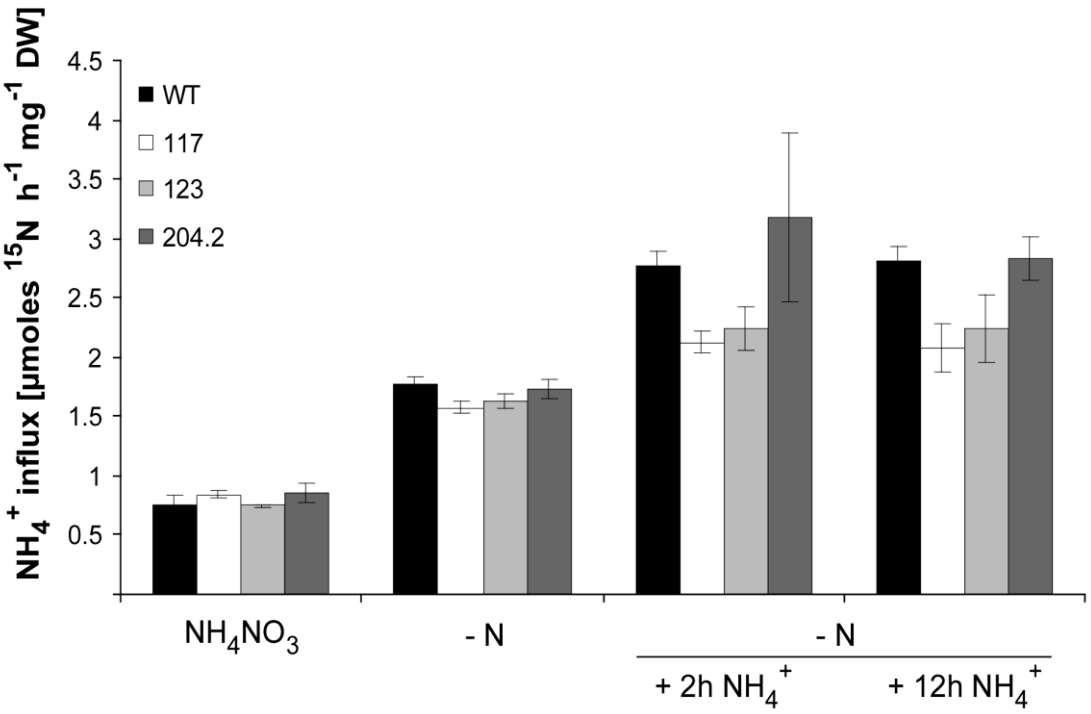

Figure 4.4-2 $^{15}NH_4^+$ influx analysis of roots from of wild-type tomato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2) with *LeAMT1;1*

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 d (-N) or N-starved for 3 d and re-supplied with 2 mM ammonium for 2 h (-N+2h NH_4^+) or for 12 h (-N+12h NH_4^+). ^{15}N-labelled ammonium was supplied at NH_4^+ 200 µm and measured in a period of 10 min. Bars indicate means± SD, n= 4.

Gene expression analysis

Since the transgenic lines did not differ significantly in their capacity for ammonium uptake, the contribution of LeAMT1;1 to the overall ammonium and N status of the plant was investigated. For this purpose, plants were grown hydroponically under N-sufficient and N-deficient conditions or subjected after 3 d of N starvation to 12 h of re-supply with 200 µM ammonium. After 3 d of starvation, some plants were further subjected to N starvation for 3 d and some of them were subjected to a daily pulse of ^{15}N-labelled ammonium at 200 µM for 2 h. These treatments should allow the distinction between the functions of LeAMT1;1 and LeAMT1;2, since the *LeAMT1;2* gene and protein were highly inducible by ammonium re-supply, whereas protein levels of LeAMT1;1 did not show this strong increase after ammonium re-supply (Borel, 2009; von Wirén *et al.*, 2000b). Transcript levels of *LeAMT1;1* were examined for each of the 4 plants of the 3 independent lines and for the WT. For a characterization of soluble N pools, the free ammonium, glutamine, glutamate and aspartate concentrations were determined in roots together with ammonium and chlorophyll concentrations in leaves, and the ^{15}N enrichment in root and leaf fractions was measured after pulse labelling.

As expected, gene expression of *LeAMT1;1* was strongly induced after 3 d of N starvation and weakly repressed after ammonium re-supply (Fig. 4.4-3). After 6 d of N starvation *LeAMT1;1* was still strongly expressed irrespective of whether the plants were additionally subjected to ammonium pulses.

Compared to the WT, *LeAMT1;1* mRNA expression was reduced in plants of the lines 117 and 123 under all conditions tested, but to a lesser extent in the plants of line 204.2 (Fig. 4.4-3).

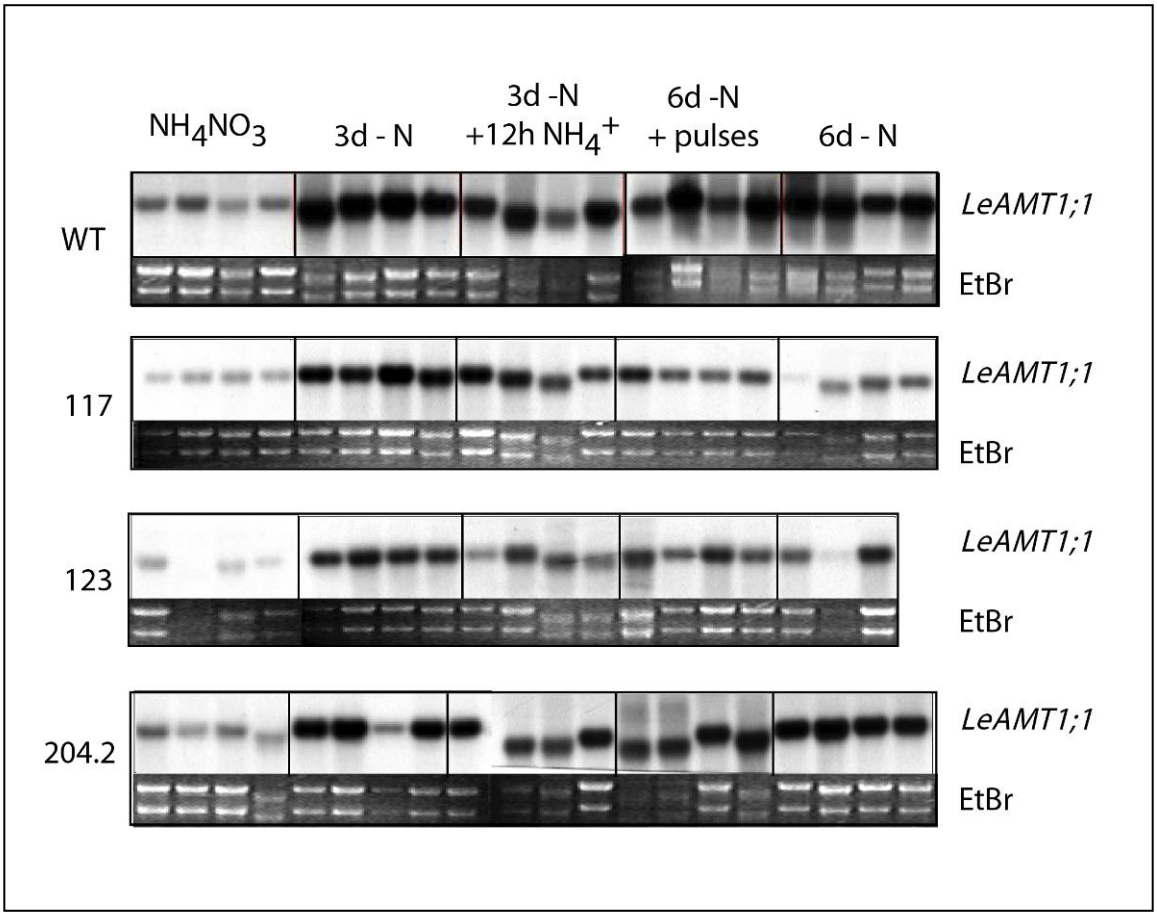

Figure 4.4-3 Nitrogen-dependent expression of *LeAMT1;1* in roots of wild-type (WT) tomato plants and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2)

RNA gel-blot analysis performed on root of 4 week-old plants hydroponically pre-cultured at 2 mM NH_4NO_3, N-starved for 3 d (3d-N) or starved for 3 d and re-supplied with 200 µM ammonium for 12 h (3d-N +12h NH_4^+), starved for 6 d (6d-N) or supplied daily with 200 µM ammonium pulses for 2 h (6d-N+pulses). Other Ethidium bromide-stained rRNA (EtBr) served as loading control.

Ammonium concentrations

In order to investigate whether LeAMT1;1 might contribute to internal ammonium accumulation, ammonium concentrations were determined in roots and shoots.

In WT plant roots, ammonium concentrations showed a strong regulation by N supply (Fig. 4.4-4 C). After 3 or 6 days of N starvation, ammonium concentrations in roots decreased to less than half of those from N-supplied plants. After 12 h of ammonium re-supply, the concentration increased 8-fold, and even a 6-fold increase was observed after successive ammonium pulses. These results indicated that the experimental setup was suitable to assess the contribution of

AMT transporters under different N regimes. However, in the 3 transgenic lines, root ammonium concentrations did not significantly differ from the WT.

Ammonium concentrations in young and old leaves from WT plants strongly responded to N supply, but not to the daily ammonium pulses (Fig. 4.4-4 A,B). Young leaves responded stronger to ammonium re-supply than old leaves. However, the response to ammonium re-supply was not as strong as in roots, probably because a part of the supplied ammonium was already assimilated in the roots and translocated to the shoots in form of amino acids. In young leaves, all 3 antisense lines showed higher ammonium concentrations than the WT under N-sufficient conditions (Fig. 4.4-4 A). In old leaves, like in the roots, no differences were observed between the ammonium concentrations from the antisense lines and the WT.

Amino acid concentration in roots

Glutamine, glutamate and aspartate concentrations in roots were determined, since these amino acids have been shown to be involved in long-distance N transport within the whole plant and might be therefore indicative for different levels of ammomium acquisition. In the WT roots, the concentrations of glutamine decreased strongly after 3 days of N starvation being 13-fold lower that under N-sufficient conditions and remained at this level even after 6 days of N starvation (Fig. 4.4-5 A). Repletion of glutamine pools resulting from ammonium re-supply was dependent on the time of exposure to ammonium. By contrast, glutamate concentrations in WT roots decreased relatively little after 3 days of N starvation and further slightly decreased after ammonium re-supply (Fig. 4.4-5 B), probably due to a depletion of glutamate for the synthesis of glutamine. Aspartate concentrations in roots showed a similar pattern as those of glutamate with a 3-fold decrease after 3 days of N starvation, which remained at the same level even after ammonium re-supply (Fig. 4.4-5 C). Although no significant differences were observed between the transgenic lines and the WT, the antisense lines showed higher aspartate concentrations than the WT in all the treatments except under adequate N nutrition conditions.

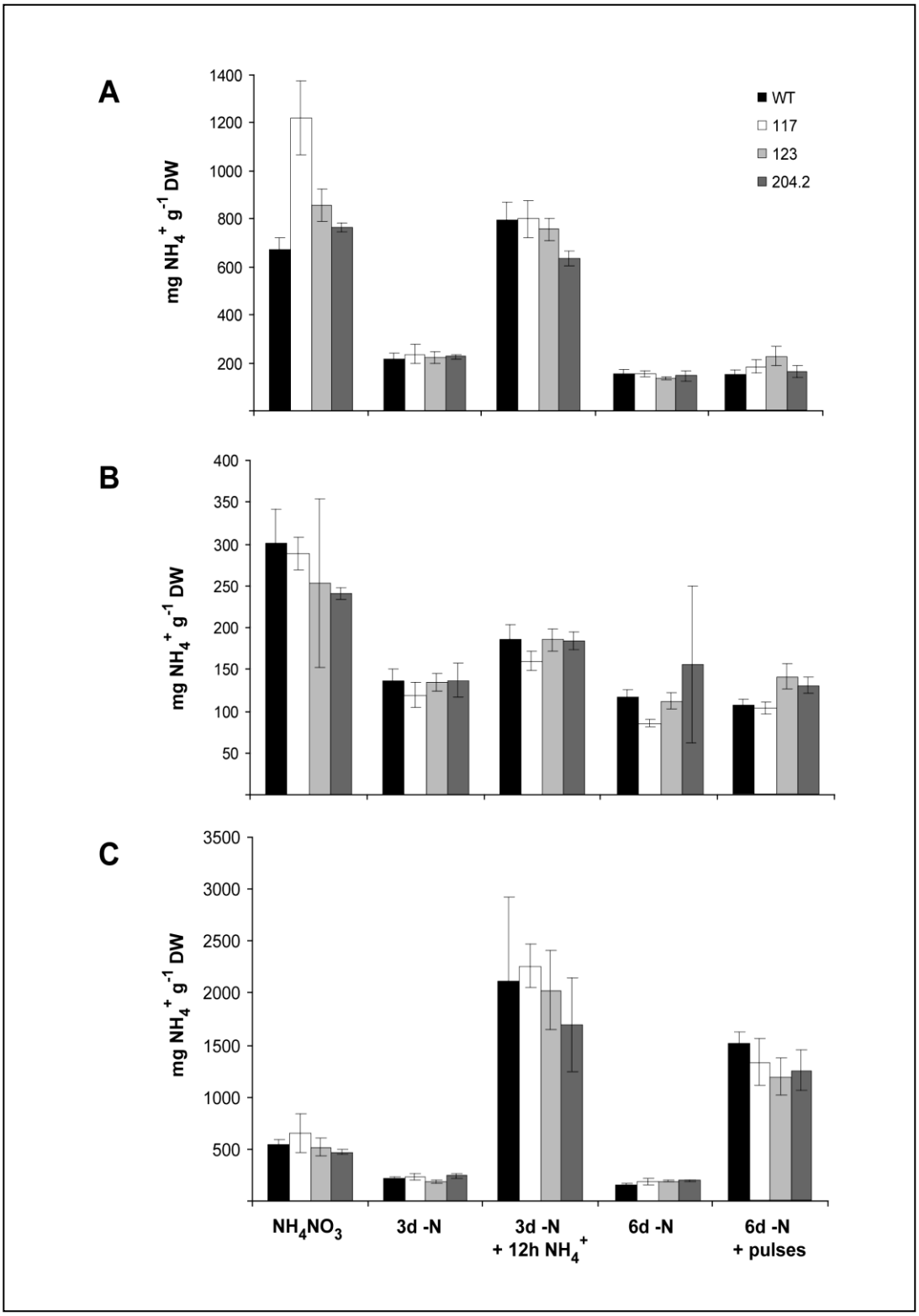

Figure 4.4-4 Ammonium concentrations in young leaves (A), old leaves (B) and roots (C) of wild-type tomtato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2)

Plants were pre-cultured for 4 weeks hydroponically with 2mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 4.

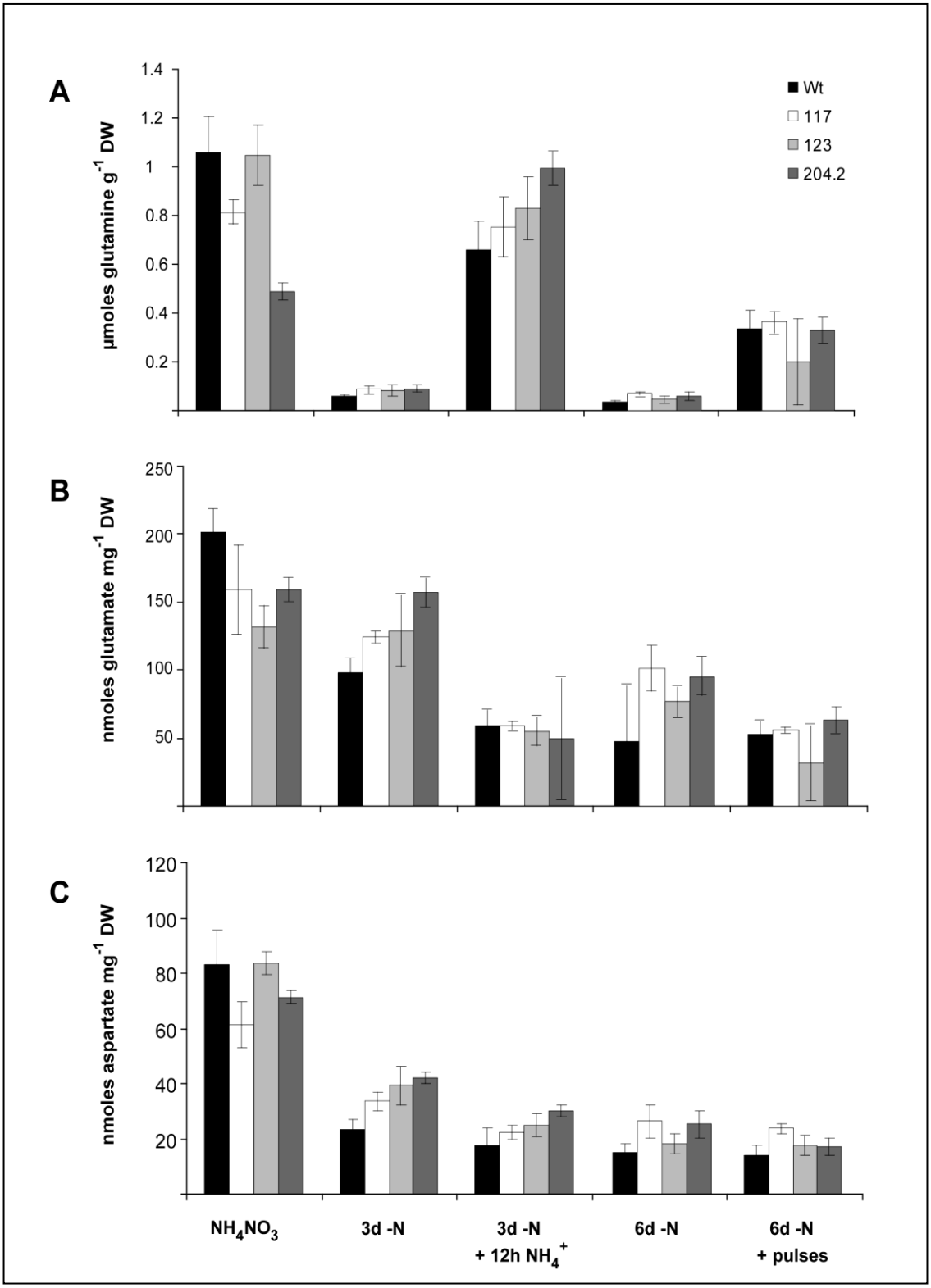

Figure 4.4-5 Amino acids concentrations in roots of wild-type (WT) tomato plants and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2). Glutamine (A), glutamate (B) and aspartate (C)

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 3.

Chlorophyll concentrations

Chorophyll concentrations were measured in WT and antisense plants as an additional indicator for the N status of the shoot (Fig. 4.4-6). In young leaves of WT plants, chlorophyll concentrations did not vary considerably between different treatments. Interestingly, in the 3 antisense lines, chlorophyll concentrations significantly decreased after 3 days of N starvation followed by ammonium re-supply, being then significantly lower than in the WT. However, after 6 days of N starvation followed by ammonium pulses, chlorophyll concentrations were higher than in the WT (Fig. 4.4-6 A).

In old leaves of WT plants, the chlorophyll concentration did not show any reduction despite 3 days starvation and for ammonium re-supply (Fig. 4.4-6 B). However, after 6 days of starvation, chlorophyll concentrations significantly decreased by about 15%. In the transgenic plants, chlorophyll concentrations significantly decreased already after 3 days of N starvation, being then significantly lower than in the WT. After 6 days of N starvation followed by ammonium pulses, only the line 117 remained significantly lower than the WT.

^{15}N accumulation in plants

Ammonium supplied in daily pulses was enriched with 10% of ^{15}N-labelled ammonium aiming at allowing later to trace small differences in N accumulation among the different lines. In the WT, a strong accumulation of ^{15}N could be observed in roots and young leaves, whereas old leaves were only weakly enriched (Fig. 4.4-7). This was most probably due to the stronger sink effect for ammonium exerted by young leaves under N starvation. No significant differences were observed between the 3 antisense lines and the WT.

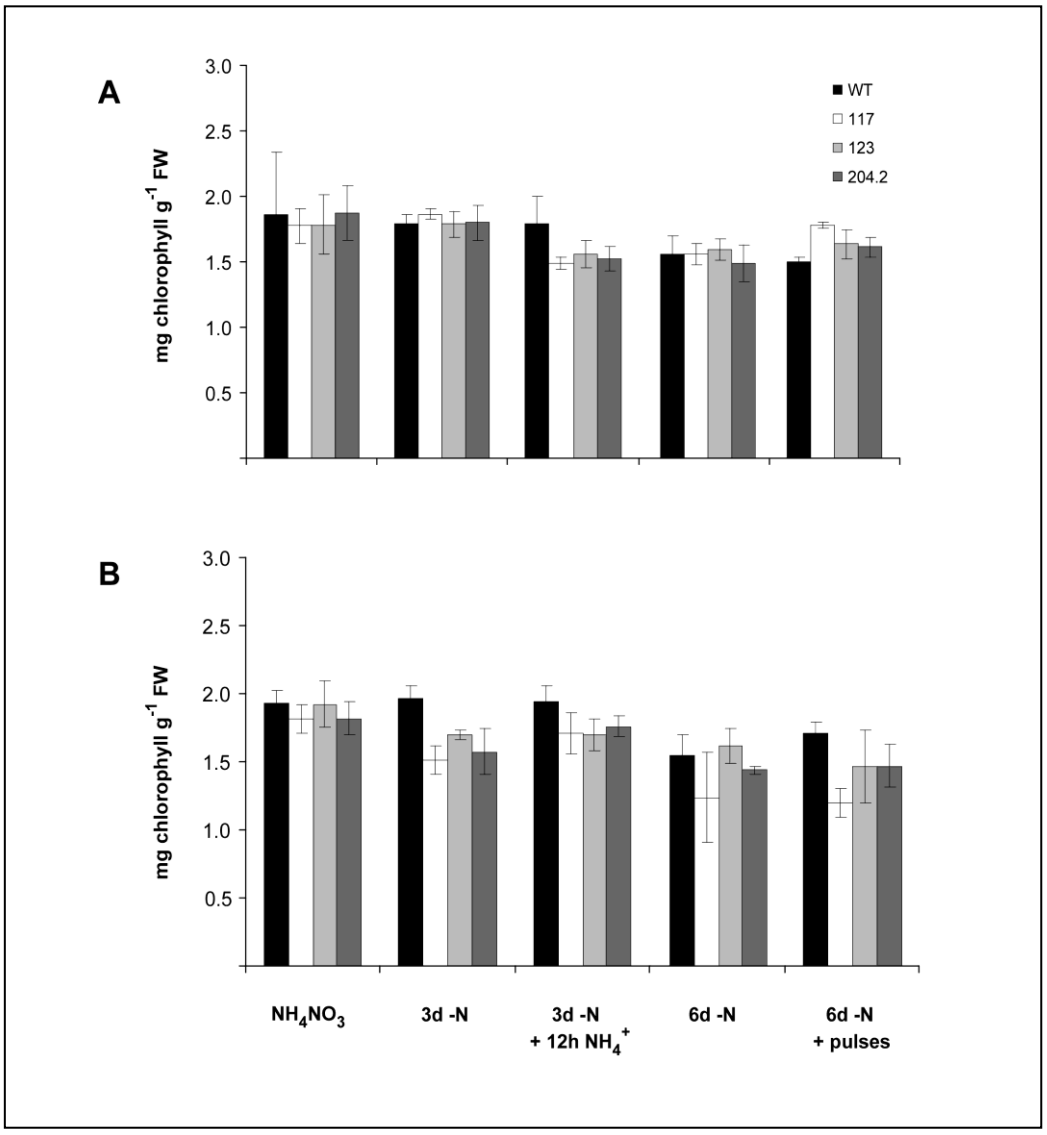

Figure 4.4-6 Chlorophyll concentrations in young (A) and old leaves (B) of wild-type tomtato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2)

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 4.

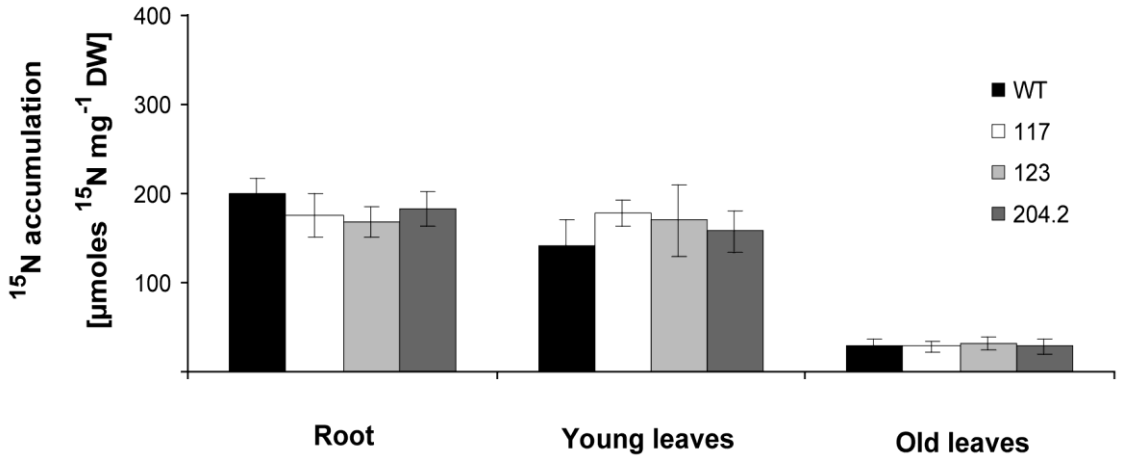

Figure 4.4-7 ^{15}N accumulation in roots, young and old leaves of wild-type (WT) tomato plants and of three independent transgenic lines transformed with *LeAMT1;1* in antisense orientation (117, 123, 204.2) after pulse-labelling with ^{15}N-ammonium

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days and then re-supplied with 200 µM ammonium in pulses of 2 h during 3 days. Bars indicate means± SD, n= 4.

To recapitulate the results of the physiological characterization of *LeAMT1;1* gene, it should be noted that the antisense repression of the *LeAMT1;1* lines was not strong, especially in the line 204.2. The antisense repression of the 3 transgenic lines did not lead to any effect on ammonium, glutamine or glutamate concentrations in roots. Only after 3 d of N starvation they showed higher aspartate concentrations. In young leaves, all 3 transgenic lines showed higher ammonium concentrations under N-sufficient conditions and higher chlorophyll concentrations after 6 d N starvation with daily ammonium pulses, whereas after 3 d of N starvation followed by ammonium re-supply the chlorophyll concentration were lower in comparison with the WT plants. Moreover, in old leaves, a decrease in chlorophyll concentrations after 3 d of N starvation was observed.

4.4.2 Characterization of the contribution of *LeAMT1;2* to ammonium uptake and the N nutritional status of tomato plants

Ammonium uptake capacity

Similar to the experiments performed with the *LeAMT1;1*, antisense lines for 3 independent antisense lines of *LeAMT1;2* (120.3, 258 and 264) were subjected to a comparison with wild-type (WT) plants. For this purpose, hydroponically-grown tomato plants were pre-cultured under adequate N conditions and then N-starved for 3 days followed by ammonium re-supply with 2 mM ammonium for 2 or 12 hours. For each treatment, 4 plants of WT and of the three transgenic *LeAMT1;2* lines were independently harvested and analysed.

The regulation of mRNA levels of *LeAMT1;2* in WT and antisense lines was examined by northern blot analysis (Fig. 4.4-8). In WT plant roots, transcript levels of *LeAMT1;2* were down-regulated after 3 d of N starvation and strongly induced already after 2 h of ammonium re-supply. This regulation was in agreement with the results previously described (von Wirén *et al.*, 2000b). In all three antisense lines, mRNA expression was lower than in WT plants under all the tested conditions. All individual plants showed the same level of repression, except the line 264 after ammonium re-supply. The extent of *LeAMT1;2* repression increased in the order 120.3 ‹ 264 ‹ 258.

In order to investigate the contribution of LeAMT1;2 in ammonium uptake in tomato plants, *LeAMT1;2* antisense lines and WT plants were subjected to short-term ammonium influx studies using 200 µM of ^{15}N-labeled ammonium as a measure for the high-affinity uptake (Fig. 4.4-9). Again, WT plants showed different ammonium uptake rates depending on the N supply. Relative to N-sufficient conditions, uptake rates increased threefold under N starvation. Ammonium uptake rates in the antisense line 258 were lower than the WT under any condition tested. However, no clear correlation was observed between the level of transcripts and ammonium uptake rates, which might point to a possible post-transcriptional regulation mechanism. It is interesting to note that ammonium uptake was high under conditions where *LeAMT1;2* is usually expressed at a relatively low level (in particular under N starvation).

Considering the large variation among the 3 antisense tomato lines, a contribution of *LeAMT1;2* to ammonium uptake is critical to be demonstrated.

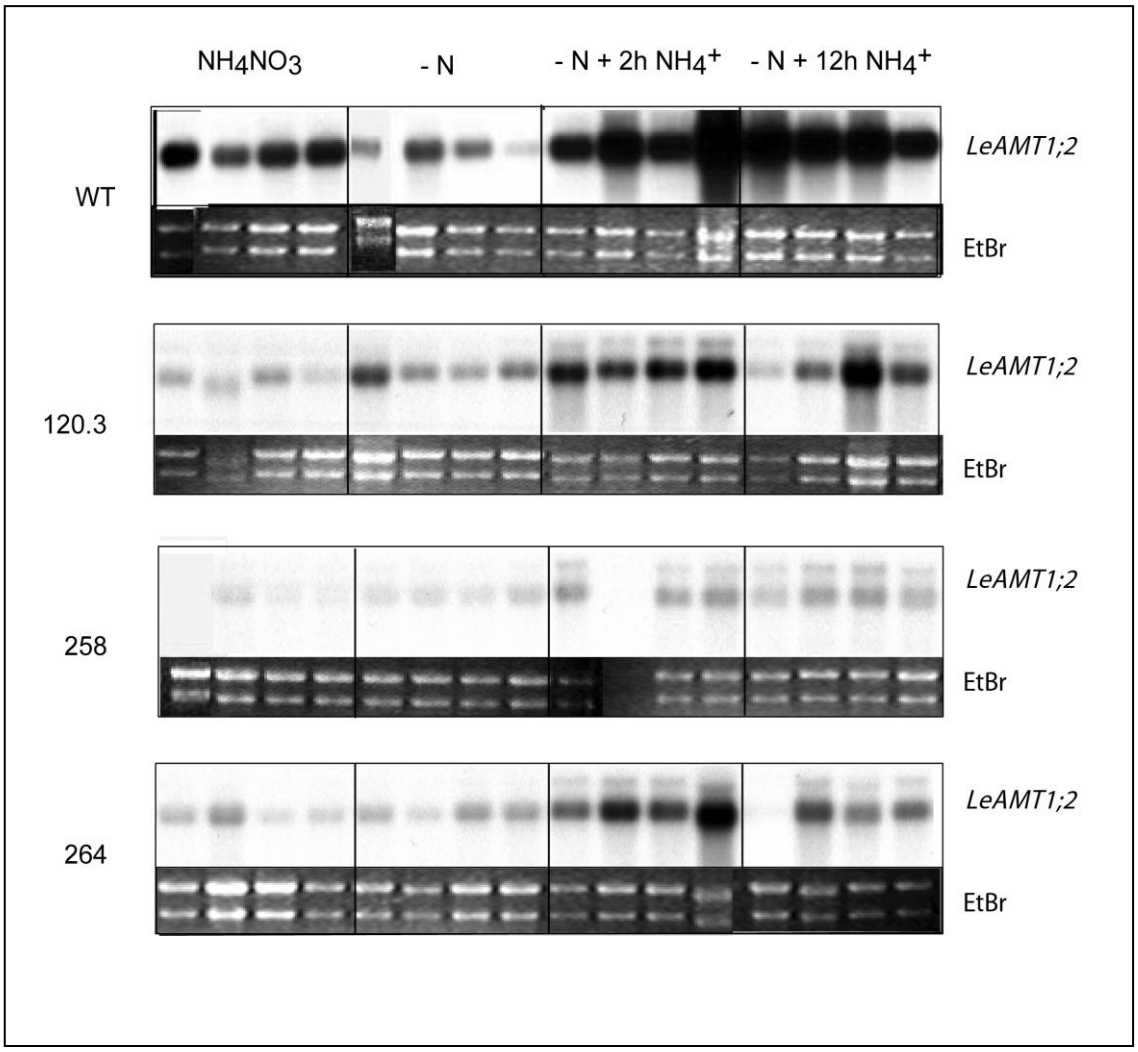

Figure 4.4-8 Nitrogen-dependent expression of *LeAMT1;2* in roots of wild-type tomato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264)

RNA gel-blot analysis performed on root of 4 week-old plants hydroponically pre-cultured at 2 mM NH_4NO_3, N-starved for 3 days (-N) or starved for 3 days and re-supplied with 2 mM ammonium for 2 h (-N +2h NH_4^+) or 12 h (-N +12h NH_4^+). Ethidium bromide-stained rRNA (EtBr) served as loading control.

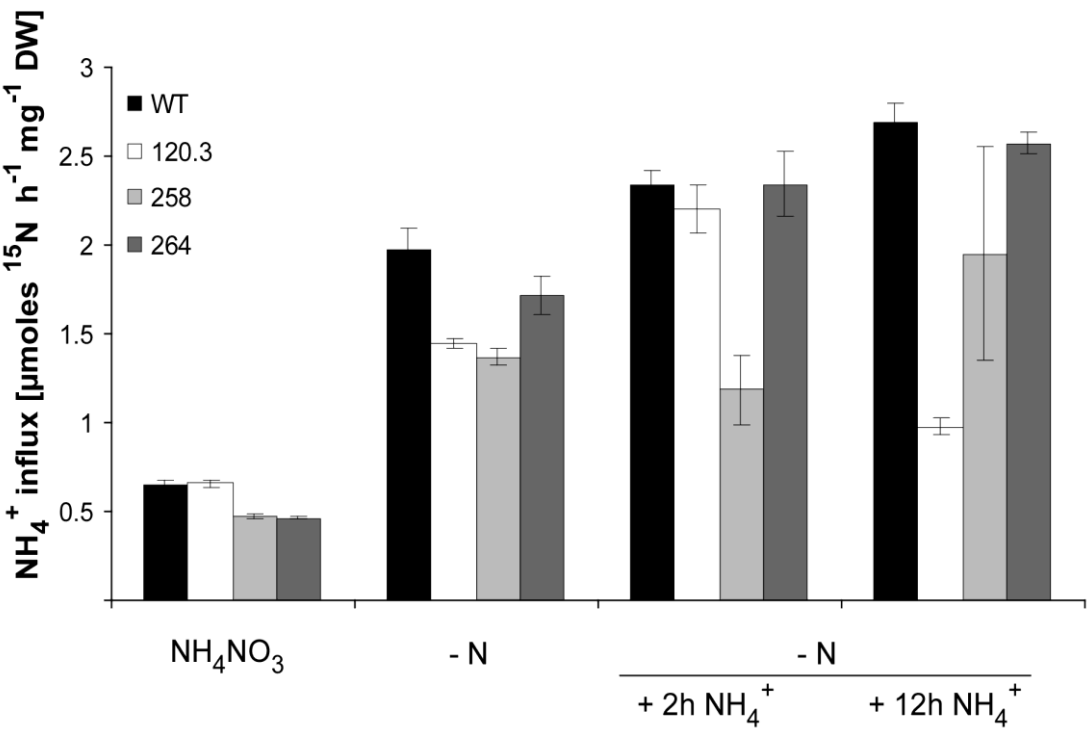

Figure 4.4-9 $^{15}NH_4^+$ influx analysis of roots from of wild-type tomato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264) with *LeAMT1;2*

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (-N) or N-starved for 3 days and re-supplied with 2 mM ammonium for 2 h (-N+2h NH_4^+) or for 12 h (-N+12h NH_4^+). ^{15}N-labelled ammonium was supplied as NH_4^+ 200 µM and measured in a period of 10 min. Bars indicate means± SD, n= 4.

Gene expression analysis

Since the transgenic lines did not differ consistently from WT plants in their capacity for primary ammonium uptake, a gene expression analysis was performed. As expected, gene expression levels of *LeAMT1;2* showed a reduction under N starvation and a strong induction after ammonium re-supply, which was in agreement with previous investigations (von Wirén *et al.,* 2000b) (Fig. 4.4-10).

In comparison with the WT plants, all antisense lines showed a reduction of *LeAMT1;2* expression levels under all the conditions tested. The variability among the 4 individual plants was quite low (Fig. 4.4-10). The extent of *LeAMT1;2* repression increased again in the order 120.3 ‹ 264 ‹ 258.

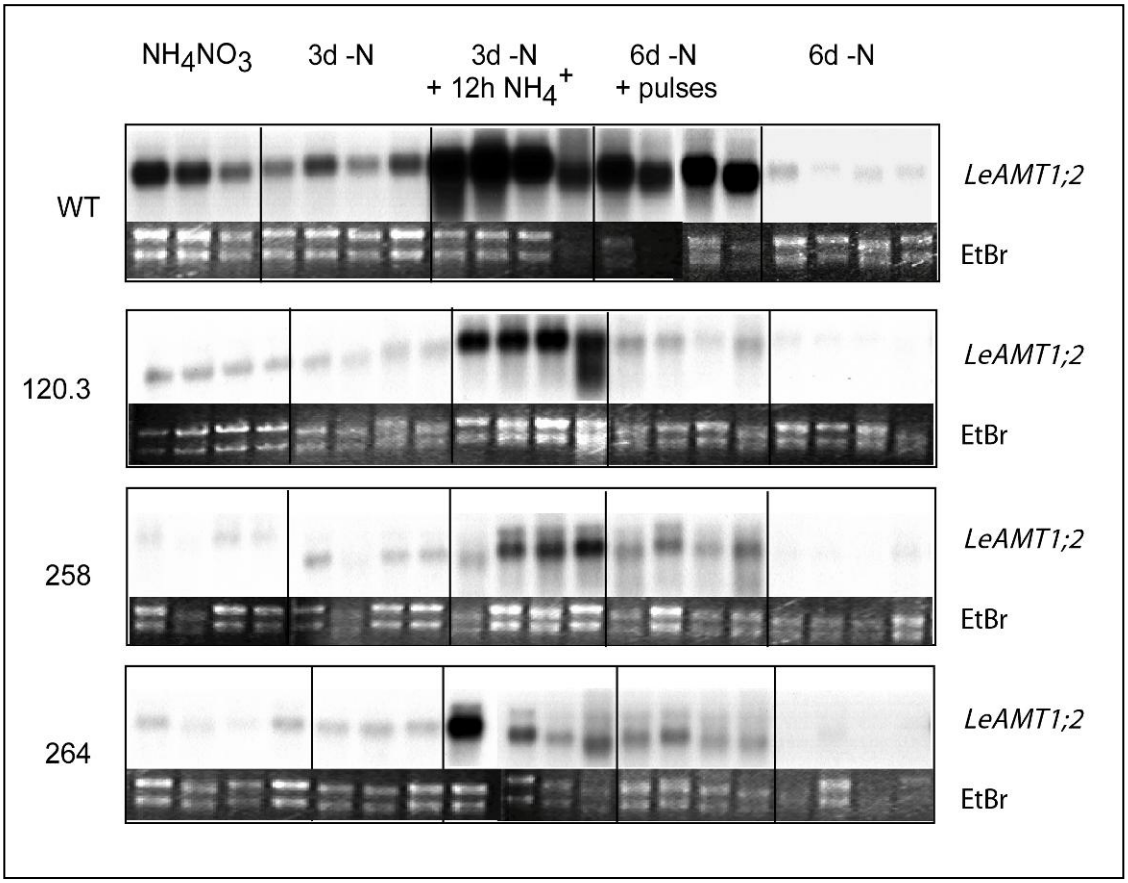

Figure 4.4-10 Nitrogen-dependent expression of *LeAMT1;2* in roots of wild-type tomato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264)

RNA gel-blot analysis performed on root of 4 week-old plants hydroponically pre-cultured at 2 mM NH_4NO_3, N-starved for 3 days (3d -N) or starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d-N +12h NH_4^+), starved for 6 days (6d-N) or supplied daily with 200 µM ammonium pulses for 2 h (6d-N+pulses). Other Ethidium bromide-stained rRNA (EtBr) served as loading control.

Ammonium concentrations

In order to investigate whether LeAMT1;2 might contribute to internal ammonium pools, ammonium concentration was determined in roots and shoots. In contrast to the results obtained by the analysis of the *LeAMT1;1* antisense lines (Fig. 4.4-4), ammonium concentrations did not differ significantly between N-sufficient and N-deficient in WT roots (Fig. 4.4-11 C). However, after 12 h of ammonium re-supply or after ammonium pulses, root concentrations increased by six to sevenfold, relative to the corresponding N-starved treatments.

Ammonium concentrations in young and old leaves from WT plants showed similar patterns (Fig. 4.4-4 A, B). Young leaves responded with a fourfold increase to 12 h ammonium re-supply, while old leaves showed only a 1.5-fold increase compared to 3 d N-starved plants. Similar to the experiment with *LeAMT1;1* antisense lines, the response to ammonium re-supply was strongest in roots, probably because ammonium was partly assimilated in the roots and translocated to the shoots in the form of amino acids. No increase was observed in leaves after ammonium pulses in 6 d N-starved plants.

In all the tissues analysed, no significant differences were observed among the ammonium concentrations from antisense lines and WT plants.

Amino acid concentration in roots

In WT roots, the concentrations of the analysed amino acids showed the same changes in response to different N regimes than in the experiment with *LeAMT1;1* antisense lines (Figs. 4.4.-5, 4.4-12). The concentrations of glutamine decreased strongly after 3 days of N starvation being 13-fold lower that under N-sufficient conditions and remained at this level also after 6 days of N starvation (Fig. 4.4-12 A). Glutamine concentrations strongly increased after ammonium re-supply. Glutamate and aspartate concentrations in WT roots decreased after N starvation, but not clear increase was observed after ammoniums re-supply (Fig. 4.4-12 B, C). Most importantly, no significant differences were observed between the transgenic lines and the WT plants.

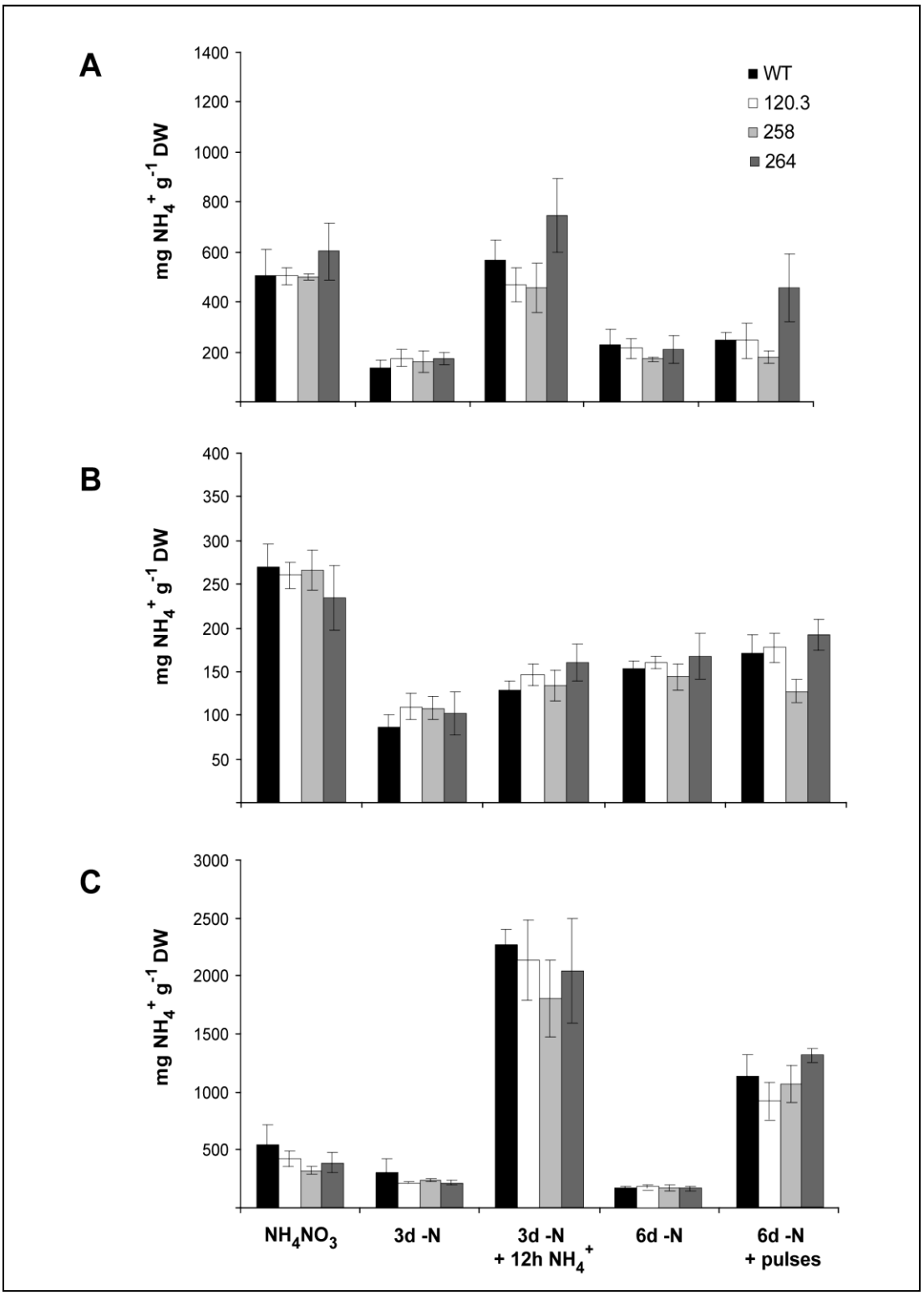

Figure 4.4-11 Ammonium concentrations in young leaves (A), old leaves (B) and roots (C) of wild-type tomtato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264)

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 4.

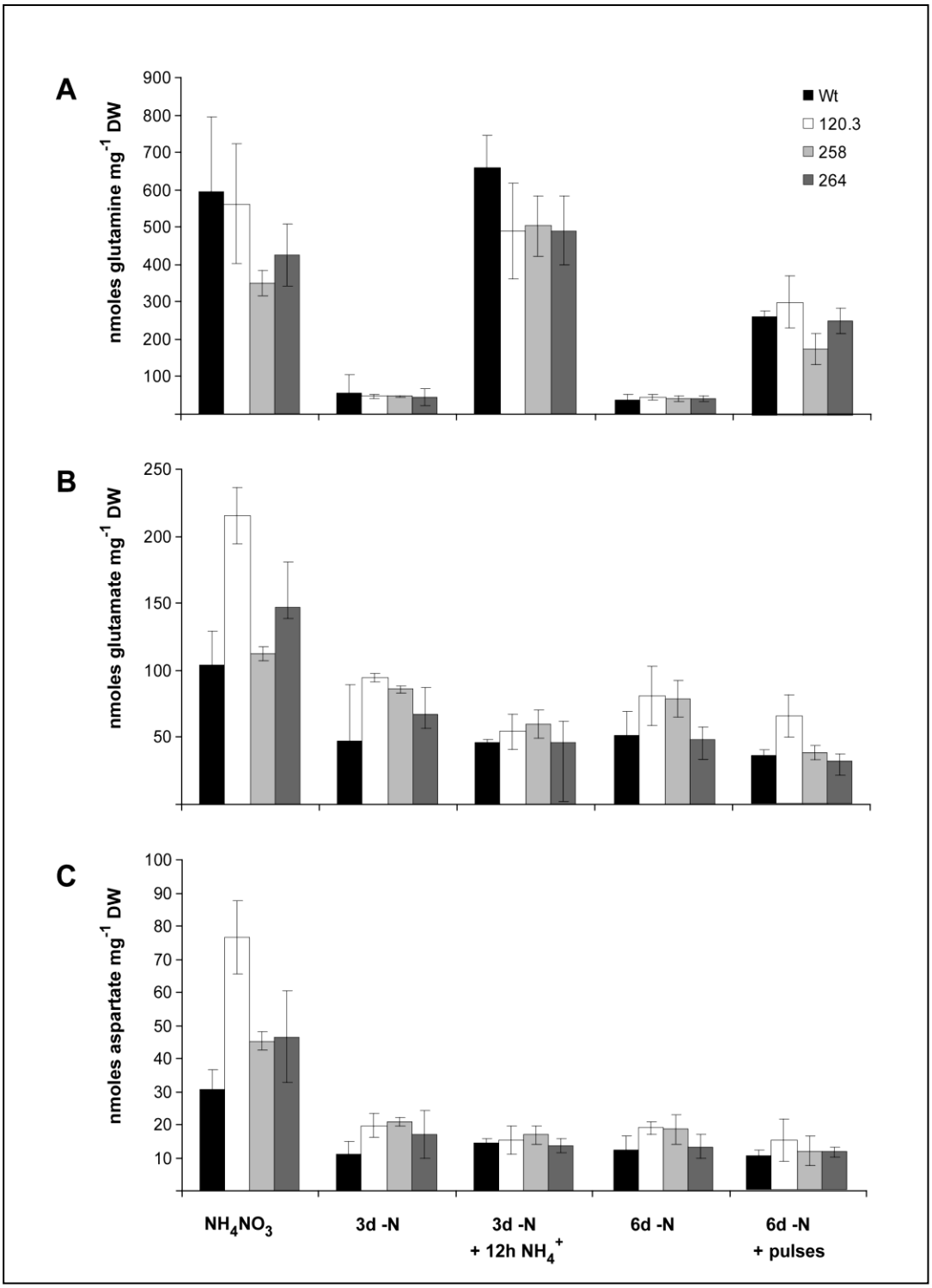

Figure 4.4-12 Amino acids concentrations in roots of wild-type (WT) tomato plants and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264). Glutamine (A), glutamate (B) and aspartate (C)

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 3.

Chlorophyll concentrations

Chorophyll concentrations in young leaves of WT plants decreased after 3 d of N starvation and remained the same level even after 6 d of starvation. After ammonium re-supply, they even decreased further (Fig. 4.4-13 A). Relative to the WT, the line 264 showed a small reduction in chlorophyll concentrations under N-sufficient and N-deficient conditions. However, no consistent differences between the 3 antisense lines and the WT could be observed.

By contrast, in old leaves of WT plants, the chlorophyll concentration was still high after 3 d starvation, decreased after 6 d of N starvation and increased after ammonium pulses (Fig. 4.4-13 B). All the antisense lines showed significantly lower chlorophyll concentrations than WT plants after 3 d of N starvation and after the ammonium pulses.

^{15}N accumulation in plants during pulses

As a consequence of daily ^{15}N-ammonium pulses, a strong accumulation of ^{15}N could be observed in roots and young leaves of WT plants, whereas old leaves were only weakly enriched (Fig. 4.4-14). This was probably due to the strong sink effect for ammonium exerted by the young leaves under N starvation. No differences in ^{15}N enrichment were observed between the 3 antisense lines and the WT.

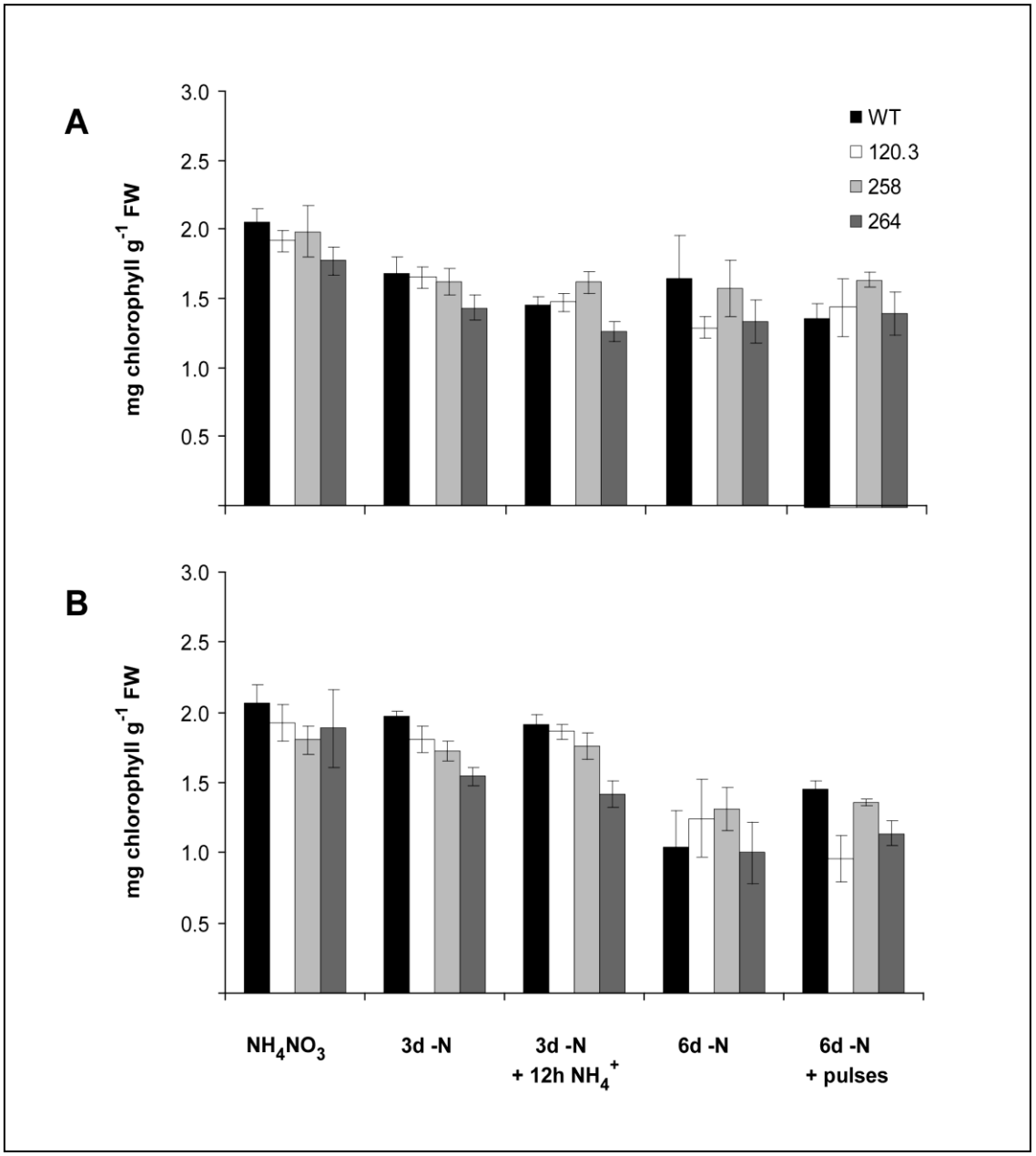

Figure 4.4-13 Chlorophyll concentrations in young (A) and old leaves (B) of wild-type tomtato plants (WT) and of 3 independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264)

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days (3d -N), N-starved for 3 days and re-supplied with 200 µM ammonium for 12 h (3d -N+12h NH_4^+), N-starved for 6 days (6d-N) or supplied with daily ammonium pulses at 200 µM for 2 h (6d-N+pulses). Bars indicate means± SD, n= 4.

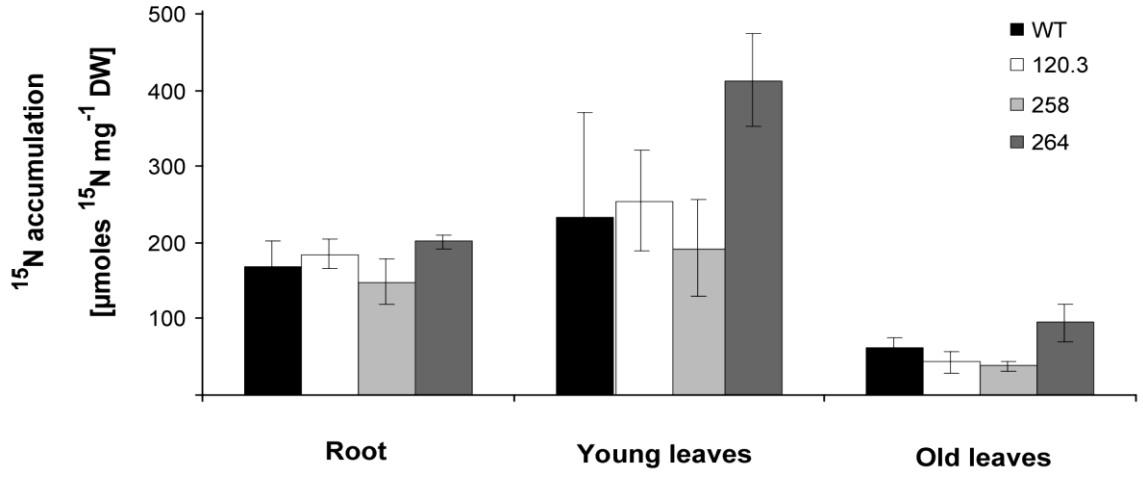

Figure 4.4-14 ^{15}N accumulation in roots, young and old leaves of wild-type (WT) tomato plants and of three independent transgenic lines transformed with *LeAMT1;2* in antisense orientation (120.3, 258, 264) after pulse-labelling with ^{15}N-ammonium

Plants were pre-cultured for 4 weeks hydroponically with 2 mM NH_4NO_3, N-starved for 3 days and then supplied with 200 µM ammonium in pulses of 2 h during 3 days. Bars indicate means± SD, n= 4.

Taken the results from the physiological characterization of *LeAMT1;2* gene together, the antisense repression of the *LeAMT1;2* lines was strong at mRNA levels. However, inconsistent differences between the independent 3 antisense lines could be observed.

The antisense repression of the 3 transgenic lines did not show in general any effect on chlorophyll concentrations, and ammonium, glutamine and glutamate concentrations in roots. However, under 3 d of N starvation they showed lower ammonium concentrations in roots and lower chlorophyll concentration in old leaves than the WT. And furthermore, a higher aspartate concentration was observed in these *LeAMT1;2* antisense lines under N-sufficient and N-deficient conditions compared to Wt.

5. DISCUSSION

5.1. Contribution of non-symbiotic rhizobacteria to nitrogen nutrition

Rhizosphere diazotrophs have already been isolated in the 1960-1970s but their contribution to the nitrogen nutrition of plants remains under debate. Unlike the *Rhizobium*-legume symbiosis, associative diazotrophs contribute to the N nutrition of their hosts to a relatively small extent. Using ^{15}N isotope dilution, it has been reported that some Brazilian sugarcane varieties can obtain up to 60-80% of their N through BNF (Lima *et al*., 1987), although the amount of fixed N is highly variable and dependent on the plant genotype and environmental conditions (Boddey *et al*., 1991). In rice, the estimated amount of fixed N_2 ranged from 1.5 to 21% (Shrestha & Ladha, 1996). In association with the rhizosphere bacterium *Burkholderia brasilensis,* BNF contributed by 11-35% to the N nutrition of rice plants grown under axenic or greenhouse conditions (Baldani *et al*., 2000). In this association, the yield increase was also shown to depend on the plant genotype (0-54% yield increment depending on the variety; Guimaraes *et al*. (2002) cited in Baldani & Baldani, 2005). The graminaceous species *Brachiaria humidicola* and *B. decumbens* have been estimated to obtain 30 and 40%, respectively, of their N from N_2 fixation, which is equivalent to an input of 30 and 45 kg N ha^{-1} $year^{-1}$ respectively (Boddey & Victoria, 1986b). However, in maize none of the endophytic non-symbiotic N_2-fixing strains tested so far were capable of alleviating the N-deficiency symptoms of unfertilised maize grown in the field or in the greenhouse (Riggs *et al*., 2001).

In the present study, which used wheat as a target crop species, there was no significant improvement of plant N acquisition in response to inoculation with different diazotrophs (Figs. 4.1-12; 4.1-13; 4.1-15; 4.1-16; 4.1-21; 4.1-22; 4.1-24; 4.1-26). As determined by the $^{15}N/^{14}N$ ratio, the ^{15}N isotop was diluted by the ^{14}N mineralized from the soil, organic matter and residual or mineralized N must have contributed to the total N uptake at all fertilizations levels (Figs. 4.1-12; 4.1-13; 4.1-24). The higher the N fertilization level, the smaller was the dilution by the ^{15}N-labelled N fertilizer (Figs. 4.1-12; 4.1-13). This observation suggests that the determination of the $^{15}N/^{14}N$ ratio had the potential to indicate a contribution of

plant N uptake via BNF in the present pot experiments. However, no differences were observed between inoculated and non-inoculated treatments. Therefore, no contribution of BNF by the tested diazotrophs to plant N nutrition could be found in this study.

Effect of soil N availability

In most cases, the application of ammonium and nitrate inhibits nitrogenase activity and therefore the BNF (Martin *et al*., 1989; Alexander & Zuberer, 1988). As intensive agricultural plant production relies on large amounts of N fertilizer input, it is not unexpected that growth stimulation by PGPR might often be suppressed. Plant growth promotion and increases in yield by *Azospirillum* inoculation have been demonstrated in subtropical and tropical latitudes but in moderate climates positive reports are rare. When positive effects have been observed in temperate regions, they were mainly restricted to low to intermediate N fertilization levels (Dobbelaere *et al*., 2001). In the present thesis, however, a particular focus was placed on the investigation of non-symbiotic N_2 fixation under conditions of moderate to high N fertilizer supply.

The apparent nitrogen mineralization of the Filderlehm soil in non-planted pots generally showed high amounts of available N, in part due to the absence of plant uptake (Appendix 1). The differences in the amounts of mineralized N most likely depended on the temperature of the period after which the soil samples were taken (Table 3.1-2). The apparent nitrogen mineralization of the Filderlehm soil in planted pots indicated a relatively low amount of N being mobilised from the soil. At the tillering stage, 12 mg N kg^{-1} soil were mobilized when no fertilizer was added; after supply of 30 mg N kg^{-1} soil only 1 mg N kg^{-1} soil was determined in the soil solution. The mineralization and immobilization of N appeared to be balanced. At the highest N level (90 mg N kg^{-1} soil) negative values were obtained in the balance indicating an immobilization of N in the soil. At the heading stage, approx. 20 mg N kg^{-1} soil were mobilized. With increasing N fertilization, higher amounts of mobilised N were found. At the ripening stage, the levels of mobilized N were comparable to the levels at heading stage. Therefore, the plants extracted all potential available N from the soil apparently already at the heading stage (Appendix 2).

A wide range of N fertilization levels (0, 15, 30, 36, 72, 60 and 90 mg N kg^{-1} soil) were tested to find an adequate N supply at which the inoculated bacteria could promote plant growth in the pot system. Finally, the minimal level of N fertilization, which still allowed the plant to grow until maturity was chosen (30 mg N kg^{-1} soil).

In this work in general, no significant effects of the inoculated bacteria were observed on plant growth, as determined by shoot and root dry weights. As expected, differences in plant growth and grain yield were found among the different N fertilization levels. Almost all yield and N measurements at high N fertilization levels (72 or 90 mg N kg^{-1} soil) showed higher values as compared to the measures at low N levels (30 or 36 mg N kg^{-1} soil). Nitrogen concentration in grains and shoots were highest at the highest N fertilization level and surprinsingly significantly higher when no N was added compared to intermediate N supply (Fig. 4.1-15 A,B). The plants grown at an intermediate N level produced more aerial biomass (Fig. 4.1-7 A,B), which led to a dilution of the assimilated N. The N concentration in grains at all N fertilization levels (0, 36 and 72 mg N kg^{-1} soil) found in plant material harvested at maturity (EC92) was around 1.0-1.8% (Figs. 4.1-11; 4.1-15 A; 4.1-21). These values suggest that the plants were grown under sub-optimal N conditions, because N concentrations were below a threshold of 2.5% as recommended by Bergmann and Neubert (1976) for spring wheat. Nevertheless, this observation may indicate that the pot-soil system and fertilization regime used was adequate to cause N deficiency in plants when harvested at maturity. On the other hand, at the 6-leaves stage (EC29), the N concentration of shoots when plants were fertilized with 30 mg N kg^{-1} soil was >3.4% (Fig. 4.1-26) being within the recommended range (3-4.3%) by Bergmann and Neubert (1976) for spring wheat. Moreover, no differences were observed in the plant biomass (Fig. 4.1-25) and the N concentration (Fig. 4.1-26) in shoots and roots of plants grown under 30 or 200 mg N kg^{-1} soil. These observations imply that the plants harvested at early growth stages did not yet suffer from N deficiency.

Despite the fact that a contribution of N_2 fixation to N nutrition has been shown in soils with low N fertilization levels (Reynders & Vlassak, 1982), there are also reports on inoculation responses at medium or high levels of N fertilization in maize, sorghum, forage grasses and wheat (Boddey *et al*., 1986a). It is probably

due to the fact that plants need a certain N amount as a starter dressing for initial development before the association takes place. Generally, plants grown under N-deficient conditions have a higher root/shoot ratio (R/S) and therefore they can better cope with drought. Similar R/S ratios have been found in plants grown without added N fertilization and at 36 mg N kg^{-1} soil, especially in the cv. Atir (Fig. 4.1-7 B,C). These results are consistent with the results obtained in the mineralisation trial, where the wheat plants could utilize all potentially available N from the soil already at the heading stage (Appendix 2), indicating that the soil used in the pot system does not supply high levels of N and therefore is suitable for N_2 fixation studies.

For the use of the ^{15}N dilution method two prerequisites should be assumed, besides a homogenous spatial distribution of the ^{15}N labelled fertilizer in the soil and a homogenous temporal distribution of the N source. The soils used in this thesis were labelled with ^{15}N fertilizer prior to sowing. The isotope was homogeneously mixed in the soil before filling the pots, but it was not temporally stabilized. The ^{15}N-labelled added to the soil inorganic N pool was continuously replenished by unlabelled N from the mineralization of the soil organic matter. The N mineralization process depended on the plant growth stage (Appendix 1 and 2) and appeared to correlate with the air temperature. The earlier the N uptake by plants, the higher was the ^{15}N enrichment in the N fraction of the soil. At early stages, the "N_2-fixing" plant and its uninoculated control could have different patterns of $^{15}N/^{14}N$ uptake from the soil, and consequently should obtain different ^{15}N enrichments in their biomass (Boddey *et al*., 1995). However, in most of the pot experiments, , plants were harvest at maturity and therefore, possible initial differences in growth between the crop and the reference plant were most probably diluted. Some authors recommend using different species as reference plants in the same experiment to mitigate the problem of the temporal distribution of ^{15}N. In the present study, only one reference plant has been used, from the same species and the same variety as the test crop but inoculated with an autoclaved inoculum as suggested by Heuwinkel (1999).

5.2. Factors influencing plant growth promotion by rhizobacteria

Azospirillum brasilense is a rhizosphere diazotroph that has often been reported to improve yield when inoculated on wheat plants. However, the growth promotion obtained by the *Azospirillum* inoculation is not consistent, only 60–70% of the field inoculations over a 20-year period produced significant increases in yield in a range of 5-30% (Okon & Labandera-Gonzalez, 1994). The mechanisms by which PGPR exert their positive effects remain unclear (Mantelin *et al*., 2006). In this context, I would like to mention the concept of “the right set of circumstances” invoked by Uren (2007). It explains that only very few root secretions can be expected to be effective in the rhizosphere unless the right set of circumstances occurs at the right time, sufficiently and frequently. The right set of circumstances may have relevance in hostile situations, when other types of root exudates become phytoactive. In the present thesis, a few circumstances have been varied that might play a role in the establishment and efficiency of PGPR.

Soil type

In the context of the EU-MicroNfix project, in which the present thesis was involved, a field trial in Israel conducted by one of the partners (Agron Ltd., www.agron.co.il) showed significant plant growth promotion of wheat plants (Table 5.2-1) when they were inoculated with the diazotroph *Paenibacillus polymyxa* in the absence of fertilizer. Following these promising field trials, one aim of this study was to reproduce these positive effects in the greenhouse experiments using the same spring wheat cultivar (Atir) and the same soil as used in Israel. Additionaly, another wheat cultivar (Paragon) and a German agricultural soil from South Germany (Stuttgart) were included.

In the present study, inoculation with *P. polymyxa* led in some cases to a weak positive promotion of plant growth. At 36 mg N kg^{-1} soil, inoculated cv. Atir plants with *P. polymyxa* showed a slight increase in the relative chlorophyll density at the tillering stage (Fig. 4.1-14). These observations were consistent with the field studies from Israel, where differences in the greenness values at early stages were observed (Table 5.2-1).

Table 5.2-1 Plant height, visual assessment and grain yield of wheat plants cv. Atir in dependence of bacterial inoculation and nitrogen fertilization in a field trial in Israel conducted by Agron Ltd. Different letters represent significant differences of the means.

Treatments	Visual assessment (1-5 scale)	Plant height (cm)	Grain yield (kg ha^{-1})
Control	2	76 D	5044
Inoculated (*P. polymyxa*)	3	82 C	5528
40 kg N ha^{-1}	4	82 C	5908
40 kg N ha^{-1}+ *P.p*.	4	85 B	5950
80 kg N ha^{-1}	4	83 BC	5989
80 kg N ha^{-1} + *P.p*.	5	88 A	6102

Most of the positive reports on inoculations have been made in Brazil, where there are mainly acidic soils. On the other hand, the two agricultural soils tested (Luvisols from Germany and Israel) have basic pH. This might have influenced the growth and establishment of the tested bacteria. Furthermore, the success of *A. brasilense* in Brazilian soils could also be due to the natural population of *Azospirillum*, which might decrease a need for competitive displacement (Boddey *et al*., 1986a). In contrast, regardless of soil texture, the number of inoculated *A. brasilense* bacteria in temperate regions has often been reduced by half already after a couple of weeks from the inoculation time (J. Vanderleyden, personal communication). It has been also reported that *Azospirillum* survives poorly in soil in the absence of a carrier material, such as alginate beads or peat (Bashan & Levanony, 1990).

Soil treatment

Soil sterilization was used to investigate a possible positive/negative influence of the native soil microbial flora on the inoculated diazotrophs and on the plants. Indeed, inoculation of sterilized soils led to different effects than inoculation of non-sterilized soils.

When Graminante®, a biofertilizer containing *Azospirillum brasilense*, was used as inoculum at high N fertilization rate, a higher number of spikes was observed in sterilized soil compared to the control (Fig. 4.1-16). By contrast, inoculation with *A. brasilense* Sp7 (10^5 CFU mL^{-1}) showed no or even an inhibitory effect (Figs. 4.1-8, 4.1-16). However, differences were small and not consistent between experiments (Appendix 4). Regardless the inoculation, a higher grain yield, shoot and root biomass (Figs. 4.1-5 A,B; 4.1-6), a higher number of spikelets per ear (Fig. 4.1-10) and a lower $^{15}N/^{14}N$ ratio (Fig. 4.1-12) were observed in plants grown on sterilized soils compared to those on non-sterilized soils. These results are consistent with results from the literature, where higher biomass and N concentrations were found in wheat plants grown in sterilized soil compared to non-sterilized soil (Saubidet *et al*., 2002). Similarly, Boddey *et al*. (1986a) found that all the tested *Azospirillum* strains (including Sp7 and isolated endophytic strains from wheat) produced similar increases in grain yield and N content in field experiments using sterilized soil, but the response to the strain Sp7 in non-sterilized soil was much smaller. It has been proposed that soil sterilization or using soil-less potting media often lead to plant growth promotion because there is a lack of competition from the indigenous soil microflora (Lugtenberg *et al*., 2001).

Several methods are currently in use for soil sterilization. It has been reported that heat sterilization (e.g. autoclaving) and chemical sterilization methods might alter the soil structure, lowering the proportion of aggregates (Utomo & Dexter, 1982) and increasing the plant availability of nutrients such as N, P and toxic elements (Johnson & Curl, 1972). By contrast, the γ-irradiation of soils seems to have only little impact on soil structure (Johnson & Curl, 1972). The general positive effects observed in the present study might be due to an enhanced acquisition of nutrients. Troelstra *et al*. (2001) observed that in γ-irradiated calcareous dune sands, P availability was significantly enhanced. In contrast, our analysis of γ-sterilized vs. non-sterilized soils did not show any significant differences (Appendix 3). In the performed experiments, it turned out that not the inoculation, but the

sterilization process was the most important factor influencing positively plant growth. Therefore, an absence of soil-borne pathogens or microbial nutrient immobilization in sterilized soil might have led to these positive effects in plant growth and development. Furthermore, less clear results from this and other studies, might be also attributed to the fact that the controls were not free of native N_2-fixing microorganisms.

The volume of soil for exploration by the plants is another critical factor that needs to be considered in PGPR-related studies. Roots from plants grown in the field usually have sufficient space to extend, for example for acquisition of nutrients and water. In the greenhouse experiments, however, relative small pots were used and the harvest of the plants was mostly at maturity. Considering that the root system of wheat plants can reach 1.5 m depth, the volume of soil available in the pot experiment might have been an important limiting factor. This is further supported by other experiments under the same greenhouse conditions, in which most of the positive effects of an inoculation were observed in deeper pots with a higher soil volume (Rubel, 2007).

Other several factors, not directly tested in this study, might also have played a role in plant growth promotion and contributed to the inconsistencies in the results. N_2 fixation by free-living diazotrophs in soils amended with straw may promote plant growth-promoting effects of rhizobacteria (Roper, 1983). An addition of straw to the soil has been found to stimulate nitrogenase activity, especially during the day in humid and warm soils. If soil moisture fell below field capacity, nitrogenase activity fell too. However, there is still a detectable enzyme activity at 50% field capacity (Roper, 1983). No straw was added in the present pot experiments and the soils had a very low content of organic matter (less than 1%). A reason for the success in the field experiments in Israel might have been in the remaining straw from the previous crop.

A common practice in greenhouse experiments and also in the pot experiments of this study, is sieving the soil through 2 mm mesh to obtain a more homogeneous structure and thereby to reduce soil structural variations among replica. At the same time, sieving alters the physical structure of the soil in a way that influences vertical water transport and oxygen diffusion. The structure of soil is important for bacteria, since bacteria are preferentially found in aggregates (Sessitsch *et al*., 2001) and in pre-existing biopores in which roots have grown before (Watt *et al*.,

2006). Additionally, soil aggregates are needed for capillary water flow and for a high water-holding capacity. Therefore, some positive effects of inoculants found in field conditions might not be reproducible under greenhouse conditions because of the loss of such structural features.

Besides this modification of the soil structure, it should also be taken into consideration that the lack of any PGP evidence could rely on the long-term storage of the soil before its use with a possibly modified natural population in regard to bacteria, protozoa, fungi and mycorrhiza that might still be present in fresh field soil.

Basal fertilization levels

In this study, growth conditions were slightly varied between experiments. The German Luvisol (Filderlehm) obtained a basal macronutrient fertilization to support growth, P (100 mg P kg^{-1} soil), K (150 mg K kg^{-1} soil) and Mg (50 mg Mg kg^{-1} soil). However, in the last 2 trials this fertilization was omitted to create less favourable growth conditions. The lack of basal fertilization led to a growth reduction by more than half of the produced total biomass (Figs. 4.1-5; 4.1-7). This suggests that the applied nutrient stress was too radical to sustain normal plant development. Indeed, in recent experiments, using the same soil system and the same wheat cultivars have shown that plants suffered from P-deficiency (0.15%), while they did not suffer from Mg (0.19%) or K (4%) deficiency (Rubel, 2007). These results have been compared with the normal nutrient requirements (0.32% P, >2.5% Mg, 2% K) proposed by Martin-Prével *et al*. (1984). Furthermore, the plants in this work received a sevenfold lower P supply compared to those from the work of Dobbelaere (2002), who obtained significant positive inoculation effects. This indicates again that P deficiency could be one of the main factors contributing to a lack of plant growth stimulation.

Plant genotype

It has been described that plant genotype has a strong impact on plant/bacteria association (Baldani & Baldani, 2005). Inoculation studies with diazotrophs have demonstrated that endophytic and homologous strains, isolated from the roots of the same crop to which they were re-inoculated, have a higher BNF potential than

heterologous associative diazotrophs (e.g. *A. brasilense* Sp7 in wheat) (Boddey *et al*., 1986a; Mostajeran *et al*., 2007; Baldani & Baldani, 2005).

In the present study, two different spring wheat genotypes (cv. Paragon and cv. Atir) with different provenience and behaviour were investigated. When no N was added, higher shoot DW (Fig. 4.1-7 B), higher plant height at harvest (Fig. 4.1-17) and higher N concentrations in grains and shoots (Fig. 4.1-15 A,B) were observed in cv. Paragon compared to cv. Atir. On the other hand, at the highest N fertilization level, higher N concentrations in shoots and roots (Fig. 4.1-15 B,C) were observed in cv. Atir.

In general, at any of the N levels tested, no significant effects were observed of inoculation with *P. polymyxa* on both cultivars. Only under moderate N supply (36 mg N kg^{-1} soil) inoculated cv. Atir plants, showed significantly higher SPAD values at the tillering stage relative to non-inoculated plants (Fig. 4.1-14).

These results resemble those obtained in the field trials in Israel with the same wheat cultivar, where inoculated plots appeared to be greener at the early stages of plant growth as assessed by visual scoring (U. Rosenberg, personal communication). Also at early stages, El Zemrany *et al*. (2007) found plant growth promotion effects in maize plants when inoculated with *A. brasilense.*

Bacteria strain

As mentioned above, homologous strains usually lead to better plant growth than non-homologous strains. In the present work, different diazotroph strains have been tested, which probably represented heterologous strains. Only 2 bacterial inoculants yielded positive effects. The inoculation with Graminante® at the highest N level in sterilized soil showed a significant positive effect on the number of spikes (Fig. 4.1-16) and the inoculation with *P. polymyxa* at an intermediate N level (36 mg N kg^{-1} soil) led to marginally higher SPAD values by 5% relative to non-inoculated plants (Fig. 4.1-14). *Azospirillum brasilense* Sp7 was used as a positive control in the first trials. However, this strain showed no positive effects on plant growth. It was realized only later that *A. brasilense* Sp7 is not an endophytic strain of wheat, such as Sp245, it was initially isolated from *Digitaria decumbens.* Likewise, plant growth promotion effects under field conditions have been reported in wheat plants inoculated with endophytic strains, such as Sp245, while

rhizospheric strains, such as Sp7, showed no effects (Baldani *et al*., 1986; Rothballer *et al*., 2005).

These results suggest that *A. brasilense* Sp245 might have been a more appropriate strain than Sp7 for the inoculation pot experiments with wheat.

Inoculation conditions: Timing

The success of inoculation responses has been reported to depend on the plant growth stage. In wheat a stimulatory effect has been reported during early vegetative growth, especially during tillering (Reynders & Vlassak, 1982). Similarly, it has been shown that inoculation of wheat seeds with *A. brasilense* Cd, at sowing or immediately after emergence, led to abundant root colonization, while colonization was reduced by 50% with each newly appearing leaf. At the 4 to 5-leaf stage most of the root system was no longer colonized (Bashan *et al*., 1986b). It has been also reported, that *Azospirillum* cells are totally dependent on the presence of roots as they survive poorly in some soils (Bashan & Levanony, 1990). Therefore, it is assumed that the plant growth stage chosen for the selected inoculation time in the present study (1 to 2-leaf stage) was not critical for the lack of plant growth promotion (PGP) by the tested diazotrophs.

Inoculation conditions: Concentration

Based on previous results showing that the inoculation density is a critical factor for PGP, different inoculum concentrations were also tested in the present study. When N was supplied at high levels, inoculation of wheat plants cv. Paragon with *A. brasilense* Sp7 at 10^5 and 10^6 CFU plant^{-1} concentrations led to a reduced grain yield (Fig. 4.1-6 A), a lower number of spikes per pot (Fig. 4.1-8) and lower relative chlorophyll values (Fig. 4.1-4). These inhibitory effects were not observed at lower N supply in any of the experiments and also at lower inoculation densities, suggesting that the bacteria caused a deleterious effect when the N_2 fixation ability of the bacteria was not needed. Similarly, it has been demonstrated in several studies that high bacterial inoculum densities, such as 10^9 CFU mL^{-1}, inhibit plant or root growth (Kapulnik *et al*., 1985; Dobbelaere, 2002); whereas a lower concentration of bacterial cells (10^5-10^6 CFU mL^{-1}) was apparently ineffective (Kapulnik *et al*., 1985). In contrast, wheat plants inoculated with *A. brasilense* at 10^7-10^8 CFU mL^{-1} showed an increased number and length of root hairs.

In sterilized soil, a lower number of spikes was observed when the plants were inoculated with *A. brasilense* at 10^5 CFU mL^{-1} compared to non-inoculated plants. However, the same or a lower inoculum density (10^3 CFU mL^{-1}) applied to non-sterilized soil did not show an inhibitory effect (Fig. 4.1-8). Furthermore, it should be noted that PGP by rhizobacteria can vary depending on the experimental system (Timmusk *et al*., 2005). For example, the optimal level of bacterial inoculation of wheat plants grown in hydroponics was found to be 10^5-10^6 CFU mL^{-1} for several bacterial strains, whereas concentrations such as 10^2-10^4 CFU mL^{-1} led to weaker positive effects, and 10^8 CFU mL^{-1} cells decreased the root surface area (Bashan *et al*., 1986b).

In the present experiments a large range of bacterial inoculation densities was used. However, in none of the treatments consistent PGP was observed. Thus, a failure of PGP as a consequence of inappropriate inoculation densities is considered to be unlikely.

Inoculation conditions: Quality

The lack of quality control in the inoculant provided was probably a major factor affecting the results of the first experiment. Only after the use of the inoculum, a relatively high level of contamination with unknown bacteria was found (J. Strauss, personal communication). In the subsequent experiments the inoculum quality was assured by strain identification and cell counting procedures. Despite these quality checks, other factors related to the quality of the inoculum could have played a role in the inconsistency of the results. For example, the growth phase in which the bacteria were harvested is a critical factor influencing the performance of the inoculated bacteria. It has been observed that bacteria can be more motile and chemotactic in the exponential growth phase rather than in the stationary phase (Adler, 1972). Bacterial movement correlates with the synthesis of flagella, which usually increase in number during the log phase, reach maximal values during the post-log phase and decrease thereafter (Amsler *et al*., 1993). Similarly, the adsorption kinetics of *A. brasilense* from the late exponential phase is higher during the first hours of contact with plants. On the other hand, bacterial cells from the stationary phase have been shown to have higher adsorption to the roots during the first 15-48 h of co-incubation (Yegorenkova *et al*., 2001). Consequently,

bacteria from the late exponential phase were harvested for preparation of the liquid inoculum.

Inoculation conditions: Delivery mode

In the literature, a large diversity in the mode of inoculum application have been reported. This diversity makes the comparison of inoculation experiments between and within field and greenhouse experiments almost impossible. Nevertheless, several studies have successfully applied liquid bacterial suspensions to seeds or young plantlets (Martinez Toledo *et al*., 1988; de Freitas *et al*., 1997). This technique has been used in the present study as well. Moreover, seed coating with inocula and application of encapsulated bacteria has been also used but without any success (Figs. 4.1-18 to 4.1-23).

Environmental conditions

After their release into the rhizosphere, root exudates are usually subjected to physical (sorption), chemical (metal oxidation) or biological (microbial degradation) processes. These processes are affected by environmental conditions and may alter PGP or BNF.

Drought stress often increases the soil mechanical impedance for plant roots, which, in turn, can stimulate root exudation (Groleau-Renaud *et al*., 1998). Contrarily, it has been demonstrated that in a certain range, the soil water content correlates positively with the nitrogenase activity (Martin *et al*., 1984), or with $^{15}N_2$ fixation in rice (Kondo & Yasuda, 2003). In this thesis, and in the master thesis of Weishaar (2007) and Rubel (2007) different water holding capacity capacities have been tested, from 60% down to 25% WHC. The last one represents the wilting point of wheat under our test conditions. This low WHC was meant to induce water stress and thereby a potentially stronger plant growth promoting effect by the inoculated bacteria under non-favourable conditions. However, no positive effects of the inoculation could be observed at any of the WHC tested. This result is consistent with observations made for *Digitaria decumbens*, which did not show any more nitrogenase activity at its wilting point (Abrantes *et al*., 1976). The experiments in this study have never been performed at the maximal WHC. Therefore, the absence of a continuous film of water might have affected the migration of our tested diazotrophs and the failure of the inoculation in our pot

experiments. Bashan *et al*. (1986a) found a correlation between migration distance and soil moisture. They tested an Israeli sandy soil, from the same area as our tested Luvisol, and the highest migration rate was found near the field capacity. Nevertheless, passive dispersion by water, especially in semiarid conditions with lack of sufficient water, cannot explain how the entire root system is colonized by *Azospirillum* cells (Bashan & Holguin, 1994). Furthermore, this factor might eliminate the potential effects of other factors, such as root exudates.

As a large proportion of the carbon released into the rhizosphere is derived from photosynthesis, changes in light intensity or quality are likely to modify the intensity of root exudation. It has been reported, that under low light conditions, legumes show less exudation, with a reduction in the BNF (Siddique & Bal, 1991), while non-leguminous plants like tomato were affected in amino acid exudation into the rhizosphere (Rovira, 1959). Moreover, high light intensities have been reported to stimulate root exudation such as the release of phytosiderophores by barley and wheat (Cakmak *et al*., 1998). Wood *et al*. (2001) reported an increase in BNF when malate was added to an axenic wheat-*Azospirillum* co-culture in nutrient solution.

The different pot experiments in the present study had to be performed in different seasons and even in different greenhouses varying in their technical equipment to control growth conditions. In particular, the plant root systems went through different temperature regimes. Except when the greenhouse was shaded, the dark coloured pots were heated up sometimes to above air temperature. To what extent the soil temperature might have contributed to a lacking PGP by the inoculated bacteria remains unclear. By contrast, greenhouse trials performed in big basins at the EMBRAPA Institute in Rio de Janeiro (Brazil) showed positive results of an inoculation of wheat with some of the strains used in the present thesis (V. Reis, personal communication). Even though Bertin *et al*. (2003) reported an increase in root exudation under stress conditions, such as extreme temperature, drought or UV exposure, which might potentially lead to a higher C availability for the rhizosphere bacteria. No improvement of non-symbiotic N_2 fixation could be observed in the present study by subjecting wheat plants to stress conditions, indicating that the applied stress conditions were too drastic for the plants growing in the established pot system.

A major problem of the present study was the uncertainty of working with a functional plant-bacterial association. As the compatibility between the plant genotype and the bacterial strain must be considered of major importance, future greenhouse experiments in similar pot systems should consider the use of a well-studied association as a control. As soon as a functional wheat-bacterial association is established, comparative studies with different wheat cultivars could help to identify those wheat genotypes that might profit most from the application of biofertilizers. Based on the results obtained by the EU Micro-N-Fix project, in which this thesis was involved, the RHIBAC project (http://impascience.eu/rhibac/) goes along these lines by testing different European wheat genotypes and bacteria strains under temperate and tropical conditions for their potential to increase BNF and/ or N fertilization use efficiency under field conditions. Furthermore, plant genotypes and soil from Brazil with basal fertilization should be tested under temperate conditions.

The intimate relationship between endophytic bacteria and their hosts could make them more suitable as PGPR, because besides the physical protection against the influences in the rhizosphere, they have direct access to the C source and they do not have any competition of microorganisms from the rhizosphere. This would also avoid the problem of selecting bacteria that display high rhizosphere competence, which is often considered obligatory for successful root colonization. As Baldani & Baldani (2005) suggested, more studies should be conducted on the endophytic association to optimize the BNF potential.

The temperature of the root system can be of high importance influencing all the rhizosphere processes. Currently, several investigations with biofertilizers tested under controlled root temperature are perfomed with promising results (Yusran *et al*., 2008). Therefore, several experiments testing different soil temperatures should be conducted, as well as testing different pot volumes, which indirectly has an influence on soil temperature.

5.3. Bacterial responses to root exudates

As no established protocols are available for screening the effects of seed or root exudates on bacterial motility, an aim of this thesis was to set up a protocol for testing bacterial migration. Many of the preliminary motility assays showed large standard deviations, which only allowed commenting on tendencies. The results from experiments on bacterial motility on semisolid media depended strongly on the experimental conditions and the inoculation method. The main factor affecting motility in soil as well as in agar-plates was moisture. Therefore, the final assays were performed in a humidified chamber resulting in smaller standard deviations. Surface moisture is a very important factor for bacterial motility. Bacteria produce surfactants and wetting agents by themselves, as for instance lipopolysaccharides (Toguchi *et al*., 2000). In this context, it is interesting to note that a new role for the bacterial flagellum has been demonstrated (Wang *et al*., 2005). It can sense external wetness to regulate its own biogenesis.

Root exudates contain signaling compounds that allow Rhizobia to find their host plants (Ferguson & Mathesius, 2003). Similarly, root exudates might be considered to play a similar role in associations between plant roots and non-symbiotic rhizobacteria. Therefore, a dual approach has been chosen. Root exudates were collected and analyzed for their composition of organic acids, which are a major fraction of compounds with chemotactic activity in root exudates (Jones *et al*., 2003) and in a subsequent step these exudates were tested for their potential to promote the migration of different rhizobacteria.

In the present study, the collection of exudates took place after 4, 6 and 8 days after sowing, corresponding to 0 d, 2 d and 4 d of N starvation. It has been reported that colonization of plant roots often takes place at the 1-leaf stage (Bashan *et al*., 1986b). Therefore, early plant developmental stages have been considered for harvesting root exudates. Furthermore, Bacilio *et al*. (2003) have found that the highest concentration and diversity of amino acids and carbohydrates in rice root exudates occurred during the first 2 weeks after seeding.

The motility assay was first tested with standard substances. The substances were chosen according to the substances that are present in the exudates of the plants used and which are known to play a role in microbial interactions (e.g. malic and malonic acid with Rhizobia) or are reported to have antibiotic effects (e.g. malonic and aconitic acid). In a second step, the exudates were tested and finally analysed. There was no more time left within the time frame of this thesis for further assays (e.g. testing lactic acid). Unfortunately, the amount of root and seed exudates collected for HPLC analysis (Fig. 4.2-1) was too low to conduct the chemotaxis experiments. Therefore, additional batches of root and seed exudates were collected, presuming that their composition remained similar to those analysed.

5.3.1 Effect of exudates on surface migration

The quantity and quality of root exudates depends on the plant genotype, age, stress factors (nutrient deficiency, water stress), light intensity, time of collection and temperature (Neumann & Römheld, 2007). Therefore, when some of these factors change, results in the bacterial motility may also change. Bacterial motility on semisolid media strongly depends on experimental conditions, such as humidity (Toguchi *et al*., 2000; Wang *et al*., 2005), temperature (Matsuyama *et al*., 1986), medium composition (Brown & Hase, 2001; Mattick, 2002; Harshey, 2003), agar quality (Brown & Hase, 2001; (Harshey & Matsuyama, 1994; Toguchi *et al*., 2000; Kirov *et al*., 2002) and agar concentration (Kirov *et al*., 2002; Rauprich *et al*., 1996; Brown & Hase, 2001; Toguchi *et al*., 2000). Furthermore, bacterial motility depends on inoculation parameters, e.g. bacterial density (Toguchi *et al*., 2000; Brencic & Winans, 2005), bacterial growth phase (Adler, 1972; Senesi *et al*., 2002; Walker *et al*., 1997; Yegorenkova *et al*., 2001; Castro-Sowinski *et al*., 2006) and colony phase (Li *et al*., 2007; Achouak, 2007); (Givaudan *et al*., 1995; Deziel *et al*., 2001; Vesper, 1987).

Bacterial motility is required as an initial step in the rhizosphere colonization. Therefore, an aim of this thesis was to set up a reproducible protocol for studying the effect of the exudates on bacterial motility and EPS production.

Root exudates may act as attractants in the colonization process, but also as a source of nutrients for the rhizosphere microorganisms (Campbell & Greaves, 1990). Therefore, a particular low amount of 1.5 µg C was supplied with a paper disc in the motility experiments (chapter 4.3). This represents a negligible amount of carbon compared to the 6000 µg of soluble C in the agar medium.

The chemotactic responses may depend on the attractant concentrations as it was already shown by *A. brasilense* (Reinhold *et al*., 1985). This additional C supply was in the concentration range generally found for LMW compounds in the rhizosphere (1-100 µM) (Jones, 1998).

An interesting observation was made during the analysis of root and seed exudates. There was a considerable change in the composition of wheat exudates when collected from seeds or roots (Fig. 4.2-1). While succinic, malic and citric acid dominated in seed exudates, lactic acid dominated in young seedlings and lactic acid together with malic and succinic acid in 4 d-old seedlings. This lactic acid exudation from roots was repressed under N-sufficient conditions. Likewise, lactic acid was also dominant in seed and root exudates of maize and tended to be more dominant under N-deficient culture. Lactic acid is a product of fermentation processes; the concentration of lactic acid is increasing during the early stages of germination due to a still inactive respiratory metabolism (Sherwin & Simon, 1969). This is in agreement with the changes of lactic acid concentration in seed and root exudates in the present investigation (Fig. 4.2-1). Moreover, lactic acid is also produced under stress conditions (Zaidi *et al*., 2003). In other studies, lactic acid has also been found in root exudates of maize (Baudoin *et al*., 2003) and other plants (Ström *et al*., 1994; Cawthray, 2003).
In preliminary experiments (data not shown) some bacteria (e.g. *Brevibacillus reuszeri* and *Paenibacillus polymyxa*) were attracted by exudates of maize and wheat but not or to a much lower extent by exudates from tomato. A particularly high motility was found for *Brevibacillus reuszeri* and *Paenibacillus polymyxa*, although the latter only showed motility (migration in all directions) but no chemotaxis (specific attraction to any of the exudates tested). On the other hand, *Azospirillum brasilense* Sp7 showed no motility at all. Since *B. reuszeri* showed

motility in the presence of all tested exudates, this strain was used as a model organism for subsequent experiments.

Among the organic acids that were used as standard substances in the motility assays, trans-aconitic acid was chosen because of its specific presence in maize (Fig. 4.2-1). It has also been reported to have allelopathic effects (Voll *et al*., 2005). Malic acid was chosen as it is exuded in wheat (Fig. 4.2-1) and highly exuded in beans, particularly under N starvation (Haase, 2008). Malonic acid was selected because of its high abundance in the exudates of bean and its possible involvement in nodule formation (Haase *et al*., 2007; Stumpf & Burris, 1981). Moreover, malonic acid has been found in large quantities in root exudates from N-starved plants of the same bean cultivar as used in this study (Haase, 2008). The organic acids tested in this study (particularly malonic and trans-aconitic acid) caused a higher motility than water or glucose. Similarly to these results, Alexander *et al*. (2000) demonstrated that organic acids such as malic, succinic, oxaloacetatic, fumaric acid, but also fructose and nitrate strongly attracted *A. brasilense*, whereas glucose was not a chemoeffector. Moreover, it has been shown that the MotPS-dependent motility, which drives flagellar movement, is greater on malate- than on the glucose-containing plates in *B. subtilis* (Ito *et al*., 2004). Although malic acid is a well-known chemo-attractant for diazotrophic bacteria, such as *A. brasilense* Cd (Reinhold *et al*., 1985), this study suggested that the concentration of malic acid present in the exudates was negatively correlated with the motility of the bacterial cells. This effect has been observed especially in older wheat seedlings, where the exudates from N-deficient plants had no malic acid and caused a high bacterial motility (Fig. 4.2-1; 4.3-3 B). These results may indicate that malic acid does not play a crucial role in regulating the motility in the *B. reuszeri*-wheat interaction and that other substance/s present in wheat exudates from plants grown under N starvation are responsible for the increase in bacterial motility. Likewise, Vande Broek *et al*. (1998) found that the presence of malate appeared to have no effect on the specific migration of *Azospirillum brasilense* towards the wheat root hair zone.

Glucose has been chosen as a control as it represents a readily available and utilizable C source for *Brevibacillus reuszeri* (Petrie *et al*., 2003). It was expected

that it would influence positively its motility, at least more than water. However, in this study, *Brevibacillus reuszeri* shows a very low motility in the presence of glucose (Fig. 4.3-2). It has been reported that sugars (fructose and glucose) cause more flocculation of *A. lipoferum* than the organic acids (Sadasivan & Neyra, 1985). Although no aggregates were visible at the macroscopic level, cell aggregation may have occurred and interfered in the motility as it has been observed many times in previous experiments (Appendix 5). Similarly, it has been reported that these sugars showed no effect on the motility of *A. brasilense* in semisolid agar plates (Barak *et al*., 1983) or towards the root hair zone (Vande Broek *et al*., 1998). Barak *et al*. (1983) observed a motility ring in plates containing other sugars, like arabinose and galactose or organic acids and amino acids.

There are several reports describing a species- and strain specific attraction of root exudates. For example, *A. lipoferum* was attracted by the mucilage of maize root tips, whereas *A. brasilense* Sp7 and strains isolated from rice were less or not attracted (Mandimba *et al*., 1986). Similarly, it has been reported that a strain isolated from wheat was attracted by sugars and amino acids, in contrast to the strains isolated from C_4 plants that were attracted by organic acids (Reinhold *et al*., 1985). Moreover, L-malic acid has been shown to have a specific chemotactic role in attracting the PGPR strain *Bacillus subtilis* FB17, whereas 6 different bacterial species, including *Azospirillum*, failed to respond to this organic acid (Rudrappa *et al*., 2008). In this study, *Brevibacillus reuszeri* showed the most pronounced motility in the presence of exudates from wheat, followed by maize and no effects have been observed in the presence of bean exudates (Fig. 4.3-3). It has been reported that strains have a higher affinity to their original host plants, allowing them to be assigned as homologous strains (Boddey & Döbereiner, 1988). The *B. reuszeri* strain used in this study has been isolated from roots of grasses growing on a golf lawn. This might explain why the most pronounced effects have been observed with exudates from wheat and maize, whereas the exudates from a dicot plant (bean) had no influence on the motility of *B. reuszeri*. Although it has been observed that wheat seedling exudates stimulated the swarming of *A. brasilense* Sp245 (Borisov *et al*., 2007), *A. brasilense* Sp7 did not show any response to root exudates in the preliminary agar plate experiments (data not shown). The lack of response of *A. brasilense* Sp7 has been also

observed in pot experiments (chapter 4.1.4). The composition of root exudates might change considerably not only with the plant species and growth conditions but also with the cultivar or genotype. Therefore, a more extended analysis is currently ongoing in the lab, in which different wheat and barley cultivars are examined for their composition of the root exudates under N-sufficient and N-deficient growth conditions.

The clearest effects on an enhanced motility were observed in the presence of exudates from wheat seedlings grown for 4 d under N starvation (Fig. 4.3-3 B). Bashan et al. (1986a) found that *A. brasilense* migration was higher towards wheat seedlings than towards non-planted soil, probably because the bacteria migrated preferentially towards an amino acid source. It might therefore be of interest to verify the composition of amino acids in the exudates of these experiments. Similarly, Haase (2008) found a higher microbial biomass (in nodules) of bean grown under N starvation. In the present study no differential mobility was observed in bean exudates in dependence of the N treatment, probably because bean seeds use more proteins as storage compounds than wheat and maize seeds, so that bean plants were not N deficient after 4 d of N deficiency treatment. The collection of bean exudates should therefore be repeated with older plants. Taken together, the effect of differential N regimes on bacterial motility is obvious, even when the treatment is applied shortly. Differential motility of *B. reuszeri* could be observed already after 2 d of treatment.

5.3.2 Effect of exopolysaccharides production on surface motility

The motility of *B. reuszeri* in the presence of glucose was significantly lower than in the presence of organic acids (Fig. 4.3-2 A). It has been reported that sugars (fructose and glucose) cause flocculation of *Azospirillum* cells and provoke the formation of a thick layer of EPS and granules of PHB in the cells (Sadasivan & Neyra, 1985), which has been reported to prevent chemotaxis to any chemoattractant (Gladys *et al*., 2000). There were no differences in the fluorescence intensity of *B. reuszeri* in the presence of water, glucose or organic acids (Fig. 4.3-2 B), only a dilution effect arising from the EPS content of the inoculum in the central spot was observed. It has been reported that the total

amount of EPS produced seems to be strongly influenced by the sugar available in the medium (Ruas-Madiedo & de los Reyes-Gavilan, 2005). In this study, a commercial medium (R2A agar from Oxoid) was used and unfortunately no exact data on its C-composition was available. Besides the C-composition, also the culture conditions and the composition of the culture medium have been reported to influence the EPS yield (Ruas-Madiedo & de los Reyes-Gavilan, 2005).

In this study, no visual differences were observed in the bacterial production of EPS, as detected by the intensity of Calcofluor-White staining, when standard substances (Fig. 4.3-2 B) or root exudates from different N regimes were added (Fig. 4.3-4). This goes along with the observation that in *A. brasilense*, no influence of Calcofluor-binding polysaccharides on primary wheat root colonization was found (Borisov *et al*., 2007; Vande Broek *et al*., 1998). In general, there was no correlation between the motility of *B. reuszeri* grown on semisolid media and its fluorescence intensity or pattern formation (Figs. 4.3-2 to 4.3-4). However, a coincidence was observed visually between the motility of *B. reuszeri* and the intensity of the EPS staining in the presence of root exudates from 8 d old wheat or maize seedlings (4 d after N starvation; Fig. 4.3-3 B,C; 4.3-4 B,C). This coincidence may indicate that some compounds in the exudates could have affected the motility of *B. reuszeri*, for example when acting as a wetting agent. It has been reported that the capacity to colonize the rhizosphere of a host plant may be modified by several components of root exudates, and some of these may induce modifications in the structure of cell-surface polymers, such as EPS (exopolysaccharides) and LPS (lipopolysaccharides), which may affect the bacterial motility (Harshey, 2003; Fischer *et al*., 2003). For instance, an accelerated swarming rate of *A. brasilense* was observed after modification of bacterial EPS and LPS by wheat seedling exudates (Borisov *et al*., 2007). Similarly, a mutant of *A. brasilense* Sp7 impaired in flocculation, showed reduced colonization of wheat roots compared to its wildtype and additionally higher N_2 fixation in wheat roots (Katupitiya *et al*., 1995). By contrast, another *Azospirillum* mutant deficient in flocculation was found to be able to colonize the host plant (Arunakumari *et al*., 1992). These studies may indicate the important role of the composition of the EPS in the flocculation and colonization processes.

Taken together, the occurrence of organic acids in seed and root exudates from wheat and maize could not be consistently correlated with the migration behavior of the model bacterium *B. reuszeri* in the presence of these exudates. This might be due to the presence of other, non-analyzed compounds in the exudate samples that played a more dominant role than the tested organic acids. However, this might also be due to the still unsatisfying reproducibility and the difficulties with standardization of the chemotaxis assay. Unfortunately, there was not sufficient time to further improve the protocol and to test more substances and more strains or even to establish more sophisticated approaches. For instance, bacterial chemotaxis is nowadays more and more determined by video imaging of the movement of individual, often fluorescence-labeled bacterial cells towards a chemoattractant (Senesi *et al*., 2002; Armitage & Packer, 1998). New modifications from the traditional capillary assay (Adler, 1972) have been developed, such as the high-throughput (Bainer *et al*., 2003) and the continuous-flow capillary assay (Law & Aitken, 2005) or a microfluidic assay (Mao *et al*., 2003). The latter has been described as a method that provides equally high sensitivity to attractants and repellents and that can be run faster (in minutes) and offers a superior performance relative to the existing assays. Employing these approaches to PGPR would certainly help identifying where positive interactions occur between root exudates and certain bacteria. As a consequence, a more systematic application of standardized chemotaxis assays might help to explain why some biofertilizers only work with certain plant species. This type of knowledge could finally promote the targeted use of biofertilizers, and thereby help to decrease the dependence of plant production on mineral fertilizer inputs.

Although malic acid is a well-known organic acid with chemotaxis effect present in wheat and maize root exudates, in this study it has been found that other substance/s should be responsible for the attraction of *B. reuszeri* under conditions of N starvation. Therefore, wheat seed and root exudates should be further fractionated (LMW / HMW) and tested.

The technique used for the collection of the exudates can highly influence the results obtained in the motility assays. For the collection of root exudates an aerated trap solution technique was used in this study. This technique is adequate for plants grown in nutrient solution. But the lack of mechanical impedance of the

substrate, which can stimulate root exudation, could be a disadvantage (Marschner, 1995). Moreover, only water-soluble exudates were collected in the solution whereas exudates adhering to the root surface (eg. mucilage) were not, or only partially, sampled by this technique. The sterile filtration of the exudates after the collection could even have eliminated all or part of the mucilage. Finally, microbial degradation might have had an impact on the recovery of root exudates, because exudates were collected under non-sterile conditions (von Wirén *et al*., 1995). On the other hand, root exudation can also be stimulated by the presence of microbes (Neumann & Römheld, 2007).

There is a spatial variation of the substances exudated along the root. The collection of the exudates of the total root system can result in solutions containing inhibitory substances that are not present in the reality and which modify the results. This problem would be avoided with the collection of root exudates from metabolically most active root zones.

The bacterial growth phase could also play an important role when establishing the chemotaxis assays. In this study, the bacteria were harvested at the late-exponential phase, which implies a risk of having undesired cells from the stationary phase. It has been observed that bacteria exhibit higher motility and a stronger chemotactic response in the logarithmic-growth phase than in the stationary phase (Adler, 1972). On the other hand, other studies have successfully used cells from the stationary phase for motility assays in semisolid agar (Senesi *et al*., 2002; Walker *et al*., 1997). Therefore, experiments should be performed using bacteria from different growth phases. It should be taken into account that the monomer composition of the *A. brasilense* EPS varies during growth phases: the bacterium produces a glucose-rich and an arabinose-rich EPS during exponential and stationary growth phases, respectively; and cell aggregation is correlated with the synthesis of the arabinose-rich EPS (Castro-Sowinski *et al*., 2006).

5.4. Possible contribution of *LeAMT1;1* and *LeAMT1;2* to nitrogen nutrition in tomato plants

Physiological role of LeAMT1;1 and LeAMT1;2 in tomato roots

So far, the physiological roles of the three tomato ammonium transporters LeAMT1;1, LeAMT1;2 and LeAMT1;3 have been deduced from their organ specific expression and their transcriptional regulation. However, one of the most direct ways to determine gene functions is to analyze the changes that occur in organism, in which the presence of the gene of interest is up- or down-regulated, representing a typical approach in reverse genetics (Emmanuel & Levy, 2002). Therefore, antisense tomato lines for the two root-expressed genes (*LeAMT1;1* and *LeAMT1;2*) were generated.

In a previous study, *LeAMT1;1* has been shown to be up-regulated in root hairs of N-deficient tomato plants, whereas *LeAMT1;2* was up-regulated after ammonium re-supply (von Wirén *et al.*, 2000b). These observations are in agreement with the results obtained in this study (Figs. 4.4-1; 4.4-8). Therefore, it was expected that the ammonium uptake capacity would increase after N starvation (due to *LeAMT1;1*) and further increase after ammonium re-supply to N-starved plants (due to *LeAMT1;2*). Indeed, ammonium uptake rates in WT plants exactly met this expectation. However, antisense lines which were expected to show lower uptake rates, showed similar ^{15}N-ammonium influx into roots as WT plants (Figs. 4.4-2; 4.4-9).

In the case of *LeAMT1;1* antisense lines, one explanation can be found in the very low repression of the *LeAMT1;1* transcript levels in the antisense lines under all tested conditions (Fig. 4.4-1). Another reason might be that the contribution of *LeAMT1;1* to ammonium uptake even in WT plants was not sufficiently strong to be detected and was masked by the activity of other transporters. Moreover, a compensation for the low *LeAMT1;1* expression levels might have occurred by the up-regulation of *LeAMT1;2*. Unfortunately, this was not reinvestigated. However, after ammonium re-supply to N-starved plants, a clear difference in ammonium

uptake rates became apparent between WT and two out of the three antisense lines of *LeAMT1;1* (Fig. 4.4-2). This difference correlated well with the transcript levels (Fig. 4.4-1).

In contrast to the *LeAMT1;1* lines, the antisense lines of *LeAMT1;2* showed a stronger reduction in the *LeAMT1;2* transcript levels (Figs. 4.4-8; 4.4-10). Despite this reduction, ammonium uptake capacities of the *LeAMT1;2* antisense lines were not lower under ammonium re-supply (Fig. 4.4-9). This observation might point to the possibility that ammonium uptake is post-transcriptionally regulated. This hypothesis is in agreement with the observation that a contribution of *LeAMT1;1* to ammonium uptake could not be shown (Fig. 4.4-2), and also that in *LeAMT1;2* antisense plants no difference was found in ammonium and amino acids concentrations (Fig. 4.4-11; 4.4-12). Surprinsingly, ammonium uptake rates were consistently lower in *LeAMT1;2* antisense lines under N deficiency (Fig. 4.4-9), although *LeAMT1;2* expression levels in WT plants are not induced under these conditions (Fig. 4.4-8).

It has been shown in long-term ^{15}N uptake experiments (K. Nielsen, personal communication) that LeAMT1;1 appears to be mainly involved in ammonium uptake at lower external concentrations (20-50 µM) rather than at 200 µM as used in the present approach. In fact, the K_m of LeAMT1;2 was shown to be approximately 40 µM in oocytes and 42 µM ammonium in root hairs, whereas the K_m of *LeAMT1;1* was determined at 8 and 16 µM respectively (Ludewig *et al*., 2003; K. Nielsen, personal communication).

Alongside a role of ammonium transporters in the primary uptake of ammonium there is a continuous passive diffusion of ammonium or ammonia out of the cell which represents an important lost of nitrogen (Wang *et al*., 1993; Rawat *et al*., 1999). It has been suggested that the *LeAMT1;1* and *1;2* could be responsible for the retrieval of ammonium leaching out of cells (von Wirén *et al*., 2000a). However, ammonium efflux is difficult to be measured and since it has been reported that the concentration of free amino acids increases with the N nutritional status of the plant (Barneix & Causin, 1996), the concentrations of free ammonium and amino acids in the root have been used as an indirect measure. In this study,

the measurements of glutamine in tomato roots reflected well the N status of the plant, decreasing after starvation and increasing after re-supply (Figs. 4.4-5 A; 4.4-12 A) and confirmed the results obtained by von Wirén *et al*. (2000). As glutamine concentrations in roots of antisense plants were not affected, the ammonium required in the cytosol for the synthesis of glutamine and glutamate was apparently not depending on the presence and activity of LeAMT1;1 and LeAMT1;2 transporters. This suggests that both transporters are not involved in ammonium retrieval under these conditions or that ammonium losses under these experimental conditions were not significant.

Physiological role of LeAMT1;1 and LeAMT1;2 in tomato shoots

The apoplastic ammonium concentration in leaf cells might be up to sixfold lower than the ammonium concentration in the cytosol suggesting the requirement for an active system, transporting ammonium from the apoplastic solution against the concentration gradient into the leaf cytoplasm. Since the cytoplasmic pH is much higher (7.0-7.5) relative to that of the apoplast (6.0), ammonia can continuously diffuse from the cytoplasm into the apoplast and get lost into the atmosphere before being recovered by an ammonium retrieval system present in the plasma membrane (Nielsen & Schjoerring, 1998).

In WT plants, ammonium concentrations in leaves (Figs. 4.4-4 A,B; 4.4-11 A,B) were not as much affected by the different N treatments as the ammonium concentrations in roots (Figs. 4.4-4 C; 4.4-11 C), probably since roots first satisfy their N requirements before ammonium is transported to the aerial organs (Kronzucker *et al*., 1998). The ammonium concentration in young leaves (Figs. 4.4-4 A; 4.4-11 A) was always twice as high as in old leaves (Figs. 4.4-4 B; 4.4-11 B), which can be explained by the elevated import of reduced nitrogen forms into young growing leaves, that act as a strong N sink. *LeAMT1;2* did not show any effect on free ammonium concentration (Fig. 4.4-11 A). On the other hand, the repression of *LeAMT1;1* coincided with elevated concentrations of free ammonium in young leaves under N sufficient conditions (Fig. 4.4-4 A). This suggests a role of *LeAMT1;1* in ammonium retrieval to the leaves cells.

Because N deficiency in plants has been shown to decrease the levels of structural photosynthetic components, in particular of chlorophyll (Delgado *et al*., 1994), chlorophyll concentrations in the leaves were measured as an alternative indication for the N nutritional status of the plant. Chlorosis is in particular observed in older leaves because N can be remobilized from older to younger leaves (Marschner, 1995). The almost unchanged chlorophyll concentrations found in younger leaves (Figs. 4.4-6 A; 4.4-13 A) across the different N treatments confirmed their action as a sink organ. The chlorophyll concentration in older leaves of WT plants decreased after 6 d of N starvation (Figs. 4.4-6 B; 4.4-13 B). In contrast, a decrease in the chlorophyll concentration of old leaves of *LeAMT1;1* and *LeAMT1;2* antisense lines was observed already after 3 d of N starvation (Figs. 4.4-6 B; 4.4-13 B) and could be explained by a lower retrieval of ammonium from the apoplast to the leaf cells. Interestingly, no accumulation of ammonium was observed under these conditions (Figs. 4.4-4 B; 4.4-11 B), suggesting that the ammonia which diffused into the apoplast and was possibly not retrieved in the antisense plants might have been emitted into the atmosphere. This conclusion would require measurements of ammonia volatilization from old leaves to elucidate a possible role of LeAMT1;1 and LeAMT1;2 in ammonium retrieval of old or even senescing leaves.

The fact that the *LeAMT1;1* antisense plants showed only a weak repression of *LeAMT1;1* transcript levels (Figs. 4.4-1; 4.4-3) complicates the determination of the physiological role of its corresponding transporter *in planta*. An alternative to the use of antisense plants would be the generation of knock-out plants. In fact, transgenic tomato plants lacking any expression of the ammonium transporters *LeAMT1;1* and *LeAMT1;2* have been created (Wu, 2005), but they did not show any phenotype in soil tests. This might indicate that other high-affinity systems contribute to the ammonium uptake in tomato roots. Another alternative would be to use the quadruple knock-out (qko) of *Arabidopsis* which is defective in four ammonium transporters (Yuan *et al*., 2007) as methodological platform to study the expression of LeAMT genes and to determine LeAMT transport activity in *planta*.

In conclusion, the failure of showing any consistent effect of *LeAMT1;1* or *1;2* repression or NH_4^+ influx might also explain why these lines are ineffective or not

suitable to investigate a possible N transfer from diazotrophic bacteria, such as *Azospirillum*, which has been shown to release ammonium (Van Dommelen *et al*., 1997). For future studies aiming at investigating whether LeAMTs play a role in ammonium uptake from the soil solution or from diazotrophic bacteria, it is recommendable first to develop a culture system most likely based on sand culture to promote the establishment of bacterial associations, in which ammonium concentration can be easily monitored and than to develop multiple knock-out lines for root expressed AMT genes, such as qko line in *Arabidopsis*.

Concluding remarks

For competitive rhizosphere colonization of PGPR strains, a wide range of factors play a role. Different environmental factors such as soil properties and N fertilization, together with internal factors, which may influence the plant-microbial association such as bacteria strain and plant root exudates, have been tested in this study. However, none of these factors have been found to be decisive for the establishment of a successful association. Furthermore, the lack of reproducibility of the experiments indicates a high specificity in the root-microbial interactions. Indeed, a differential profile of seed and root exudates has been found among the tested plant species, together with a differential bacterial motility. Therefore, more investigations with the putative N_2-fixing bacteria are needed before field experiments can be performed.

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7. APPENDIX

Appendix 1. Apparent nitrogen mineralization rates (Napp) in non-planted German Luvisol (Filderlehm) at different nitrogen fertilization levels

The soil was incubated without plants and assayed at the same time points as in the Table A2. Nitrogen balance of N input (N fertilization, N mineral in soil, biological N fixation and N gases from air) and N output (N mineral in soil and N leaked). Watering: 60% WHC. Substrate: Filderlehm + Perlite mixture, data represent mean values, n=2.

	Tillering stage (EC25)			*Heading stage (EC51)*			*Ripening stage (EC92)*		
N INPUT									
N fert (mg N kg^{-1} soil)	0	30	90	0	30	90	0	30	90
N min (mg N pot^{-1})		31			31			31	
BNF		0			0			0	
N air		0			0			0	
N OUTPUT									
N min (mg N pot^{-1})	52	142	207	40	70	131	98	116	278
N losses		0			0			0	
N app (mg N kg^{-1} soil)	**14**	**45**	**29**	**6**	**-3.5**	**-22.5**	**45**	**27**	**77**

Appendix 2. Apparent nitrogen mineralization rates (Napp) in planted Luvisol German (Filderlehm) at different nitrogen fertilization levels

Seeds of wheat (*Triticum aestivum* L. cv. Paragon) were sown directly on soil and harvested at 3 different plant developmental stages. Nitrogen balance of N input (N fertilization, N mineral in soil, biololgical N fixation, N gases from air) and N output (accumulation in plants, N mineral. N leaked). Watering: 60% WHC. Substrate: Filderlehm / Perlite mixture, data represent mean values, n=2.

	Tillering stage (EC25)			*Heading stage (EC51)*			*Ripening stage (EC92)*		
N INPUT									
N fert (mg N kg^{-1} soil)	0	30	90	0	30	90	0	30	90
N min (mg N pot^{-1})		31			31			31	
BNF		0			0			0	
N air		0			0			0	
N OUTPUT									
N plant (mg N pot^{-1})	5	7	7	6	11	16	8	14	21
N min (mg N pot^{-1})	3	2	6	2	2	1	2	2	2
N losses		0			0			0	
N app (mg N kg^{-1} soil)	**12**	**1**	**-39**	**16**	**20**	**36**	**16**	**17**	**23**

Appendix 3. Filderlehm-Perlite soil analysis. Sterilized soil vs. non-sterilized soil

	Sterilized soil	Non-sterilized soil	Replica
water extractable phosphorus (mg P kg^{-1} soil)	111.4± 7.7	101.3±5.8	n=4
ammonium (mg N kg^{-1})	1.60	0.80	n=2
nitrate (mg N kg^{-1})	16.50	17.00	n=2

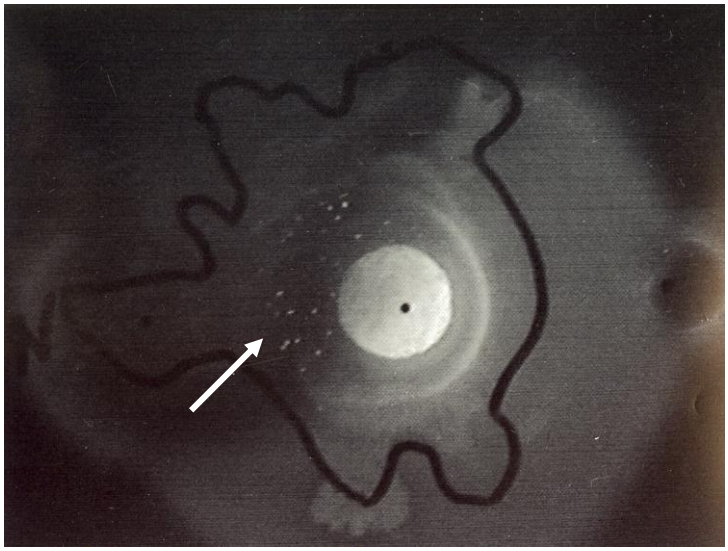
Appendix 4. Statistically significant differences of means by Fisher's LSD test

Results showing significant differences tested with Fisher's LSD method. N = mg N kg^{-1} soil. Inoc = inoculated treatments. Significant differences found with other methods are shown in the last column (p≤0.05).

Trial I				
Shoot DW	90N	Graminante	sterilized soil	(inoc>control)
Shoot DW	90N	Graminante	non-sterilized soil	(control>inoc)
Grain DW	90N	Graminante	non-sterilized soil	(control>inoc)
No. of spikes	90N	Graminante	non-sterilized soil	(control>inoc)
No. of spikes	90N	Graminante	sterilized soil	(inoc>control) Tukey
Trial II				
SPAD (EC31-32)	60N	*A.b.* 10^3	non-sterilized soil	(inoc>control)
No. of spikes	60N	*A.b.* 10^5	sterilized soil	(control>inoc) Tukey
Grain DW	60N	*A.b.* 10^6	non-sterilized soil	(control>inoc) Tukey
Grain DW	60N	*A.b.* 10^5	sterilized soil	(control>inoc) Tukey
Trial V				
$^{15}N/^{14}N$ roots	30N	*A.b. vs A.b.* nif^-		(control>inoc)
$^{15}N/^{14}N$ roots	30N	*P.p.*		(control>inoc)
$^{15}N/^{14}N$ roots	30N	*X.v.*		(control>inoc)
$^{15}N/^{14}N$ shoots	30N	*X.v.*		(control>inoc)
Trial VI				
SPAD (EC25)	36N	cv. Atir	non-sterilized soil	(inoc>control) t-test
Plant height	36N	cv. Atir	non-sterilized soil	(inoc>control)

Appendix 5. Flocculation of *Brevibacillus reuszeri* in the presence of wheat root exudates

Paper discs (5 mm diameter) were soaked in different exudates (1 µg C $disc^{-1}$) and placed at 2 cm distance left and right of the central inoculation spot. Bacterial colonies were incubated on semi-solid agar medium (5-fold diluted R2A-agar medium, Oxoid) containing Calcofluor during 4 days.



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Work experience

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Sep 1996 - May 1997 Leonardo da Vinci scholarship (EU) holder at "Bezirksstelle für Naturschutz und Landschaftspflege", Regierungspräsidium Tübingen (Regional goverment, Germany)

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1993 - 1996 Forestry Engineering (University of Lleida, Spain). Focus: Environmental Management. Master Title: "Indexing of the ecological values of a semiarid area in Baix Segre"

1992 - 1993 Garden and Landscape Architecture (Postgraduate study at Polytechnic University of Catalonia, Barcelona, Spain)

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