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Study the possible mechanisms of plant growth promotion by wheat diazotrophic bacteria grown in Uzbekistan soil

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Abstract

Plant growth promoting bacteria (PGPB) are ubiquitous in both plant root and shoot, and are important contributors to the nitrogen-input of plants exerting their positive effects on plant growth directly or indirectly through different mechanisms. The present work focuses on a) the isolation of PGPB, which promotes the growth of different plant cultures and controls plant diseases caused by Fusarium species, b) the prospects of PGPB to solve plant nutritional problems, c) developing new molecular methods for the assessment of their diversity and activity.

A total of 780 bacterial strains were isolated from root, rhizosphere and phyllosphere of wheat grown in soil Syrdarya and were tested for their ability to promote the growth of other plants resulting in several universal PGPB strains.

Contributions of PGPB to plant nutrition were investigated with wheat, and several vegetable plants such as cucumber, tomato, paprika, and cauliflower on quartz-sand substrates. Bacterial colonization, plant dry weight, and N concentrations in plants were measured.

Bacterial inoculation effects on plant N nutrition in cucumber and tomato plants were determined by exposing bacterial and non bacterial plants to two, low and high $NH^{4+}NO^{3-}$ supply.

In the frame of this thesis, the methods for the description of the diversity of root colonizing PGPB have been developed and improved to provide links between introduced PGPB abundance and activities. The approach used was based on the sensitive real – time PCR detection/quantification of introduced PGBP and the nitrogenase reductase gene (*nif*H), which served as a marker gene for potential diazotrophs.

The amplified 16S-23S ISR sequences of studied bacteria were subjected to strain – specific primer design and a highly specific bacteria quantification protocol were developed. The bacteria quantification protocol was based on real – time PCR using strain specific primers in order to evaluate the colonization ability of studied bacteria, which were inoculated to plant roots.

The application previously used universal *nif*H primers to the real – time PCR improved the detection of less abundant diazotrophs in dry land plant root. The protocols were tested and optimized using pure cultures of diazotroph reference strains, and subsequently applied to the analysis of two vegetable plant roots. Real – time analyses of PCR products obtained from plant root DNA extracts revealed that the new *nif*H PCR protocol differentiated between the diazotroph populations in different plants.

The developed methodology was applied to study *nif*H abundance of *Bacillus licheniformis* and *Xanthomonas sp.* inoculated to cucumber and tomato growing in non – sterile quartz sand. Treatments with nitrogen limiting conditions resulted in more diazotrophic bacteria abundance, as well as, *nif*H gene pool while nitrogen excess suppressed diazotrophic bacteria abundance in both inoculated and non-inoculated plants. Furthermore, the *nif*H gene abundance was significantly correlated with measurements of N amount taken by the plant and inoculated bacteria density showing direct contribution of introduced bacteria to plant N nutrition. The results presented in this thesis have shown that monitoring of *nif*H amount in plant root is a suitable and promising approach to link inoculated diazotrophic bacteria abundance and its potential activity. The study of *nif*H gene abundance in plant offers the opportunity to identify key players in asymbiotic nitrogen fixation, to study short-term community responses in changing environments, or to analyze the effect of regulation *in situ*.

Keywords

16S-23S *ISR* quantification – asymbiotic diazotrophic bacteria – cauliflower – cucumber – *nif*H gene quantification – nitrogen uptake – paprika – real-time PCR – tomato – wheat

Inhaltsangabe

Das Pflanzenwachstum fördernde Bakterien (PGPB) kommen ubiquitär sowohl an der Wurzel als auch am Spross der Pflanzen vor und sie können über direkte oder indirekte Mechanismen einen bedeutenden Beitrag zur Stickstoffernährung der Pflanzen leisten. Die vorliegende Arbeit umfasst a) die Isolierung von PGPB, welche das Wachstum verschiedener Pflanzenarten fördern und durch Fusarien verursachte Pflanzenkrankheiten bekämpfen, b) die Analyse der Möglichkeiten Probleme der Pflanzenernährung durch den Einsatz von PGPB zu lösen, c) die Entwicklung neuer molekularbiologischer Methoden zur Messung der Diversität und Aktivität der PGPB.
780 Bakterienstämme wurden aus der Wurzel, der Rhizosphäre und Phyllosphäre von Weizen, der auf Boden der Syrdarya wuchs, isoliert. Daraus wurden universelle PGPB Stämme ausgewählt, die das Wachstum verschiedenster Pflanzen förderten. Der Beitrag dieser PGPB zur Pflanzenernährung wurde an Weizen und verschiedenen Gemüsepflanzen, wie Gurke, Tomate, Paprika und Blumenkohl in Quarzsand Modellversuchen analysiert. Bakterienbesiedlung der Pflanzen, Pflanzen Trockenmasse und N-Konzentration in den Pflanzen wurden gemessen und bewertet.
Der Bakterieneinfluß auf die pflanzliche N-Ernährung von Gurke und Tomate wurde bei geringer und hoher NH_4NO_3 Versorgung an mit Bakterien inokulierten Pflanzen und nicht inokulierten Kontrollpflanzen analysiert.
Im Rahmen dieser Arbeit wurden Methoden zur Beschreibung der Diversität von rhizosphären PGPB entwickelt und verbessert um Verbindungen zwischen applizierten PGPB und deren Aktivitäten zu prüfen. Die sensitive quantitative real-time PCR Methode wurde zur Quantifizierung bzw. zum Nachweis der inokulierten PGPB und zum Nachweis des nitrogenase-reduktase Gens (*nif*H), des Markergens für potentiell diazotrophe Bakterien, genutzt.
Bakterienart spezifische Primer wurden aus dem Sequenzvergleich der 16S-23S ISR ausgewählter Bakterienstämme selektiert und Protokolle zur Quantifizierung dieser Bakterienarten erarbeitet. Die Protokolle basierten auf der real-time PCR Methode und dem Einsatz der selektierten artspezifischen Primer. Ziel der Untersuchungen war die Besiedlungsfähigkeit inokulierter Bakterien an Pflanzenwurzeln zu analysieren. Die Anwendung der früher selektierten universellen *nif*H Primer in der quantitativen real-time PCR verbesserte die Nachweisgrenze von diazotrophen Bakterien signifikant. Somit konnten diazotrophe Bakterien, die nur in geringer Zellzahl an Pflanzenwurzeln in trockenen Regionen vorkommen, entdeckt und quantifiziert werden. Die Protokolle wurden unter Einsatz von diazotrophen Referenzstämmen getestet und optimiert und nachfolgend zur Messung an zwei Gemüsearten angewendet. Die Ergebnisse der real-time PCR Messungen, die an DNA Extrakten aus Pflanzenwurzeln von Gurke und Tomate durchgeführt wurden, zeigten, dass das vorliegende *nif*H PCR Protokoll zur Differenzierung der Bakterienpopulationen diazotropher Bakterien zwischen verschiedenen Pflanzenarten geeignet ist.
Die neu entwickelten Methoden wurden zum Studium des *nif*H Vorkommens und der Prüfung der Besiedlungsfähigkeit von *Bacillus licheniformis* und *Xanthomonas sp.* an Tomaten Wurzeln eingesetzt, die in nicht sterilisiertem Quarz Sand wuchsen. Unter Stickstoff limitierten Bedingungen waren sowohl die Anzahl diazotropher Bakterien als auch der *nif*H Genpool erhöht, während bei hoher N-Versorgung der Pflanzen die Anzahl diazotropher Bakterien sowohl in inokulierten als auch nicht inokulierten Varianten reduziert waren. Außerdem bestand eine enge signifikante Korrelation zwischen *nif*H Gen Vorkommen und N-Aufnahme der Pflanzen und die Dichte der inokulierten Bakterien zeigte einen direkten Beitrag der applizierten Bakterien zur N-Ernährung der Pflanzen.
Die Ergebnisse dieser Arbeit zeigten, dass das Monitoring des *nif*H Gen Vorkommens in Pflanzenwurzeln eine vielversprechende Methode ist, um die potentielle Luftstickstoffbindungsaktivität inokulierter diazotropher Bakterien zu analysieren. Die *nif*H Gen Quantifizierung an Pflanzen eröffnet die Möglichkeit Schlüsselorganismen in der assoziativen biologischen Luftstickstoffbindung zu identifizieren und kurzfristige Reaktionen der Bakteriengesellschaften auf Umweltveränderungen und Regulationsmechanismen *in situ* zu analysieren.

Stichworte

16S-23S ISR Quantifizierung – assoziative diazotrophe Bakterien – Blumenkohl – Gurke – *nif*H Gen Quantifizierung – Stickstoffaufnahme – Paprika – real-time PCR – Tomate – Weizen

Contents

Abbreviations

ANF - Asymbiotic Biological Nitrogen Fixation
ANOVA - ANalysis Of VAriance
BLAST - Basic Local Alignment Search Tool
BNF - Biological Nitrogen Fixation
CFU - Colony-Forming Unit
CLUSTALW - General purpose multiple sequence alignment program for DNA or proteins
DW – Dry Weight
FASTA – DNA and protein sequence alignment software package
FW – Fresh Weight
GPA - Glycerin Peptone Agar
ISR – Intergenic Spacer Region
MPA - Meat Peptone Agar
MPB - Meat Peptone Broth
NL – Number Of Leaves
PCR - Polymerase Chain Reaction
PDA - Potato-Dextrose Agar
PGP – Plant Growth Promoting
PGPB – Plant Growth Promoting Bacteria
PGPR - Plant Growth Promoting Rhizobacteria
RDP - Ribosomal Database Project
RDW – Root Dry Weight
RFLP - Restriction Fragment Length Polymorphism
RFW – Root Fresh Weight
RIDOM - Ribosomal Differentiation of Microorganisms
RL – Root Length
SDW – Shoot Dry Weight
SE - Standard Error
SFW – Shoot Fresh Weight
SL – Shoot Length
TBA - Thiobarbituric Acid
TEF - Thyrotrophic Embryonic Factor

Chapter 1. General introduction

1 GENERAL INTRODUCTION

1.1 Objectives and outlines of the thesis

1.1.1 Objectives: Towards a better understanding of Plant Growth Promoting mechanisms of diazotrophic bacteria to improve plant N nutrition

The need for a better understanding of the mechanisms that affect plant N nutrition in natural systems is recurrent theme in the literature discussing the potential application of biological nitrogen fixation (BNF) as performed by free-living diazotrophs. The real-time PCR based quantification has been successfully used to quantify *nif*H gene pool in soil (Wallenstein 2004) and is applicable to the plant environment as well. However, this method requires implementation of an internal control that prevents the miscalculation of the quantified gene pool due to the presumed different DNA extraction efficiencies from environmental samples. Moreover, this study only used quantification results to describe the effect of environmental factors on *nif*H gene abundance in soil and did not link the results to N-fixation activity. Methods of molecular ecology are capable of significantly extending our understanding of this process in plant. The establishment of the real-time PCR approach for the quantification of *nif*H gene abundance in plant will add a possible tool to study the factors that influence BNF in much more detail. The *nif*H gene quantification parallel with the considered bacteria enumeration, and plant N nutrition makes possible to determine the potential of inoculated bacteria to fix atmospheric nitrogen. Moreover, it will become possible to determine how individual members of the diazotroph community react to environmental factors, and to study some elements of gene regulation *in situ* in the soil environment. The knowledge gained will allow a focused search for methods of enhancing BNF in the field by inoculation with specifically selected microorganisms. Furthermore, this technology will also allow detailed monitoring and control of the effectiveness of such future applications.

The objective of this thesis is to improve, develop and to apply a number of key methods that are necessary for a successful application of the real - time approach to free-living plant diazotrophs. These methods should provide a sound basis for quantitative studies in the plant environment to obtain new information on the PGP mechanisms and dynamics of inoculated diazotrophs.

1.1.2 Research focus and hypotheses of the thesis

This thesis investigates the growth promoting effects of asymbiotic diazotrophic bacteria, in combination with fertilizers to contribute to growth, nutrient uptake of agricultural plants.

Four main topics are included in the work:

a) Isolating local PGPB

   Soil nutritional conditions, microbial diversity may effect a growth promoting efficiency of inoculated bacteria. Therefore, in this study, PGPB were isolated from wheat grown in a nutrient deficient calcisol soil in Syrdarya, Uzbekistan.

b) Universal plant growth promoting bacteria

   The growth-promoting activity of some bacteria may be highly specific to certain plant species, cultivars and genotypes. Therefore, one of the effective strategies for initial selection and screening of PGPB is the consideration of host plant specificity. In this study, the bacterial strains shown to have a plant growth promoting effect on wheat plants were tested for their stimulatory effect on other plants, such as cauliflower, cucumber, paprika and tomato.

c) Effect of inorganic N availability on diazotrophic bacteria colonisation in plant

   Understanding the factors involved in controlling the colonisation/distribution of diazotrophic bacteria in the environment may allow identifying the factors controlling N_2-fixation in the environment. Therefore it is important to evaluate the response of inoculated bacteria to fertiliser supply to the plant in the presence of N in different levels.

d) Linking structural and genomic data

   Molecular measurements of the functional gene abundance, as a potential of activity, can link structural and genomic data. To evaluate the direct contribution of the N_2-fixing plant-inhabiting diazotrophic bacteria to plant nutrition, the abundance of a marker gene for biological nitrogen fixation, *nif*H gene can be investigated.

Several approaches based on quantitative or semi-quantitative PCR has been suggested for the quantification of *nif*H gene abundance in environmental samples or pure culture DNA. All these approaches have their advantages and disadvantages. Real-time PCR-based methods developed by Wallenstein (2004) have shown to be a powerful tool to quantify N2-fixing genes in soil. However, these methods require implementation of an internal control that prevents the miscalculation of the quantified gene pool due to the presumed different DNA extraction efficiencies from environmental samples. Therefore, it was aimed to develop a new method to accurately quantify *nif*H gene copy numbers in plant DNA.

From these main topics the flowing hypotheses were generated:

There is a significant correlation between asymbiotic diazotrophic bacteria abundance in plant and N content of plants.

- In the low level of N availability, diazotrophic bacteria are more abundant than in high N supplied conditions, and that even in high N availability, the application of diazotrophic PGPB strains can increase the diazotrophic population allowing increased potential for plant N nutrition.
- Correlations between plant N content and applied bacteria cell numbers and quantified *nif*H gene abundance in plant tissue indicate/evaluate the capacity of the applied diazotrophic bacteria to fix atmospheric nitrogen.

1.1.3 Outline of the thesis

Chapters, three, four, five and six consist of the content of a manuscript which has been published, has been submitted for publication, or which is nearly ready to be submitted for publication in a refereed academic journal.

Chapter two describes the isolation, phenotypic characterisation and screening the plant growth promoting bacterial strains from wheat plant for producing plant growth promotion in series of plate and pot experiments. Inoculation experiments under greenhouse conditions provides the basis for studies of plant growth promoting effect of used bacteria allowing determination of their effect mechanisms.

Chapter three of this thesis focuses on conventional and molecular based identification of selected bacterial strains. The disagreement between conventional identification and

phylogenetic sequence analysis of 16S-23S ISR rDNA amplified from two diazotrophic plant growth promoting bacteria strains was discussed.

Chapter four details the development of strain-specific primers that provide the basis for an accurately evaluation of inoculated bacteria colonization ability in plant root by quantitative detection of bacteria after inoculation.

Chapter five describes the newly developed method for *nif*H gene quantification that provide the basis for more sensitive and more quantitative the detection of *nif*H templates in plant root by real-time PCR with universal *nif*H primers. The laboratory experiment in this study provided data on the *nif*H gene abundance in plant root growing in non-sterile quartz sand under different conditions, which was significantly positively correlated to inoculated bacteria abundance (chapter 4). The methods described and discussed in chapter 5 are based on peer-reviewed article published in Canadian Journal of Microbiology under the title of 'Detection and quantification of the *nif*H gene in shoot and root of cucumber plants' (Juraeva et al. 2005).

Chapter six presents some preliminary results on the application of the developed methodology that employed to quantify the abundance of considered bacteria after inoculation (as described in chapter 4) parallel with *nif*H gene quantification (as described in chapter 5) using real – time quantitative PCR, to the study of the capacity of the inoculated diazotrophic bacteria to fix atmospheric N in plant roots and furthermore contains a comprehensive discussion of the achieved results, open questions, and future research opportunities.

1.1.4 Current challenges

1.1.4.1 Improving detection methods for nifH.

*nif*H primers in different level of specificity were used to amplify *nif*H gene fragments from both pure culture DNA and environmental samples (Ueda et al. 1995). The application of PCR to mixed assemblages of diazotrophic organisms requires an unbiased amplification of all *nif*H gene fragments. Because of the huge phylogenetic differences of *nif*H genes (Zehr et al. 2003), universal *nif*H primers (Ueda et al. 1995) have a high degree of sequence degeneracy. Using universal *nif*H primers developed by Ueda et al. (1995) was successful to amplify *nif*H genes in rice root. However, this method was not sensitive enough to amplify *nif*H genes in the dryland plant roots (maize, soybean) suggesting that many less diazotrophic bacteria exist in dryland plant roots than in rice (Ueda et al. 1995). Nowadays, developed molecular approaches and high quality reagents which make approach more sensitive allow amplifying *nif*H gene even in bulk soil (Wallenstein 2004). The application of previously published primers to more sensitive

approaches, such as real-time PCR based direct quantification may allow a more sensitive detection of diazotrophs in dryland plant roots as well. Furthermore, since, quantitative data on *nif*H abundances obtained with such methods using degenerated primers should be interpreted with caution; the development of real-time PCR protocols with real-time control of PCR product would reduce the risk of PCR biases, making the results more reliable and more amenable to quantitative interpretation of the data.

1.1.4.2 Development of methods to examine N_2 fixation by certain bacteria in complex environments.

While the capability of diazotrophs to fix nitrogen *in vitro* can be demonstrated easily, efforts to quantify nitrogen fixation in natural associations with plants have produced widely varying results. In the past 30 years many crop inoculation studies, coupled to acetylene reduction measurements, N balance and ^{15}N isotope dilution experiments, have been conducted with root associated bacteria to determine whether the bacteria supply significant amounts of nitrogen to cultivated plants (Boddey et al. 1999; James 2000). A major drawbacks of the acetylene reduction assay, is that it only measures nitrogenase activity and reveals no information on whether the fixed N is incorporated into the plant (Boddey et al. 1995). N balance experiments have the disadvantage that the plant N is not necessarily derived from the air but might also result from improved nutrient uptake by the inoculated plant. Nowadays, the most useful methods for examining N_2 fixation in the field and large greenhouse experiments are still the ^{15}N isotop dilution and ^{15}N natural abundance techniques (James 2000).

Assaying the genetic potential of inoculated bacteria for nitrogen fixation, i.e. *nif*H gene quantification in combination with N-content analysis may be a possible tool to evaluate the direct contribution of the N_2–fixing plant-colonizing diazotrophic bacteria to plant N nutrition. Due to stringent regulation of gene is regulated at both pre- and posttranslational levels (Dean and Jacobson 1992) under unfavorable conditions; gene abundance does not always mean the activity of nitrogenase. However, it is an indicator of potential of inoculated bacteria to fix atmospheric nitrogen. Furthermore, the study of gene abundance under environmental conditions is in itself an interesting objective, as the conditions in natural environments (e.g. plant roots) are vastly different from the optimum growth conditions used in laboratory cultures.

Tab. 1: Comparison of methods of estimating nitrogen fixation

Methods	Advantages	Disadvantages	Sensitivity
1. Total N balance	Simplest	Low sensitivity including other inputs.	Lowest
2. $^{15}N_2$ incorporation	Most direct	Expensive, only for short period	High-moderate
3. Acetylene reduction	Simple, highly sensitive	Indirect, semi-quantitative	High
4. ^{15}N dilution	Throughout growing season	Only N Fixation in plant Varies with reference plants	High-low
4a. Natural abundance	Simple, no disturbance to system	Only slight difference in ^{15}N content	Low
4b. Substrate addition	Difference in ^{15}N content is large	Change of ^{15}N in time and space in soil	Moderate

Note: Adapted from Watanabe 2000.

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Chapter 2. Isolation, phenotypic characterization and screening of wheat inhabiting bacteria for their plant growth promoting effect

2 ISOLATION, PHENOTYPIC CHARACTERISATION AND SCREENING OF WHEAT INHABITING BACTERIA FOR THEIR PLANT GROWTH PROMOTING EFFECT

2.1 Abstract

The aim of this study was the isolation of bacteria, which promotes the growth of different plant cultures and controls plant diseases caused by *Fusarium* species. A large number of bacteria were isolated from root, rhizosphere and phyllosphere of wheat grown in Syrdarya. Determination of phenotypic traits was used to evaluate the presence of the bacterial isolates with similar phenotypic characteristics. Three hundred sixteen strains were discarded due to their similar phenotypic characteristics. In addition, 18 strains isolated in previous studies were screened for their PGPB effect. Based on the plant-inoculation experiments performed on plates, 74 and 154 strains were discarded as potential pathogens and shoot and/or root growth inhibitors, respectively. Of the remaining strains, 111 appeared to have a positive plant growth effect on wheat. These PGPBs were also tested for their ability to inhibit pathogen *Fusarium* growth selecting 24 isolates. Conventional identification methods identified a number of new plant growth promoting strains as *Bacillus* sp., *Pseudomonas. sp., Azotobacter* strains and *Micrococcus*. Laboratory experiments conducted on wheat under gnotobiotic conditions demonstrated increases in root elongation (up to 50%), root dry weight (up to 31%), shoot elongation (up to 47%) and shoot dry weight (up to 48%) of inoculated wheat seedlings. Based on growth-promoting activity, four isolates were selected and designated as plant growth promoting bacteria. Sand-based seed inoculation with selected PGPB isolates exhibited stimulatory effects on the growth of vegetables, namely cauliflower, cucumber, paprika and tomato with varied response with different plant and PGPB strains, resulting in two universal plant growth promoting bacterial strains, *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148.

Key words

PGPB screening - cauiliflower – cucumber – paprika – tomato – wheat

2.2 Introduction

The microbe-plant interaction in the root, the rhizosphere or phyllosphere can be beneficial, neutral, variable, or deleterious for plant growth. Rhizobacteria that exert beneficial effects on plant development are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978, Kloepper et al. 1986). These bacteria significantly influence plant growth by increasing nutrient uptake, suppressing pathogens and may be used in agriculture to minimize the utilization of chemical pesticides and fertilizers (Hartmann and Bashan 2009, Díaz-Zorita and Fernández Canigia 2009). Bacteria species including *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Klebsiella, Enterobacter, Xanthomonas* and *Serratia* have been shown to promote plant growth. During the last couple of decades, the use of PGPB for sustainable agriculture has increased. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPB has been reported (Ruppel 1987, Díaz-Zorita and Fernández Canigia 2009). Biological N_2 fixation provides a major source of nitrogen for plants as a part of environmentally friendly agricultural practices. Apart from fixing N_2, PGPB can affect plant growth directly improving nutrient uptake, by the synthesis of phytohormones and vitamins, inhibiting plant ethylene synthesis, enhancing stress resistance, solubilising inorganic phosphate, and mineralising organic phosphate (Dobbelaere et al. 2003, Lucy et al. 2004). Plant growth benefits due to the addition of PGPB include increases in germination rate, root growth, yield, leaf area, chlorophyll content, nitrogen content, protein content, tolerance to drought, shoot and root weight, and delayed leaf senescence (Dobbelaere et al. 2003). Siderophore-producing bacteria promote plant growth by an iron uptake resulting in the limited iron in the rhizosphere, especially in neutral and alkaline soils, and thereby reduce its availability for the growth of pathogen (Winkelmann 2002).

Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivars and genotypes (García de Salomone and Döbereiner 1996, Dobbelaere and Okon 2003, Sala et al. 2007, Behl et al. 2007).

This study focuses on the screening of effective PGPB strains on the basis of their potential for plant growth promoting activity and to study the early phases of bacterial inoculation effect using *in vitro* methods.

2.3 Materials and methods

2.3.1 Bacteria isolation from root, rhizosphere and phyllosphere of wheat

2.3.1.1 Soil and Plant

Wheat (*Triticum aestivum* cv. Ziklon) was grown on soil sampled from Syrdarya province, Uzbekistan. Syrdarya soil chemical properties also determined as described in Egamberdieva et al. (2002): soil samples (0-30 cm depth) were taken with a soil probe (3.5 cm diameter). Soil samples were pooled, and sieved (<2 mm mesh) directly after collection. Air-dried samples were analyzed for contents of total C, N, P, and K. Soil chemical analysis was as follows (per 100 g d.w.): 100 mg C; 0.6 mg N; 3.0 mg P; 12 mg K; pH was 7.8. Total C was identified by elementary analysis while total N was determined by Kjeldahl method. The molybdenum blue method was used to determine total P. Potassium was determined using the flame photometric method (Riehm 1985). Soil pH value was measured in H_2O (water: soil solution ratio 1:2.0) with a potentiometric glass electrode (measuring range of 0-14 pH with resolution and accuracy of 0.1 pH). The soil water content after planting was approximately 12% water holding capacity and was kept nearly constant throughout the experiment. All soil was sieved (mesh width 3 mm; mesh length 6 mm) prior to use. The soil was placed in 350 ml pots to a bulk density of 1.0 g/cm^3.

Wheat seeds were obtained from the University of Agriculture of Uzbekistan, Tashkent.

2.3.1.2 Collecting samples

Five plants were harvested. To collect samples, 21 days after sowing plant in soils (Syrdarya, Uzbekistan), and the plants were removed from the soil. Subsequently, the plant was shaken carefully and soil still tightly adhering to the roots was defined as rhizosphere soil. Roots were washed in running tap water to remove adhering soil, cut into 1 cm pieces and surface sterilised in 0.7% NaOCl solution for 30 minutes. To isolate phylosphere bacteria, 1 g of youngest part of the leave and stem of seedlings were cut. All samples were kept separated in Erlenmeyer flasks.

2.3.1.3 Bacteria isolation

To isolate the root or phylosphere microorganisms samples were placed in Erlenmeyer flasks containing 95 ml of 0.1% sterile sodium pyrophosphate solution and 10 g grit and shaken on a rotary shaker at 200 rpm for 20 min. Tenfold-serial dilutions of the suspensions were made with

0.1% sodium pyrophosphate and plated in triplicate on glycerin peptone agar for total bacterial counts. To count the total number of bacteria, 100 µl of resulting suspensions were sprayed over the surface of glycerin peptone agar (GPA). The plates contained cyclohaximide in a concentration of 100 mg l^{-1} to inhibit fungal growth. The total number of bacteria was established after 7 days of incubation. The bacterial isolates grown in "master plate" were transferred to fresh Petri dishes containing the same medium. This process was repeated 3 times to purify diazotrophic bacteria isolates and they were then stored in tubes containing GPA medium for further examination. Determination of diazotrophic bacteria was performed using nitrogen free medium Ashby agar. Ten serial dilutions of bacterial isolates were sprayed on the Ashby agar. Survived isolates were then stored in tubes containing Ashby agar medium and regarded as diazotrophic bacterial isolates.

2.3.1.4 Morphological characterization and identification of bacteria

The identification of strains relied on standard biochemical and physiological tests according to the classification of Bergey (Holt and Krieg 1984). Cultures were grown on nutrient broth and nutrient agar for morphological characterization, i.e. gram staining, study colonial forms, motility. Gram reaction of each culture was determined by the rapid KOH test (Ryu 1938). Colony morphologies were examined after 24 h, 48 h, and 72 h growth on glycerin peptone agar at 28^{o}C. Cell morphologies were examined with phase contrast microscopy or after staining with methylene blue. The activity of catalase was tested by suspending a loopful of cells in a 10% (vol/vol) H_2O_2 solution. Formation of a fluorescent pigment was observed on King B medium. The oxidation and fermentation of glucose was performed according to the method of Hugh and Leifson (1953).

2.3.2 Screening of bacterial isolates for their effect on wheat growth

Wheat (*Triticum aestivum* cv. Ziklon) seeds were obtained from of Tashkent Agriculture University, Uzbekistan. In addition to the isolated strains, we used diazotrophic isolate BL43 (identified as *Bacillus licheniformis* in our study). These strains were obtained from Microorganism collection Institute of Microbiology, Uzbekistan Academy of Sciences.

2.3.2.1 Bacteria suspension preparation

Pure cultures of the diazotrophic bacterial isolates were grown in Ashby broths on a rotary shaker (150 rpm) at 28°C for 72h. The pH of medium was adjusted to 7.0 before autoclaving. The bacterial suspensions were centrifuged at 7.000 rpm for 10 min. Growth medium was

discarded and the bacterial pellet was resuspended in 0.05M NaCl buffer. Cell densities of bacterial suspensions used for seed inoculation were counted by dilution plating and CFU counts. The bacterial cell densities in the inoculant material were 10^7-10^8.

2.3.2.2 Screening in Petri dishes

Petri dish and pot experiments were conducted on wheat to screen the diazotrophic isolates for their effect on wheat root and shoot growth, respectively. The first screening was performed in Petri dishes. Wheat seeds were surface sterilized by momentarily exposing to 95% ethanol and immersing in 0.2% $HgCl_2$ solution for 3 min. The seeds were then subjected to six washings with sterile distilled water. Thoroughly washed seeds of wheat were sown on sterilized filter paper sheets placed in Petri plates. Six seeds were sown in each Petri dish with four replicates. Two ml of bacterial suspension were applied on seeds present in each dish with the sterilized pipette. The control group seedlings were immersed in sterile 0.05M NaCl. Sterilized distilled water (10 ml) was added to each Petri dish to wet the filter paper sheets and the seeds were covered with another sterilized filter paper sheet. The dishes were incubated in a growth room at 24°C. After 2 weeks, the wheat seedlings were examined for shoot, root growth (length, fresh weight). The isolates which have shown significant plant growth promoting effect were selected for the further screening step.

2.3.2.3 Screening in pot experiments

Based on the performance of rhizobacteria in the Plate experiments, nine effective plant growth promoting isolates (WR101, WR2, WR9, WR22, BL43, WPh45, WPh138, WR109, Xs148) were selected and used in pot trials. For pot experiment, a loamy soil sample with pH 7.7 was collected, air-dried, sieved (2-mm) before filling the pots. Inoculation suspension preparation and seed inoculation were performed as described for Petri experiments. Four inoculated and non-inoculated seeds of wheat were sown in pots (400 g soil per pot). Plants were supplied with half-strength Hoagland solution (Hoagland and Arnon 1950) receiving nutrient inputs of potassium/nitrogen/phosphorus in a ratio of 0.4:1:0.6. Two seedlings were maintained in each pot after germination. The pots were arranged in complete randomized design with six repeats. Plants were grown under greenhouse conditions with a temperature of 26°C to 28°C during the day and 16°C to 18°C at night. Four weeks after germination, plants were sampled to assess effects of inoculation. Six replications were harvested, roots and shoots were separated and soil particles were carefully removed from the roots under a gentle stream of tap water and were used to measure plant fresh mass, total root length and plant dry mass.

2.3.3 Antagonistic activity of bacteria isolated from wheat root, rhizosphere against pathogenic *Fusarium* isolates

2.3.3.1 Bacterial and fungal isolates.

Bacterial isolates originated from wheat root and rhizosphere, altogether 111 isolates, which have shown the stimulatory effect on wheat growth in plate experiments, were included in screening for their antagonistic activity.

In this study, *Fusarium culmorum, Fusarium solani,* and *F. avenaceum* were used as pathogenic *Fusarium* isolates. Monoconidial cultures of these isolates were stored in sterile soil tubes at 4°C. Active cultures were obtained from small aliquots of a soil culture plated on potato-dextrose agar (PDA). Fungal cultures were incubated at 25°C.

2.3.3.2 Selection of bacteria for ability to inhibit in vitro growth of Fusarium culmorum.

A total of 111 bacterial isolates were assayed in dual cultures on PDA for their ability to inhibit *in vitro* growth of *F. culmorum*. All bacteria-fungi combinations were examined on 15 ml of PDA in 9 cm Petri dish with 3 replications. A bacterial isolate per plate were spotted 1 cm from an edge of the plate, and was first incubated in the dark at 28°C. After 48 h, a 6-mm plug from the leading edge of a 5-day-old culture of *F. culmorum* on PDA was placed in 1 cm from the opposite edge of the plate. For control, PDA agar was inoculated with pathogen alone. Plates were incubated at 28°C. After 5 days, the length of hyphal growth toward the bacteria (Tinoc) and that on a control plate (Tcontrol) were measured. Inhibition of fungal growth was recorded as the relative growth ratio R = Tinoc/Tcontrol (Hamdam et al. 1991). There were 3 replicated plates in a completely randomized design for each bacterium–fungal isolate combination.

2.3.3.3 Specificity of bacterial antagonistic activity against Fusarium isolates.

Bacterial isolates selected from dual-culture assay with *F.culmorum*, were used to determine the degree of their antagonistic specificity against *Fusarium solani* and *F. avenaceum*. Fungal growth inhibition was assayed in a dual culture experiment on PDA as described previously.

2.3.4 The influence of beneficial bacteria isolated from wheat rhizosphere on growth promotion of some vegetable plants

2.3.4.1 Plants and bacterial strains

The experiments were carried out using quartz sand. Cauliflower (*Brassica oleracea L. cv*. Fremont), cucumber (*Cucumis sativus L.* cv. Corona F1), paprika (*Capsicum annuum L. cv.* Rosita F1), and tomato (*Lycopersicon esculentum* [Mill] L. cv. Counter F1) were used as test plants for the inoculation experiments. Seeds of these plants were obtained from the Institute of Vegetable and Ornamental crops, Grossbeeren, Germany.

Bacterial strains *Bacillus licheniformis* BL43, *Xanthomonas sp.* Xs148 and *Bacillus sp.* WR2, *Bacillus sp.* WR9, and *Bacillus sp.* WR22 were tested for their effect on plant growth of vegetable plants listed above.

2.3.4.2 Pot experiment setup

Based on the performance of bacterial isolates in the pot experiments with wheat, five effective PGPB isolates (WR2, WR9, WR22, BL43 and Xs148) were selected and used to test the effect of PGPB isolated from wheat on vegetable plants. Surface-sterilized seeds were sown on trays for 10 days. Uniformly germinated seeds were selected for transplantation to the pots containing sand to eliminate the variation in growth contributed by different endogenous germination rate/potential of the seeds. For pot experiments, plastic containers were filled with 800 g sand and half-strength Hoagland solution (Hoagland and Arnon 1950) was applied to provide nutrition to the plants.

Plant seedlings were divided into six treatment groups. Seedling roots were inoculated with respective bacteria inoculation suspension for 2 minutes. Control seedlings received 1 ml 0.05M NaCl solution. The inoculation treatments were set-up in a randomized design with six replicates. The seedlings were re-inoculated by applying 1ml bacterial suspension the plants rhizosphere soil 2 days after transplantation. Two seedlings were planted per pot and after germination; plants were thinned to one per pot. The pots were incubated in the growth chambers. The pots were placed on plates and, thus, nutrient loss through leaching was prevented. A climate chamber conditions were set as given Tab. 2, and a relative humidity of 70% day/80% night. Light intensity provided by lamps (Agro Son T 400, Phillips, Hamburg, Germany) was between 450 and 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at different positions in the chamber. Pots were re-arranged in regular intervals. Pots were always arranged in a completely randomised design.

Tab. 2: Physical conditions of the pot experiment in growth chambers.

	Light intensity in 24 hour				Temperature C°	
	2 h	8h	2h	12h	Day	Night
Cauliflower	200	400	200	-	20	18
Cucumber	400	600	400	-	25	20
Paprika	400	600	400	-	25	20
Tomato	300	500	300	-	23	18

2.3.4.3 Preparation of inoculation material

The bacteria were grown in glycerol-peptone-medium. Tubes were secured on a rotary shaker (120 rpm; 28°C) and agitated for 48 h. Tubes were centrifuged for 4 min at 7000 rpm/min.
The flow-through was discarded and the cells were washed with 0.05M NaCl solution three times. Bacterial cells were resuspended with 0.05M NaCl. Seedlings of these plants were inoculated with 1 ml of the bacterial suspension that resulted in an inoculum's density of 10^8 – 10^9 CFU ml^{-1}.

2.3.5 Harvest and plant analysis

Eight weeks after transplanting, the roots were separated from shoot, washed from the substrate with running cold water using a set of sieves (smallest sieve size 1 mm). The fresh weight (FW), length of plant shoot and root were recorded. Both shoot and root are dried at 80°C for two days, and dry weight (DW) was recorded separately.

2.3.6 Statistical analysis

Comparison of mean values of six replicates plant growth measurements, respectively, was performed using Student's *t* - test at a *P*-level of ≤ 5 %. All statistical analyses were performed using STATISTICA 6.0 (StatSoft, Tulsa, OK, U.S., 2001).

2.4 Results and Discussion

2.4.1 Bacteria isolation from root, rhizosphere and phyllosphere of wheat

2.4.1.1 Isolation and phenotypic characterization of diazotrophic bacteria

Basic phenotypic tests based on the colony formation at the surface of medium, cell shape, colony type, pigmentation on different media discarded 316 apparently sibling isolates (the isolates apparently belonging to same species) out of 780 isolates counted on Master plate. Isolates showing plant growth promoting effect were further identified based on utilization of specific carbon substrates. Natural colonisation of the studied isolates in wheat root, phylosphere and rhizosphere is given in Tab. 3. Studies based on the cultivation techniques provides useful information regarding microbial diversity in environmental samples, such as plant root, rhizosphere and phyllosphere samples, however, this studies suffers from bias, resulting from the media and cultivation condition applied, and from the inability to grow and isolate approx. 99% of the natural microbial community. These limitations have been overcome to some extent by using bacteria rRNA gene analysis for microbial population and colonization analysis. In our further studies, we have studied the colonization ability of certain bacteria in plant root and shoot using real-time PCR approaches (chapters 4, 6).

We identified the strains at genus level only. Majority were found to be facultative anaerobic, catalase positive spore-forming rods and identified as *Bacillus* sp. The remaining isolates were identified as *Pseudomanas*, *Burkholderia, Klebsiella* and others. Diazotrophic *Bacillus sp.* and *Pseudomonad sp.* are commonly isolated from wheat roots (Nelson et al. 1976, Ruppel 1987). Trials with plant growth promoting *Bacillus* species showed yield increases in rice (Sudha et al. 1999), wheat (de Freitas 2000), canola (de Freitas et al. 1997), maize (Pal 1998), sugar beet (Cakmakcı et al. 1999), sugarcane (Sundara et al. 2002), and conifer species (Bent et al. 2002).

Tab. 3: Natural colonisation of the studied isolates in wheat root, phylossphere and rhizosphere as counted on the master plate with a visual inspection.

Isolate	CFU 10^6	Origin	Used as
Bacillus sp. WR2	4	root	PGPB
Bacillus sp. WR9	15	rhizosphere	BCA/PGPB
Pseudomonas sp. WR12	1	rhizospere	BCA
Pseudomonas sp. WR14	4	rhizosphere	BCA
Bacillus sp. WR17	8	rhizospere	BCA
Bacillus sp. WR22	3	rhizosphere	PGPB
Pseudomonas sp. WPh45	1	phylossphere	PGPB
Azotobacter sp. WRh101	1	rhizosphere	PGPB
Pseudomonas sp. WR109	1	root	PGPB
Pseudomonas sp. WPh138	1	phylossphere	PGPB
Xanthomonas sp. WR148 (Xs148)	2	root	PGPB

Bacillus strains increased root and shoot dry weight, as well as total nutrient uptake, including N, by plants (Canbolat et al. 2006) by different plant growth promoting mechanisms, such as nitrogen fixation (Coelho et al. 2003), P solubilisation (de Freitas et al. 1997), antibiotic production (Rosado and Seldin 1993), cytokinin production (Timmusk et al. 1999), and increased root and shoot growth (Sudha et al. 1999). Some reports have shown the effect of plant growth promoting substances, such as phytohormones, produced by PGPB (Gutierrez Manero et al. 2001). Inoculation with *Bacillus megaterium* reduced the required P fertilisation of sugarcane by 25% (Sundara et al. 2002). *Pseudomonas* inoculants significantly increased root dry weight in spring wheat (Walley and Germida 1997) and promoted the growth of spinach (Urashima and Hori 2003).

A group of N_2-fixing bacteria isolated from the surface sterilized roots of wheat were identified as *Xanthomonas* based on a 114-bp 16S rRNA and 240-bp 16S-23S ISR sequences (chapter 3). Although *Xanthomonas* is generally regarded as a potential plant pathogen (Van den Mooter and Swings 1990, Van Sluys et al. 2002, Succstorf and Berg 2003), species of *Xanthomonas* have shown positive effect on growth effect on sunflower (*Helianthus annus* L.) growth (Fages and Arsac 1991). Moreover, xanthan produced by *Xanthomonas sp.* was reported to improve aggregate formation (Chaney and Swift 1986).

2.4.2 Screening of bacterial isolates for their effect on wheat growth

A series of plate and pot experiments were conducted to assess the potential of various wheat bacterial isolates for improving growth and yield of wheat (*Triticum aestivum* L.).

2.4.2.1 First screening of isolates for their effect on the plant growth.

First screening results of all bacterial isolates picked from Petri dishes with Ashby agar showed that 24 % (111 out of 464) bacterial isolates significantly increased plant growth with variable degree of stimulation (30% higher growth patterns: shoot and loot length, fresh mass) compared to non-inoculated plants and 25% (116) of tested bacterial strains showed inhibitory effect on plant growth. Before testing for plant growth promoting activities of 111 isolates using saline Syrdarya soils, they were screened for their ability to inhibit plant-pathogen *Fusarium* species in dual-culture assay.

Tab. 4: The effect of bacterial strains on plant growth and development

Effects	Proportion of bacterial isolates
Bacterial strains, used for study	100% (464)
Stimulators	24 (111)
Inhibitors	
Shoot	12 (56)
Root	13 (98)
Neutral	34 (158)

2.4.2.2 Antagonistic activity of bacteria isolated from wheat root, rhizosphere against pathogenic Fusarium isolates.

Antagonistic root-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soilborne pathogens (Weller 1988, Sørensen 1997). The goal of this work was to test if wheat-growth-simulating bacteria have also the ability to protect the plant from plant pathogens, namely F*usarium* species. Our reasoning is that plant growth promotion by bacteria can be partly due to their ability to protect the plant of pathogenic organisms in plant rhizosphere. In order to test the bacterial isolates of wheat for their ability to inhibit the growth of soilborne pathogens of wheat, 111 root-associated bacteria were evaluated using a combination of two screening steps.

2.4.2.3 Selection of bacteria for ability to inhibit in vitro growth of Fusarium culmorum.

As a result of the first screening, isolates were found to produce detectable inhibition zones against *Fusarium culmorum* on agar. 22% (24 of the 111) bacterial isolates from the wheat root and rhizosphere inhibited the *in vitro* hyphal growth of *F. culmorum* with R values lower than 0.7, while the majority had a neutral effect. The production of clear inhibition zones in dual culture screens is due to the production of antibiotics, toxic metabolites or siderophores as mechanisms for biological control (Swadling and Jeffries 1996). The presence and size of the zone of inhibition have been used as evidence of the production of antibiotics by the bacteria (Rothrock and Gottlieb 1981, Jackson et al. 1991, Crawford et al. 1993). Another possibility is that the bacterial isolates depleted the nutrient in the agar surrounding them and thereby inhibited the growth of *F. culmorum*. However, the PDA medium used for dual cultures is rich in nutrients and thus competition for them might be excluded. These observations from bioassays in dual cultures suggest that production of antibiotics and/or other antifungal substances by these bacteria may be involved in the inhibition of mycelial growth of fungal isolates. In most cases, bacteria effective as biocontrol agents of fungal plant diseases belong to the genera *Bacillus*, *Pseudomonas* and *Streptomyces* (Edwards et al. 1994). The 24 antagonistic bacterial isolates of the above belonged to genus *Bacillus* (11 isolates), *Micrococcus* (3 isolates), *Pseudomonas* (5 isolates) and others (5 isolates). These bacterial isolates were selected as antagonists for subsequent assays. In our study, the bacterial isolates from the wheat root and rhizosphere with the greater inhibitory capacity against *F. culmorum* are *Bacillus* sp., while isolates of *Pseudomonas sp.* showed a lesser inhibitory capacity against *F. culmorum* in dual cultures. Isolates showing a lesser ability to inhibit *F. culmorum* were not selected for further assays.

2.4.2.4 Specificity of bacterial antagonistic activity against isolates of Fusarium.

The antagonism of selected isolates towards other fungal pathogens was assessed because under field conditions synergistic interactions of pathogens occurred (Scholte and Jacob 1989). Biological control agents which can control more than one pathogen are extremely interesting. In our study, the majority of the bacteria tested in the second step was also active against other fungal pathogens, only a small number of selective *F. culmorum* antagonists was found (Tab. 5).

Tab. 5: Relative inhibition of growth of *Fusarium* species by selected strains.

Isolate	*F. culmorum*	*F. avenaceum*	*F. solani*
Pseudomanas sp. WR12	0.77	0.56	0.65
Pseudomanas sp. WR14	0.73	0.77	0.65
Bacillus sp. WR9	0.83	0.88	0.55
Bacillus sp. WR17	0.92	0.93	0.83

Note: Inhibition of fungal growth is expressed as the ratio of the radius of mycelial growth in the direction of the bacteria relative to the radius of growth on a control plate on which no bacteria were spotted. The values shown are means of 3 plates.

Pseudomonas sp. (WR12, RW14) and *Bacillus* sp. (WR9, WR17), which inhibited *in vitro* growth of *F. culmorum*, also inhibited growth of *F. avenaceum, F. solani* and nonpathogenic *Fusarium*. Bacterial isolates differed in the extent of growth inhibition of the fungal isolates, with *Bacillus* sp. WR17 showing the strongest activity. In our other studies, isolates *Pseudomonas* sp. WR12 and RW14 showed the strong antagonistic activity against tomato *Fusarium* dry rot *in vivo* (Juraeva et al. 2004) and tomato *Fusarium* wilt caused by *F.o. lycopersici* (unpublished data). Antagonistic bacteria may effect against a beneficial rhizosphere fungi. Fravel (1988) discussed the possibility of deleterious effects of antibiotic and antibiotic-like compounds, produced by biocontrol agents, on beneficial microorganisms. The inhibitory effect of antagonistic bacteria that inhibit pathogenic *F. culmorum* on suppression of beneficial/nonpathogenic *Fusarium* should be investigated.

2.4.3 Screening of bacterial isolates for their plant growth promoting effect

The data summarised in Tab. 6 demonstrate that seed inoculation with nine selected diazotrophic PGPB isolates significantly effected the growth of wheat under greenhouse conditions (Tab. 6). All the treatments, except for *Pseudomonas WR109 and Pseudomonas* WPh138, enhanced shoot dry weight as compared to the control. Shoot weight enhancement was greatest in response to *Xanthomonas sp.* Xs148 (46% more than the control) and *Bacillus sp.* WR9 (44% more than the control) whereas maximal root weight resulted from *Xanthomonas sp.* Xs148 (31% more than the control) followed by *Bacillus licheniformis* BL43 (28% more than the control) (Tab. 2). Of the bacterial inoculations, *Xanthomonas sp.* Xs148 inoculation produced the highest total weight (39% more than control) followed by *Bacillus sp.*

WR9*, Bacillus licheniformis* BL43, *Bacillus sp.* WR2, *Bacillus sp.* WR22, all increasing root dry weight significantly compared to non inoculated plants.

A number of studies (Abdel-Wahab and El-Sharouny 1979, Abdel-Waheb 1980, Rennie and Larson 1979, Rennie et al. 1983) found that wheat specifically harbored nitrogen fixing *Bacillus* in the rhizosphere. They found that nitrogen fixation by this bacterium and *Azospirillum* could account for 14% - 63% of the plant N, as tested by non-isotopic methods in vitro and in the field, and by isotopic methods in vitro. Some of these yield increases, however, may not be due to nitrogen fixation, but to bacterial production of plant growth substances (Gutierrez-Manero et al. 2001, Dobbelaere et al. 2003). The present experiment revealed that inoculation with diazotrophic bacteria *Xanthomonas sp.* Xs148, *Bacillus sp.* WR9, *Bacillus licheniformis* BL43, *Bacillus sp.* WR2, *Bacillus sp.* WR22 was an effective treatment for improving the parameters measured, especially with reference to the increase in shoot and root dry weight in nonsterilised soil. The plant growth promoting ability of these bacteria is further tested with vegetable plants.

In the present study, we investigated the diazotropic bacterial isolates that are positively tested from the wheat experiments for their effect on different vegetable plants growth. All parameters for all test plants inoculated with certain bacteria used in this study were increased relative to control. The significant effect of inoculation on plant growth differed depending on plant type. The most effective inoculation effect on all test plants were observed with *Xanthomomas* sp. Xs148 (Fig. 1).

Tab. 6: The effect of inoculation of wheat with diazotrophic PGPB on the length and weight of shoots and roots in nonsterile soil 4 weeks after planting. Plants were either non-inoculated with bacteria, or were inoculated with one of the bacteria given in the list. Effects of the bacterial treatment were tested with one-way ANOVA. Asterisks (*) denote significant differences between means of non-inoculated plants as determined by the Student-Newman-Keuls test ($P<0.05$). Values are means of 6 observations ± SE.

Bacterial strains	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	Shoot d. wt (g plant^{-1})	Root d. wt (g plant^{-1})
Control	17.60 ± 0.73	14.07± 0.92	0.031± 0.0020	0.0170± 0.0014
Bacillus sp. WR2	24.46± 0.87*	20.44± 1.64*	0.039± 0.0014*	0.0214± 0.0013*
Bacillus sp. WR9	23.93± 0.96*	21.00± 0.48*	0.045± 0.0018*	0.0204± 0.0011*
Pseudomonas WPh45	23.40± 1.12*	19.18± 2.37*	0.039± 0.0019*	0.0190± 0.0019
Azotobacter sp. WR101	23.93± 1.07*	14.98± 2.39	0.040± 0.0018*	0.0196± 0.0016
Bacillus sp. WR22	25.87± 0.27*	19.88± 2.03*	0.040± 0.0007*	0.0194± 0.0009*
Pseudomonas WPh138	22.70± 0.98*	19.74± 1.04*	0.033± 0.0022	0.0170± 0.0012
Xanthomonas sp. Xs148	25.69± 1.87*	17.50± 0.51*	0.045± 0.0017*	0.0223± 0.0014*
Pseudomonas WR109	20.24± 1.92	14.84± 3.55	0.033± 0.0023	0.0207± 0.0024
Bacillus licheniformis BL43	24.77± 1.33*	20.33± 1.12*	0.039± 0.0019*	0.0215± 0.0011*

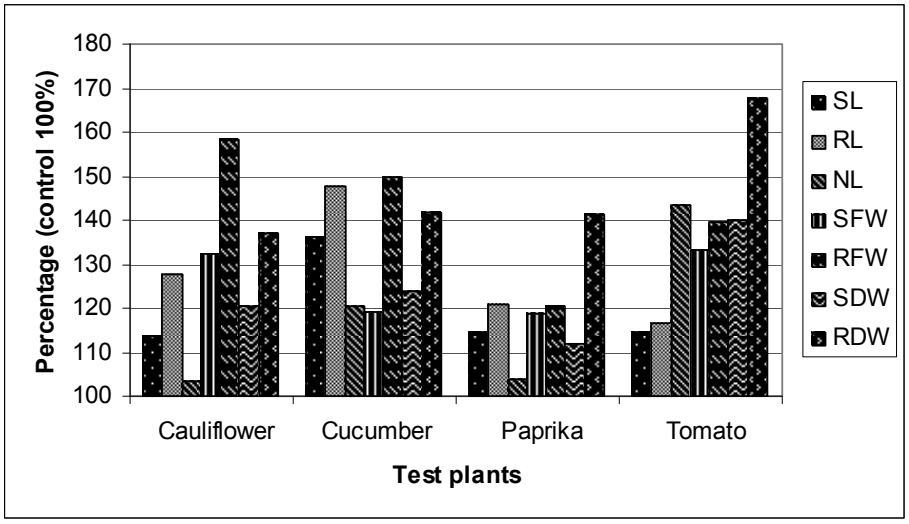

Fig 1: The effect of diazotrophic bacteria *Xanthomonas sp. Xs148* on shoot root length, fresh and dry weight of vegetables 8 weeks after planting. SL – shoot length, RL – root length, NL – number of leaves, SFW – shoot fresh weight, RFW – root fresh weight, SDW – shoot dry weight, RDW – root dry weight.

2.4.4 The influence of beneficial bacteria isolated from wheat rhizosphere on growth promotion of some vegetable plants

In all test plants, all growth parameters tested shown to be significantly increased (Tab. 7-10). This indicates that the bacterium does not distinguish between plants. Only exception is that the number of leaves in cauliflower, cucumber and paprika was not significantly different than control plants. Also, root length of tomato was not significantly different than control plants. The second most effective strain was *Bacillus licheniformis* BL43. Bacterial inoculation resulted in significantly increased growth of plant for all tested plants (Fig. 2). In cauliflower, cucumber and paprika, shoot length, shoot fresh weight and number of leaves, respectively, did not show significant response to the inoculation (Tab. 7 -10).

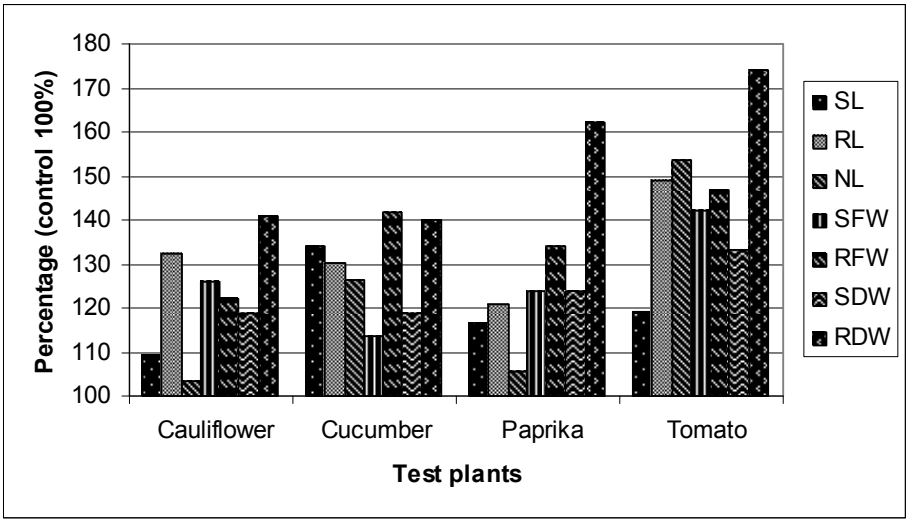

Fig. 2: The effect of diazotrophic bacteria *Bacillus licheniformis* BL43 on shoot root length, fresh and dry weight of vegetables 8 weeks after planting. SL – shoot length, RL – root length, NL – number of leaves, SFW – shoot fresh weight, RFW – root fresh weight, SDW – shoot dry weight, RDW – root dry weight.

Inoculation with *Bacillus* sp. WR2 showed the best effect on cauliflower growth (Fig. 3). Except number of leaves, all measured growth parameters reached the significant level (Tab. 8). Paprika plants also showed the significantly positive response to the inoculation with *Bacillus* sp. WR2 (Fig. 3). For cucumber plants, no significant effect on plant root was observed (Tab. 8). In tomato plants, plant shoot weight increased relatively to control, the rest of the traits tested were significant (Tab. 10).

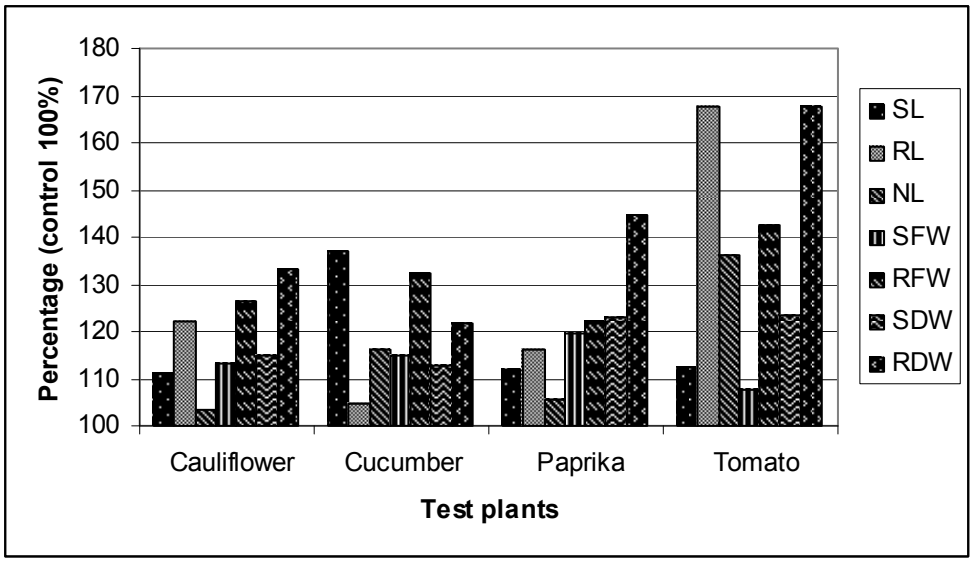

Fig. 3: The effect of diazotrophic bacteria *Bacillus sp.* WR2 on shoot root length, fresh and dry weight of vegetables 8 weeks after planting. SL – shoot length, RL – root length, NL – number of leaves, SFW – shoot fresh weight, RFW – root fresh weight, SDW – shoot dry weight, RDW – root dry weight.

The inoculation with *Bacillus* sp. WR9 was the most effective on paprika and tomato increasing all growth parameters significantly, except tomato shoot fresh weight (Fig. 4). In cauliflower plants, only increases in root growth of cauliflower were statistically significant (Tab. 7). On the contrary, cucumber plant root growth stayed unaffected by inoculation (Tab. 8).

The influence of the inoculation with *Bacillus* sp. WR22 was the most effective for paprika plants resulting in significantly increased growth pattern (Fig. 5). The least effected plant was cucumber showing only significant difference from control plants in shoot length and number of leaves (Tab. 8). In cauliflower, plant fresh and dry weight was shown to be significantly increased (Tab. 7). The response of tomato plants to inoculation was shown in root growth (length, fresh and dry weight) (Tab. 10).

Tab. 7: Cauliflower shoot, root length and dry matter 8 weeks after planting. Plants were either non-inoculated with bacteria, or were inoculated with one of the bacteria given in the list. Effects of the bacterial treatment were tested with one-way ANOVA. Asterisks (*) denote significant differences between means of non-inoculated plants as determined by the Student-Newman-Keuls test (P<0.05). Values are means of 6 observations ± SE.

Parameters	Control	*Bacillus* sp. WR2	*Bacillus* sp. WR9	*Bacillus* sp. WR22	*B. licheniformis* BL43	*Xanthomonas sp.* Xs148
Shoot length (cm plant^{-1})	19.1 ± 0.43	21.2* ± 0.30	21.2 ± 0.75	21.1 ± 0.64	20.9 ± 0.55	21.7* ± 0.71
Root length (cm plant^{-1})	16.6 ± 0.92	20.3* ± 0.70	19.7 ± 0.62	20.0 ± 0.85	22.0* ± 1.00	21.2* ± 0.84
Number of leaves (>3 cm)	5.80 ± 0.20	6.00 ± 0.00	6.20 ± 0.20	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Shoot f. wt (g plant^{-1})	14.6 ± 0.92	16.5* ± 0.26	16.0 ± 0.26	18.7* ± 0.67	18.4* ± 0.66	19.3* ± 0.52
Root f. wt (g plant^{-1})	2.53 ± 0.07	3.20* ± 0.15	3.45* ± 0.27	3.16* ± 0.17	3.09* ± 0.07	4.00* ± 0.17
Shoot d. wt (g plant^{-1})	1.70 ± 0.08	1.95* ± 0.02	1.87 ± 0.04	2.03* ± 0.09	2.02* ± 0.07	2.05* ± 0.08
Root d. wt (g plant^{-1})	0.27 ± 0.01	0.36* ± 0.01	0.39* ± 0.01	0.33* ± 0.01	0.38* ± 0.01	0.37* ± 0.02

Tab. 8: Cucumber shoot, root length and dry matter 8 weeks after planting. Plants were either non-inoculated with bacteria, or were inoculated with one of the bacteria given in the list. Effects of the bacterial treatment were tested with one-way ANOVA. Asterisks (*) denote significant differences between means of non-inoculated plants as determined by the Student-Newman-Keuls test (P<0.05). Values are means of 6 observations ± SE.

Parameters	Control	*Bacillus* sp. WR2	*Bacillus* sp. WR9	*Bacillus* sp. WR22	*B. licheniformis* BL43	*Xanthomonas sp.* Xs148
Shoot length (cm plant^{-1})	31.4 ± 2.44	43.0* ± 2.60	42.7* ± 2.95	40.4* ± 3.69	42.1* ± 1.90	42.7* ± 3.33
Root length (cm plant^{-1})	20.5 ± 1.51	21.5 ± 1.78	23.4* ± 1.88	22.5 ± 1.28	26.7* ± 1.30	30.3* ± 1.65
Number of leaves (>3 cm)	9.8 ± 0.58	11.4* ± 0.24	11.6* ± 0.24	11.2* ± 0.37	12.4* ± 0.24	11.8 ± 0.49
Shoot f. wt (g plant^{-1})	39.9 ± 2.11	45.8* ± 2.55	46.5* ± 2.00	42.8 ± 3.18	45.3 ± 1.42	47.6* ± 2.15
Root f. wt (g plant^{-1})	6.94 ± 0.87	9.17 ± 0.68	8.35 ± 0.81	9.28 ± 0.59	9.84* ± 0.76	10.4* ± 0.72
Shoot d. wt (g plant^{-1})	4.05 ± 0.31	4.57 ± 0.23	4.70 ± 0.11	4.35 ± 0.29	4.80* ± 0.16	5.02* ± 0.13
Root d. wt (g plant^{-1})	0.65 ± 0.08	0.79 ± 0.10	0.71 ± 0.03	0.75 ± 0.04	0.91* ± 0.05	0.92* ± 0.04

Tab. 9: Paprika shoot, root length and dry matter 8 weeks after planting. Plants were either non-inoculated with bacteria, or were inoculated with one of the bacteria given in the list. Effects of the bacterial treatment were tested with one-way ANOVA. Asterisks (*) denote significant differences between means of non-inoculated plants as determined by the Student-Newman-Keuls test (P<0.05). Values are means of 6 observations ± SE.

Parameters	Control	*Bacillus* sp. WR2	*Bacillus* sp. WR9	*Bacillus* sp. WR22	*B. licheniformis* BL43	*Xanthomonas sp.* Xs148
Shoot length (cm plant^{-1})	12.6 ± 0.29	14.1* ± 0.47	14.3* ± 0.01	14.9* ± 0.78	14.7* ± 0.32	14.4* ± 0.25
Root length (cm plant^{-1})	14.4 ± 0.40	16.7* ± 0.14	18.0* ± 1.34	15.8* ± 0.66	17.4* ± 0.50	17.4* ± 0.74
Number of leaves (>3 cm)	10.8 ± 0.20	11.4 ± 0.24	11.4 ± 0.24	12.4 ± 0.92	11.4 ± 0.24	11.2 ± 0.20
Shoot f. wt (g plant^{-1})	9.95 ± 0.35	11.9 ± 0.70	12.5* ± 0.74	11.9* ± 0.22	12.3* ± 0.25	11.8* ± 0.19
Root f. wt (g plant^{-1})	2.69 ± 0.14	3.29* ± 0.12	3.23* ± 0.09	3.45* ± 0.18	3.60* ± 0.07	3.24* ± 0.07
Shoot d. wt (g plant^{-1})	1.35 ± 0.04	1.66* ± 0.09	1.62* ± 0.06	1.63* ± 0.03	1.67* ± 0.03	1.51* ± 0.02
Root d. wt (g plant^{-1})	0.29 ± 0.01	0.42* ± 0.01	0.41* ± 0.01	0.42* ± 0.01	0.47* ± 0.01	0.41* ± 0.01

Tab. 10: Tomato shoot, root length and dry matter 8 weeks after planting. Plants were either non-inoculated with bacteria, or were inoculated with one of the bacteria given in the list. Effects of the bacterial treatment were tested with one-way ANOVA. Asterisks (*) denote significant differences between means of non-inoculated plants as determined by the Student-Newman-Keuls test (P<0.05). Values are means of 6 observations ± SE.

Parameters	Control	*Bacillus* sp. WR2	*Bacillus* sp. WR9	*Bacillus* sp. WR22	*B. licheniformis* BL43	*Xanthomonas sp.* Xs148
Shoot length (cm plant^{-1})	18.7 ± 0.60	21.0* ± 0.82	21.2* ± 0.37	21.4* ± 0.92	22.3* ± 0.69	21.4* ± 0.50
Root length (cm plant^{-1})	16.8 ± 0.72	28.2* ± 2.93	27.3* ± 0.73	28.3* ± 1.09	25.0* ± 1.89	19.6 ± 1.36
Number of leaves (>3 cm)	21.6 ± 1.86	29.4* ± 1.43	28.6* ± 0.40	33.2* ± 1.68	33.2* ± 0.73	31.0* ± 2.58
Shoot f. wt (g plant^{-1})	13.3 ± 1.65	14.3 ± 0.89	16.6 ± 0.46	17.5 ± 1.07	18.9* ± 0.72	17.7* ± 0.13
Root f. wt (g plant^{-1})	3.94 ± 0.53	5.62* ± 0.35	5.56* ± 0.16	5.51* ± 0.15	5.78* ± 0.26	5.50* ± 0.23
Shoot d. wt (g plant^{-1})	1.53 ± 0.19	1.89 ± 0.10	2.07* ± 0.05	1.91 ± 0.07	2.04* ± 0.03	2.14* ± 0.05
Root d. wt (g plant^{-1})	0.31 ± 0.03	0.52* ± 0.04	0.57* ± 0.02	0.48* ± 0.02	0.54* ± 0.03	0.52* ± 0.04

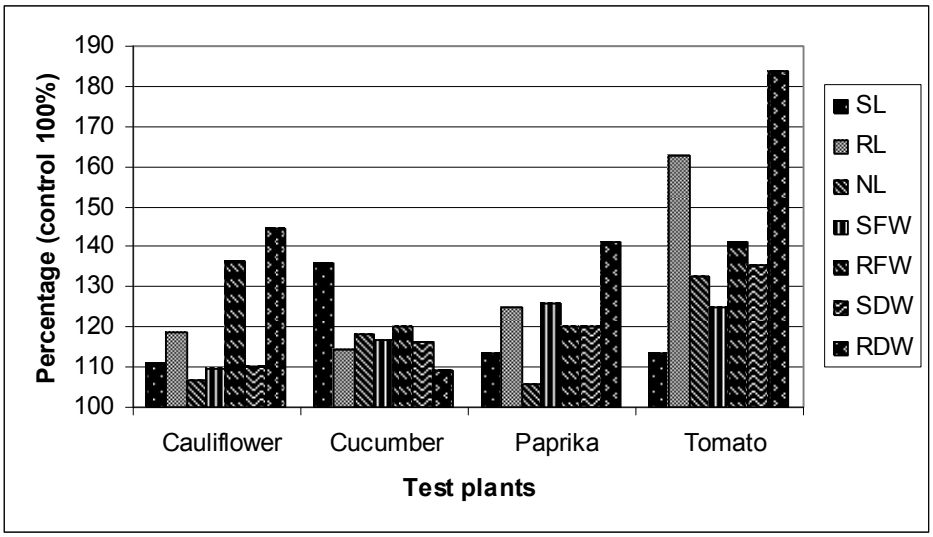

Fig. 4: The effect of diazotrophic bacteria *Bacillus* sp. WR9 on shoot root length, fresh and dry weight of vegetables 8 weeks after planting. SL – shoot length, RL – root length, NL – number of leaves, SFW – shoot fresh weight, RFW – root fresh weight, SDW – shoot dry weight, RDW – root dry weight.

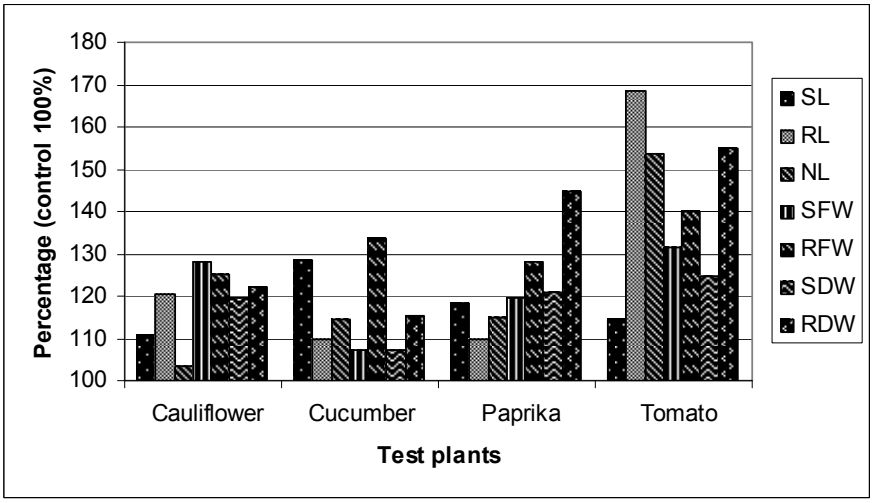

Fig. 5: The effect of diazotrophic bacteria *Bacillus* sp. WR22 on shoot root length, fresh and dry weight of vegetables 8 weeks after planting. SL – shoot length, RL – root length, NL – number of leaves, SFW – shoot fresh weight, RFW – root fresh weight, SDW – shoot dry weight, RDW – root dry weight.

The different responses of plants to PGPB include simulation of root branching and root hair development, stimulation of total nutrient uptake (especially that of nitrogen), and an increase in biomass accumulation.

It has been suggested that certain PGPB are plant-species-specific (Chanway and Holl 1993). And therefore, one of the effective strategies for initial selection and screening of rhizobacteria

is the consideration of host plant specificity. Inoculation with the strains used in this study has been carried out because of its capacity to increase the growth of wheat. Bacterial inoculation affected the early plant growth of tomato, cucumber, paprika and cauliflower grown in quartz sand. Paprika seedlings were more responsive to treatment with all used bacteria than were other test plants (Tab. 8). The establishment of inoculated PGPBs in the root system, showing a closer interaction between the bacteria and paprika roots, is a precondition for beneficial plant growth-promoting effects (Lucas-Garcia et al. 2003, Lugtenberg et al. 2001, Wiehe and Höflich, 1995). Characteristic quantifiers and qualifiers of root exudation play a fundamental role in the colonisation, as do the root structure/architecture and dynamics (e.g. flat rooting versus deep rooting) (Brimecombe et al. 2001, Neumann and Römheld 2001, Uren 2001). In this respect, the root system of paprika presents more surface contact than the root system of other plants tested, example tomato, which develops a main root with fewer branches.

Since, plants were grown in sand and supplied with half-strength Hoagland solution, it emphasizes that the bacteria tested can influence plant growth even in the presence of a nutrient solution. These findings suggest that plants may be grown with lower amounts of applied fertilizers and implies (1) a reduction in the cost associated with growing plants and (2) a reduction in the pollution associated with agricultural practices.

The importance of bacterial strain *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 is particularly significant regarding its beneficial effects on the plant growth and suppression the growth of plant pathogens. Therefore, bacterial isolates *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 are selected for further studies.

2.5 Conclusion

A set of wheat bacterial isolates was characterized based on their effect on plant growth promotion and pathogen growth inhibition. The isolates that tested positive for wheat plant growth were further studied for their effect on some vegetable plants. Thus the characterization and screening of PGPR has helped in the selection of *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 as potent strains in stimulating growth promotion in both cereal and vegetables.

Based on the results described above it can be concluded that the bacterial strains isolated in this study mainly belong to two genus, namely *Bacillus* and *Pseudomonas*, and that they stimulate the growth of wheat, and that PGPBs of wheat tested were not strictly plant-specific stimulating the different vegetable species.

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Chapter 3. Evaluation of 16S rRNA and 16S-23S ISR sequence-based analyses as part of a polyphasic approach to identify plant-inhabiting bacteria

3 EVALUATION OF 16S rRNA AND 16S-23S ISR SEQUENCE-BASED ANALYSES AS A PART OF A POLYPHASIC APPROACH TO IDENTIFY PLANT-INHABITING BACTERIA

3.1 Abstract

The potential of 16S rRNA and 16S-23S rRNA intergenic spacer region (ISR) sequence-based analyses to identify bacteria as part of a polyphasic approach was evaluated. These approaches were used to assess whether the taxonomies of plant-inhabiting diazotrophic bacteria belonging to the Pseudomonads and genus *Bacillus* as previously identified by phenotypic methods could be further defined. Bacterial isolates were identified to genus level and then their 16S rRNA and 16S-23S rRNA ISR gene segments were sequenced and analysed as a prelude to sequence-based identification. For one genus, however, bacterial identification was complicated because, according to GenBank entries, *Bacillus licheniformis* and *B. subtili* had up to 100% similar 16S rRNA gene sequence. Furthermore, significant sequence variability within and between strains of the same species also led to difficulties in identification. However, we found that 16S-23S rRNA ISR sequence-based analysis provided more specific identification when 16S rRNA sequence-based identification failed; although the potential of ISR analysis for bacterial identification is likely to depend on species characteristic sequence stretches of the ISR or on the bacterial species studied. Moreover, we performed similarity searches using different databases (EMBL-Bank, RDP-II and RIDOM) and programs (FASTA, BLAST and RDP-II) to evaluate their potential for bacterial identification. Here, we report that molecular identification methods may also lead to misidentification unless they are performed in combination with basic phenotypic tests. Finally, we conclude that 16S-23S rRNA ISR sequence-based identification in conjunction with analysis of 16S rRNA sequences has a great potential in future bacterial identification studies only when reliable and enough sequence entries in database are provided.

Key words

bacteria identification – BIOLOG test – sequence-based identification – 16S rRNA – 16S-23S rRNA ISR.

3.2 Introduction

Harmless bacterial isolates, for which the turnaround time to identification is less critical in comparison to clinical isolates, can generally be identified with conventional tests like morphological, physiological and chemotaxonomic characterization. However, when conventional methods are used to identify bacteria, interpretation of test results involves substantial subjective judgement (Stager and Davis 1992). Several commercial identification systems, such as the carbon source utilisation system developed by Biolog, Inc., offer computer-assisted identification of a wide variety of bacterial isolates (Miller and Rhoden 1991, Holmes et al. 1994, Tang et al. 1998). However, while such systems may reduce subjectivity and safe labour time, they still rely on phenotypic identification (Drancourt et al. 2000). Since some microorganisms share phenotypic profiles, but are segregated based on polymorphisms in their genome (Nakamura et al. 1999), the application of only morphological-biochemical-based approaches can lead to inaccurate bacterial identification. Moreover, even these approaches are sometimes unable to identify bacteria to the species level.

Nowadays, with the advent of molecular biology-based techniques, investigations based on comparative DNA sequence analysis of genes that carry phylogenetic information have become commonplace in microbiology as a tool for classification of microbial organisms. In contrast to phenotypic identification methods, identification using molecular techniques provides two primary advantages: (1) they are faster and (2) their accuracy in identification is improved (Springer et al. 1996, Patel et al. 2000). Two of the most useful and extensively investigated taxonomic marker molecules for bacterial identification studies are the 16S rRNA gene (Mahenthiralingam et al. 2004, Clarridge 2004) and ribosomal intergenic spacers, e.g. 16S-23S rRNA ISR (Drebot et al. 1996, Roth et al. 1998, Blackwood et al. 2004). However, some researchers found that for certain species of the genus, rRNA intergenic spacer regions do not contain sufficient stretches of identical sequence to allow identification at the phylogenetic level (Yoon et al. 1997, Yoon et al. 1998, Kuwahara et al. 2001). On the other hand, phylogenetic analysis of the 16S-23S ISR gene sequences of some species was confirmed by 16S rRNA gene-based phylogenic identification and had the added benefit of providing a higher resolution (Leblond-Bourget et al. 1996, Aakra et al. 1999, Goncalves and Rosato 2002, Song et al. 2004). Thus, the evaluation of the potential of ISR sequence-based analysis as part of a polyphasic approach for bacteria identification is both important and necessary.

The most common molecular-based method of bacterial identification is the amplification of marker genes, followed by either probe hybridisation, restriction fragment length polymorphism analysis (RFLP) or sequencing. All the aforementioned molecular-based methods have become

both routine and more affordable and offer the most accurate level of bacterial identification (Turenne et al. 2001). Although molecular identification of bacteria based on sequencing of taxonomic marker genes is regarded as the best method to date (Patel et al. 2000), it is dependent on the quality of available sequence databases, and currently, many of them are not optimal for this purpose. For example, the presence of faulty and/or redundant sequence entries (due to error prone sequencing techniques used earlier, e.g. reverse transcriptase sequencing), ragged sequence ends (resulting in wrong 'best' matches in similarity searches), non-characterised entries, outdated nomenclature, absence of quality control of sequence entries, and finally, a lack of type strains pertaining to many important microorganisms. Therefore, it is imperative to assess the quality of the database before using it for bacterial identification purposes.

In this article, conventional tests (like morphological, physiological and chemotaxonomic characterization) and the use of commercial identification systems (like, carbon source utilisation system BIOLOG) are referred to as phenotypic identification methods. In this study, bacterial isolates were identified to genus level by morphological characterization, and additional phenotypic investigations were not performed, but rather, 16S rRNA gene (Mahenthiralingam et al. 2004, Clarridge 2004) and 16S-23S rRNA ISR sequence analyses were used as a complement to phenotypic identification. We compared our sequences to those deposited in public databases, such as EMBL Bank (Cochrane et al. 2006), RDP-II (Maidak et al. 2001) and RIDOM (Harmsen et al. 2003). Although the RIDOM database was developed specifically for the identification of clinical microorganisms, we were interested to also use this database to identify harmless bacterial isolates, some of which may have clinical counterparts. The results presented here emphasize the need to take a polyphasic approach, i.e. phenotypic and sequence-based approaches, when identifying bacterial isolates, particularly in cases when correct assignment of the bacteria to the species level is required.

3.3 Materials and methods

3.3.1 Bacteria isolation

Wheat plants were uprooted 21 days after sowing in salty soil (Syrdarya, Uzbekistan). Roots were washed in running tap water to remove adhering soil, cut into 1 cm pieces and surface sterilised in 0.7% NaOCl solution for 30 minutes. In tubes containing 20 ml sterile water, 1 g root pieces were placed and macerated by vigorous shaking for 2 hours. The suspension was then spread on Petri dishes containing Ashby medium (Methods of soil microbiology and

biochemistry, 1991) to allow endophytic bacteria growth. After 4 to 7 days' incubation, single colonies were transferred to fresh Petri dishes containing the same medium. This process was repeated 3 times to purify bacterial culture – strain 148 - that were then stored in tubes containing Ashby agar medium. Isolate BL43 was taken from the Culture Collection of the Institute of Microbiology, Uzbekistan Academy of Sciences, Tashkent.

3.3.2 Phenotypic characterisation of bacterial isolates

The Gram reaction was performed as described previously (Suslow et al. 1982) using a 3% KOH test in parallel with traditional Gram staining (Gram 1884). According to their Gram-type, carbon source utilisation patterns of bacterial pure cultures were analysed using the BIOLOG® test as described below. Single colonies were picked, subcultured on BUGM (Biolog Universal Growth Medium) and incubated overnight at 28°C. A homogenous suspension of inoculum was made in 0.85% saline and diluted to a transmittance of 55 to 60% at 590 nm. From this suspension, 150 µl was dispensed into each well of the Gram-negative (GN2) or Gram-positive (GP2) MicroPlates™ (Oxoid GmbH, Wesel, Germany), which were then incubated for 24 h at 28°C. Colour development was measured at 590 nm at 4 and 24 h with a computer-controlled MicroPlate reader (Miller and Rhoden 1991, Holmes et al. 1994, Tang et al. 1998). The purified bacterial isolates were identified by comparing their substrate utilisation patterns with those found in the MicroLog System 2 database, release 4.01B (BioLog, Inc., Hayward, CA).

Further identification was performed using morphological characterisation and basic biochemical tests. After 24 and 48 h of growth on PA (Peptone Agar) at 28°C, colonies of purified bacterial isolates were characterised for the following traits: colour, shape, length, breadth and width, surface, opacity and texture. Motility, cell morphology, size and division mode were also evaluated by performing phase-contrast microscopy. Respiration type was determined by growth of bacteria in MPB (Meat Peptone Broth) at 28°C (for 3 d), temperature of growth was determined in MPA (Meat Peptone Agar) medium incubating at 4°C (for 14 d) and 50°C (for 5 d). Oxidase activity was tested using Bactident-Oxidase test strips (Merck) according to the manufacturer's instructions. The activity of catalase was tested by suspending a loopful of cells in a 10% (vol/vol) H_2O_2 solution.

Plant growth-stimulating effects of isolates were analysed for different agricultural crops, as described in previous studies (Egamberdiyeva et al. 2003, 2004).

3.3.3 Extraction of bacterial DNA

Pure bacterial cultures were grown at 28°C in standard I (Merck, Darmstadt, Germany) nutrient broth for 48 hours and bacterial DNA was extracted using MO BIO Ultra Clean™ Microbial DNA isolation kit (MO BIO laboratories, Inc. Hamburg, Germany) according to the manufacturer's instructions. Concentration and purity of DNA samples were measured at optical densities of 260 and 280 nm using an Eppendorf spectrophotometer. DNA concentration was adjusted to 20 ng μl^{-1} by diluting in deionised rRNA-free H_2O and stored at -20°C.

3.3.4 16S rRNA gene amplification and sequencing

16S rRNA genes were amplified by PCR using the primer set 27f and 1492r (Martin-Laurent et al., 2001) (Tab. 11). PCR reaction mixture contained 12.5 µl Master Mix (Qiagen, Hilden, Germany), 2.5 µl of each 10 µM primer and was brought to a final volume of 25 µl by addition of 5 µl of H_2O. 2.5 µl of bacterial DNA (approximately 50 ng) was used as template. Sterile water was used for the no-template negative control. PCR amplification was carried out in 96-well PCR plates with a Bio-Rad iCycler as follows: 94°C for 15 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min 15 s. A single final extension step consisted of 72°C for 10 min. Resulting PCR products were examined by agarose gel electrophoresis (2%) using GeneRuler™ DNA ladder mix, Marker SMO 0328 (MBI, Fermentas, St. Leon-Rot, Germany) as size standard (Fig.6). PCR products were purified using MiniElute™ PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced (the value read, MWG-Biotech).

3.3.5 16S-23S *ISR* amplification and sequencing

To obtain the 16S-23S rRNA ISR sequence information of selected bacterial strains, nested PCR was performed using universal prokaryotic primers (Tab. 11). The intergenic spacer regions were first amplified using primers 785 and 422. This was followed by a second nested PCR using primers 3-17R and EricM. PCRs were performed as described by Rumpf et al. (1999) with the following exceptions: (i) 300 nM of each primer was added to each reaction mixture and (ii) as information about the annealing temperature of the primer pair was not mentioned in the original protocol, the second amplification primers were optimally annealed at 60°C as determined experimentally using gradient real-time PCR in this study (data not shown). The reaction mixture contained 12.5 µl QuantiTect mastermix (Qiagen, Hilden, Germany) and 2.5 µl of each 300 nM primer. As template, for the first and the nested PCR, 2.5 µl of pure bacterial DNA (approximately 20 ng μl^{-1}) along with a 1:10 or 1:100 dilution of purified product of the first PCR were used, respectively. In both cases, the total reaction mixture was

brought to a final volume of 25 µl by the addition of 5 µl RNA-free H_2O (Qiagen, Hilden, Germany). The templates for the second PCR were prepared as follows: (i) the first PCR product DNA fragments were separated by electrophoresis in 2.5% agarose in TBA (thiobarbituric acid), (ii) gels were stained with ethidium bromide, (iii) gel pieces containing the desired-size DNA band (2000 bp) were cut and (iv) were cleaned with Mini Elute Gel purification Kit (Qiagen, Hilden, Germany). The amplified nested PCR products were excised from a 1% agarose gel after electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced (the value read, MWG-Biotech).

3.3.6 Sequence data analysis

The Internet tools CLUSTAL W, BLAST and FASTA3 provided by the European Bioinformatics Institute (http://www.ebi.ac.uk) and the RDP-II (Sequence Match, version 9.0, provided by the

Tab. 11: Specificity and nucleotide sequences of PCR primers used in this study.

Primer sequences	Target gene	Product length (bp)	Source
27f: 5'-AGAGTTTGATCCTGGCTCAG-3' 1492r: 5'-TACCTTGTTACGACTT-3'	16S rRNA	~1490	Martin-Laurent et al. 2001
1) 785: 5'-GGATTAGATACCCTGGTAGTC-3' 317R: 5'-GGCTGGATCACCTCCTT-3'	16S rRNA 16S rRNA	~2000	Rumpf et al. 1999
2) EricM: 5'-GCCAAGGCATCCACCG-3' 422: 5'-GGAGTATTTAGCCTT-3'	23S rRNA 23S rRNA	~830	Rumpf et al. 1999

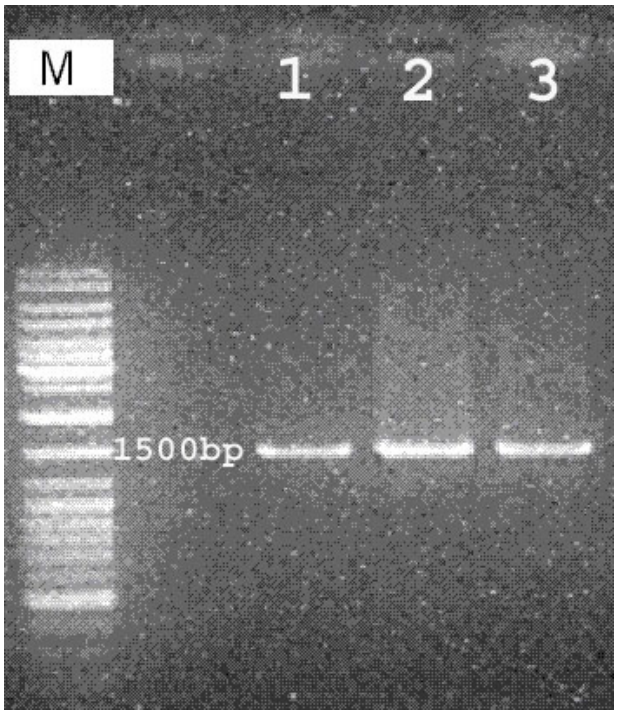

Fig. 6: Gel electrophoreses analysis of amplified 16S rRNA from bacterial isolates BL43 and Xs148. Lane M: Marker SMO 0328; lane 1 – *E.coli* (positive control), lane 2 – isolate BL43, lane 3 – isolate Xs148.

Ribosomal Database Project (Maidak et al. 2001; http://rdp.cme.msu.edu/)) were used for identifying the isolates. The sequencing data were analysed as follows: (i) assembly of the reverse and forward sequences into a consensus sequence; (ii) comparison of the consensus sequences with sequences deposited in EMBL-Bank (Release 85, December 2005), RDP-II and RIDOM databases and the basic local alignment search tools BLAST, FASTA and RDP-II. The newly determined sequences were aligned with their related sequences retrieved from EMBL-Bank by using the CLUSTAL W (1.8) graphical multiple alignment The DNA sequences determined for the strains BL43 and Xs148, both 16S rRNA and 16S-23S rRNA ISR sequences have been deposited in the EMBL-Bank database and given accession numbers EF601575 – EF601582.

3.3.7 Criteria for bacterial isolate identification

To define identification at the genus or species level, the following similarity score values were used: (i) when the comparison of the determined sequence with a reference sequence of a classified species deposited in the databases yielded a similarity score ≥ 99%, the unknown isolate was assigned to this species; (ii) when the score was < 99% and > 96%, the unknown isolate was assigned to the corresponding genus; and (iii) when the score was < 96% and > 92%, the unknown isolate was assigned to a family (Bosshard et al. 2003).

3.4 Results

3.4.1 Conventional bacterial identification

In this study, two bacterial isolates were tested: isolates 43 and 148. In the KOH test that lyses Gram-negative bacteria, isolate Xs148 was observed to lyse; thereby indicating that this isolate could be Gram negative. Considering the results of commercial BIOLOG test-based identification, Gram-positive isolate BL43 did not respond to the BIOLOG test, while isolate Xs148 was most similar to *Burkholderia glumae* showing an identity score of 0.590 (Tab. 12a). Using a range of common bacteriological tests, as listed in materials and methods, classic bacteriological profiles of bacterial isolates were obtained and both bacteria were classified accordingly to genus level. The Gram-negative strain, namely isolate Xs148, showed key classic characteristics of the *Pseudomonads*, e.g. the strain was aerobic and motile. Moreover, the cells are rod-shaped, approximately 0.5 - 1.0 μm by 1 – 1.5 μm. The strain grows best between 25°C – 37°C, but poorly at 45°C and produces beehive-shaped colonies on GPA medium after 48 hours of incubation at 28°C with a predominant amber-coloured colony type approximately 2 - 3 mm in diameter. In addition, the strain proved positive in the oxidase and catalase assays. Isolate BL43 was of the genus *Bacillus*. The strain was a facultative aerobe and motile. It was also rod-shaped (approximately 0.5 – 0.6 μm by 1.2 – 1.5 μm). Moreover, isolate BL43 was oxidase and catalase positive and capable of growth on N-free Ashby medium. The strain is also capable of forming endospores. Finally, the strain grows between 25°C – 37°C, but poorly at 45°C and forms colonies that are mucoid, slimy and tend to spread at 28°C. Both bacteria were capable of growth in N-free Ashby medium.

3.4.2 16S rRNA sequence-based bacterial isolate identification

The value of comparing 16S rRNA sequences derived from unknown bacterial isolates to EMBL-Bank and RDP-II databases is addressed in Tab. 12a and Tab. 12b. In these databases, the degree of relatedness is expressed differently. EMBL-Bank's main measure is percent identity and RDP-II's measure is a relatedness value somewhat close to (but lower than) the EMBL-Bank percent identity (Clarridge 2004). The identification of bacterial isolates based on 16S rDNA gene sequencing was determined by choosing the species of bacteria with the highest identity match. Searching for sequence similarity against the EMBL-Bank database was performed using the FASTA and BLASTN programs. The identification of bacteria was complicated due to numerous ambiguous results. For example, searching the EMBL-Bank database based on BLAST search using the BLASTN program showed that isolate BL43 was

99% identical to four species (among 100 hits obtained in BLAST searches) of *Bacillus subtilis* group: *B. licheniformis, B. subtilis*, *B. mojavensis* and *B. vallismortis* and also *B. amyloliquefaciens*. Moreover, BLAST searches showed 99% identity of the sequence in query to a micro-organism belonging to another family, *Pseudomonas* sp. (EM_PRO:AB211031). When the FASTA program was used, almost the same results were obtained: isolate BL43 yielded an ambiguous result showing 99.72% identical percent similarity to two species *B. licheniformis* and *B. subtilis*, while it was 99.37% identical to *B. mojavensis*. For isolate Xs148, different results were obtained even when the sequence was compared against the same database (EMBL-Bank) by using different programs (FASTA and BLASTN), resulting in the assignment of different identities: using BLAST searches resulted in the highest matches to the *Pseudomonas* genus, while using the FASTA program, isolate Xs148 was closest in identity to the *Xanthomonas* species (Tab. 12a). Analysing the search results obtained from FASTA searches for isolate Xs148 in more detail revealed that this isolate was 96% identical to *Xanthomonas oryzae pv. oryzae* and *Xanthomonas campestris,* and 95% identical to a different genus, *Pseudomonas*. To demonstrate the quality and accuracy of results provided from different programs and databases, searches for sequence similarity analysis were further performed using RDP-II (Sequence Match, version 9.0). Using RDP-II with dataset-2 (consisting of both type and non-type strain and isolate sequences), the best match for isolate Xs148 sequences was *Azotobacter salinestris* with similarity scores of 0.776 (Tab. 12b), while the highest match to *Xanthomonas oryzae pv. oryzae* (sequence similarity – 96%) and *Pseudomonas* sp. (sequence similarity – 97%) was obtained using FASTA and BLASTN, respectively. Considering the results of the two data sets of the RDP-II database used, dataset-1 showed highly ambiguous results, most probably, due to the short target sequence or due to the limited number of sequences included in the sequence match search (Tab. 12b); whereas, dataset-2 had almost 17 times more sequences in the search.

3.4.3 16S-23S ISR-based bacterial isolate identification

For isolate Xs148, 16S rRNA (based on the FASTA analysis) and 16S-23S rRNA ISR designations offered a consensus at the genus level (98.53% similarity to EM_PRO:DQ003220) showing that this bacterium belongs to the *Xanthomonas* genus. Among 100 hits obtained from BLAST searches for isolate Xs148 sequence (240 bp) similarities, 93% of the 100 hits obtained belonged to the genus *Xanthomonas,* mainly to *Xanthomonas oryzae* and *Xanthomonas campestris*, and 7% belonged to members of other genera, such as *Stenotrophomonas maltophilia* (5%), *Pseudomonas* (1%), rice phylosphere bacterium (1%) and uncultured bacteria

(1%). Considering that isolate Xs148 was identified as *Burkholderia glumae* by the BIOLOG test, we performed CLUSTAL W alignment analysis of 16S-23S rRNA ISR sequences of randomly selected *Burkholderia* genus strains with isolate Xs148-derived ISR sequences. The alignment results showed only 76-78% similarity scores to the 16S-23S rRNA ISR partial sequence of isolate Xs148, while *Xanthomonas* genera has a 100% similar sequence composition; thereby, strongly suggesting that from the molecular point of view, this bacteria belongs to the *Xanthomonas* genera. We must note however that among BLAST search isolate Xs148 (Rice phyllosphere bacterium (EM_PRO:AY485407)) with a sequence similarity of 99.63%, since this entry was not fully characterised.

The ISR sequence analysis of isolate BL43 showed that among 100 hits, there was only three matches to the Bacillus genera, B. subtilis (EM_PRO:BSTGRG16), B. licheniformis (EM_PRO:CP000002), and B. clausii (EM_PRO:AP006627), showing 88%, 88% and 75% identity, respectively, while the 16S rRNA sequence of this bacteria was above 99% similar to different Bacillus species (Tab. 12b). Since, one of the mostly related species B. amyloliquefaciens was not in 100 hit list, the CLUSTALW analysis were performed comparing the isolate BL43 and B. amyloliquefaciens 16S-23S ISR sequences (EM PRO:AF478079). The result showed low similarity of 47%. The rest of the hits included microorganisms belonging to the same order of *Firmicutes* (order *Bacillales,* class *Bacilli*) as our selected bacteria with very low similarity: *Staphylococcus haemolyticus (*81% identical), *Listeria innocua* (74% identical) and *OceanoBacillus iheyensis* (71% identical). This did not provide enough information to be able to identify isolate BL43 based on 16S-23S rRNA ISR sequences alone. If we consider the previous reports on the maximum level of ISR divergence between the strains belonging to the same species, e.g. 13% for *Bifidobacterium* species, (Leblond-Bourget et al. 1996), 16% for Saccharomonospora (Yoon et al. 1997) and *Nocardioides* (Yoon et al. 1998), the 12% difference between EMBL-Bank deposited *Bacillus licheniformis* and *Bacillus subtilis* and the studied isolate BL43 is in the scope of acceptable divergence. According to both FASTA and BLAST, target bacteria sequences showed 43 out of 100 hits for the Gram-negative bacilli (Tang et al. 1998) genus *Acinetobacter* sp. with identity ranging from 61 to 98%. Since the *Acinetobacter* genus belongs to Gram-negative bacteria, respective hits were excluded from identification analysis. Moreover, when 16S rRNA partial sequences of *Acinetobacter baumannii* (AJ247197) deposited in EMBL-Bank and isolate BL43 derived in this study aligned using CLUSTAL W, these two organisms showed only a 63% score of identity.

Tab. 12a: Results obtained from phenotypic and molecular biological methods used to identify plant-inhabiting bacteria.

Isolate	Phenotypic identification	BIOLOG identification		16S rRNA sequence-based identification					
	Family/genus	Genus/species	Score	Sequence length bp	Programs and databases used with EMBL database				
					NCBI-BLASTN 2.2.13 / EMBL database			NCBI-FASTA version 3.4t / EMBL database*	
					Genus/species	ID %	Included sequences	Genus/species	ID %
43	*Bacillus sp.*	No ID	-	1448	*B. licheniformis* *B. subtilis* *B. mojavensis* *B. vallismortis*	99	3.711.827	*B. licheniformis* *B. subtilis*	99.724
148	*Pseudomonads*	*Burkholderia glumae*	0.590	100	*Pseudomonas sp.*	97	3.711.827	*Xanthomonas oryzae*	96.000

Tab. 12b: Results obtained from phenotypic and molecular biological methods used to identify plant-inhabiting bacteria.

* - the number of sequences included in the similarity search was not provided.

Isolate	16S rRNA sequence based identification				16S-23S ISR sequence based identification				
	Seq length bp	Program and dataset used with RDP-II database			Seq length bp	Programs and databases used with EMBL database			
		SeqMatch - Dataset 2				NCBI-BLASTN 2.2.13 /EMBL database*		NCBI-FASTA version 3.4t /EMBL database*	
		Genus/species	Score	*# of included sequences*		Genus/species	ID %	Genus/species	ID %
43	1448	*B. licheniformis* *B. subtilis*	0.987	*74498*	389	*B. licheniformis* *B. subtilis*	88.0	*B. licheniformis* *B. subtilis*	88.0
148	100	*Azotobacter salinestris*	0.766	*29791*	273	*X. oryzae* *X. campestris*	100.0	*Xanthomonas sp.*	98.5

3.5 Discussion

The exact taxonomic affiliation of some microorganisms can only be ascertained by using a polyphasic taxonomic approach. The present study genetically characterised two bacterial isolates isolated from plant endorhizosphere and compared these results against those derived from bacteriological and metabolic identification techniques to assess the need for a polyphasic approach to identify bacteria. Although the BIOLOG system has been evaluated as having the largest database (Holmes et al. 1994), this system's identification did not always agree with biochemical criteria belonging to some genera including *Bacillus* (Tang et al. 1998). Therefore, failure of the BIOLOG system to identify isolate BL43 could be explained by the fact that this isolate belongs to the genera *Bacillus* as established using rRNA sequence tests. Moreover, there are examples where reliance on only biochemical-based identification could lead to inaccurate bacterial identification because some closely related microorganisms within the *Bacillus* genus can share phenotypic properties, but have previously been classified as different species based on DNA re-association values (Nakamura et al. 1999). Furthermore, inaccurate conventional identification of *Bacillus* species due to a result of unmatched Gram and biochemical profile determination as well as growth requirements was established using 16S rRNA sequence-based identification (Drancourt et al. 2000). In this study, genotypic methods based on rRNA sequence analysis improved the identification of plant-inhabiting diazotrophic bacteria; thereby completing the identification results obtained using conventional biochemical methods and the BIOLOG® test.

3.5.1 16S rRNA sequence-based bacteria identification and conflicting results

To identify microorganisms correctly, sequences deposited in databases must be correct and appropriately named. To date, there are only a few reports on the quality of commonly used databases, e.g. GenBank-EMBL, RIDOM (Harmsen et al. 2003) and RDP-II (Maidak et al. 2001) as well as user preference of these databases along with programs concerning the quality and/or number of sequences (Turenne et al. 2001, Cloud et al. 2002). As sequences can be deposited in the GenBank-EMBL and RDP-II databases without undergoing any checks, it is not surprising that errors do occur as regards species assignment (Harmsen et al. 2003). For example, among EMBL-Bank deposited sequences of *Pseudomonas* spp., we found one that we believe is misidentified; EM_PRO: AB211031 "*Pseudomonas* sp. SSCS3 gene for 16S rRNA, partial sequence (1479 bp)" shows 99% total 16S rRNA similarity to *Bacillus subtilis*, but 72-78% to sequences derived from strains belonging to *Pseudomonas* genus. Therefore, EM_PRO:

AB211031 is most probably the sequence of a strain belonging to the *Bacillus* genus. This obviously incorrect database entry highlights the common presence of faulty sequence entries.

Turenne et al. (2001) reported that the quality and/or the number of the sequences derived from a certain group of bacteria in the RIDOM database are higher. However, although the RIDOM database has a particularly good collection of mycobacterial sequences, this does not extend to all other bacterial categories. We found, for example, that the RIDOM database is not useful for identification of the bacterial strains investigated in this study although Blackwood et al. (2004) reported that 16S rRNA gene sequences for 65 (of all 83) type strains of the *Bacillus* genus have been submitted to the RIDOM database at http://rdna.ridom.de/. It is therefore likely that submission of all these sequences is either not yet complete or that the database has not been updated. Note that it was last modified on 05.08.2005.

In this study, the 16S rRNA-derived sequences were analysed by comparing the results from the RDP-II database with those of the EMBL-Bank. We suggested that since RDP-II offers an opportunity for comparing user submitted sequences to the RDP-II database using data subsets (data sets) available for sequences from type material, it allows avoiding using taxonomically misidentified and non-cultured bacteria sequences in searches that may result in ambiguous identification. Therefore, we performed searches in RDP-II for sequence similarities to strains 43 and 148 using two data subsets consisting of sequences of (i) type strains (data set-1) and (ii) both type and non-type strains and isolates (data set-2).

For isolate BL43, the search results of three programs (BLASTN, FASTA and RDP-II) were in agreement when the RDP-II search performed with data set-2. However, with data set-1, we obtained results that were not in agreement with the FASTA and BLASTN results, and hence, these were discarded. Therefore, we suggest that although performing sequence similarity searches with the sequences of type strains provided by the RDP-II program should allow avoiding inaccurate identification, it is likely that there are not enough type strain sequence entries in the current RDP-II database. Hence, the observed difference in results for the two data sets of RDP-II may be partly explained by the vastly lower number of sequences included in searches with data set-1 compared to that of data set-2 (Tab. 12a). Hence, these results were not considered in this study.

Because the 16S rDNA gene is highly conserved, determination of species and strain distinctions relies upon the resolution of only small differences between sequences. Moreover, as two distinct species may possess identical 16S rRNA sequences including *Bacillus*, gene sequence identification is not foolproof (Ash et al. 1991, Fox et al. 1992, Clarridge 2004). Using BLAST searches in the EMBL database, since the identity match value of isolate BL43

to different species (*Bacillus licheniformis* and *Bacillus subtilis*) was equal; it was not possible to distinguish this isolate to species level. Furthermore, since isolate BL43 was assigned to *Bacillus licheniformis* and *Bacillus subtilis* with sequence similarities of 99.72% and the second classified species in the scoring list (*B. mojavensis and B. vallismortis*) showed less than 0.5% additional sequence divergence, isolate BL43 was marked as a "*Bacillus licheniformis* or *Bacillus subtilis* with low demarcation to the next species, such as *B. mojavensis and B. vallismortis*" (Bosshard et al. 2003). Finally, besides ambiguous results regarding related species, search results also showed the highest matches to non-respective Gram-type microorganisms. Therefore, we were careful to only consider matches with the target bacteria Gram-type. Taken together, the results presented here emphasise that a number of basic biochemical tests alongside sequence-based analysis are of critical importance.

3.5.2 Comparison of 16S rRNA sequence-based identification results and conventional bacteria identification

Considering previous studies, both *Pseudomonas* and *Xanthomonas* species are regularly misidentified as *Burkholderia* species (Burdge et al. 1995, Urakami et al. 1994). Recent studies based on rRNA sequence showed that *Burkholderia* species sharing characteristics in common with members of the genus *Pseudomonas* are distinct and separate, and therefore, some species of the *Burkholderia* genus were transferred from one genus to other (Kersters et al. 1996). Our results for isolate Xs148 were in agreement with the aforementioned studies reporting conflicting results between conventional and sequence-based identification: the BIOLOG test resulted in *Burkholderia glumae* being identified, whereas 16S rRNA sequence-based analysis using FASTA, BLAST and RDP-II (with data set-2) showed the highest matches to *Xanthomonas oryzae pv. oryzae*, *Pseudomanas* sp. and *Azotobacter salinestris*, respectively. Although the best sequence matches given by these three programs were different, the EMBL-Bank sequences of *Burkholderia* were not even shown on the BLAST match list due to too low similarity (identity score by multiple alignment is 77%) between the sequences; thus, we strongly suspect that this strain was misidentified by conventional methods.

For some genera or species, the conflict between sequence-based and phenotypic identification is simply due to too few entries deposited in the databases, so that the similarity level for a particular query does not exceed to species (99%) or even, genus level (96%) (Drancourt et al., 2000). However, we found that there are more than 600 entries for cultured *Burkholderia* species in the EMBL-Bank (January 2006). Such a number should be enough to determine the sequence in question, at least to the genus level, if the target bacteria belonged to this genus. On the other hand, it may be argued that the conflicting results obtained for isolate Xs148 in this

study may be due to the very short sequence length analysed. However, Wilck et al. (2001) reported that DNA sequences of even less than 200 bp were enough for identification purposes. Although only partial sequence of isolate Xs148 was used, it suggests that this isolate belongs to a different genus as identified by conventional methods. Therefore, taken together, we suggest that 16S rRNA-based identification of strains of some genera at the species level is not reliable enough and requires additional tests. Finally, reliance on a single molecular method for species definition, such as 16S rRNA gene sequencing, cannot take into account small evolutionary changes, such as point mutations (Stackebrandt et al. 2002). Thus, in practice, a polyphasic approach including alternate gene targets performed in parallel with the examination of a number of phenotypic properties is necessary for definitive species identification.

3.5.3 16S-23S ISR-based bacteria identification

Since 16S rRNA-based identification did not enable us to identify isolate Xs148 or to classify isolate BL43 to the species level, the potential of alternate genes was suggested. A review of current taxonomic molecular marker genes for bacteria identification prompted us to examine the use of the 16S-23S rRNA ISR (Harrel et al. 1995, Dong and Cote, 2003) for further sequence-based identification. The 16S-23S rRNA ISR proved to be the best alternate target to 16S rRNA because we found a high consensus between results from 16S rRNA and 16S-23S rRNA ISR sequence-based identification for isolate Xs148. Goncalves and Rosato (2002) reported that among *Xanthomonas* species, 16S-23S rRNA ISR sequence similarity ranged from 63 to 99%; however, the topology of the 16S-23S rRNA ISR phylogenetic tree was very similar to that of the higher level of the diversity among the ISR (ITS) sequences (16.2%) compared with the 16S rDNA sequences (1.8%). In this study, both 16S rRNA and 16S-23S rRNA ISR analyses showed that studied bacterial isolate Xs148 fell into Cluster I of *Xanthomonas* species (as determined by Goncalves and Rosato, 2002) showing the highest similarity to *Xanthomonas oryzae* and *Xanthomonas campestris*. For isolate Xs148, direct sequence determination of 16S-23S ISR fragments represented a highly accurate method for bacterial identification to the species level, even when the species in question was notoriously difficult to identify by 16S rRNA sequence-based identification. This is because 16S-23S rRNA ISR similarities reflect phylogenetic relationships and has more discriminative nucleotide stretches, which allow identifying in species; even to the strain level (Blackwood et al. 2004).

The 16S-23S rRNA ISR-based approach failed to identify isolate BL43 producing differing data to both the basic bacteriological and 16S rRNA sequence-based identification results. We suggest that the potential of ISR analysis for bacteria identification is likely to depend on

specific nucleotide stretches of the ISR or on the bacteria species studied (Yoon et al. 1997, Yoon et al. 1998, Kuwahara et al. 2001).

3.6 Conclusion

Since no identification method is able to identify all microorganisms and each of them has its advantages and disadvantages, we tested the potential of 16S rRNA and 16S-23S rRNA ISR sequence-based molecular identification methods in combination with conventional identification ones (morphological-biochemical method and phonological BIOLOG test) as a part of a polyphasic approach to identify two plant root colonizing bacteria as *Bacillus subtilis* and *Xanthomonas sp.*

The present study is unique in that (i) results derived from 16S rRNA and 16S-23S rRNA ISR sequence-based identification were compared to better allow further species definition and (ii) two bacterial strains belonging to different taxonomic groups were examined to ensure that the findings reported here are applicable to a wide range of bacteria.

From our analyses, we conclude that (i) rRNA sequence-based identification should be performed in conjunction with traditional biochemical bacteriological tests that provide basic information about the microorganism in query, (ii) for similarity scores of different species belonging to the same genus of less than 1 % dissimilarity, the FASTA program displays more accurate values, i.e. to 3 decimal places, than BLAST and (iii) 16S rRNA in conjunction with 16S-23S rRNA ISR sequence-based identification can be used to identify and differentiate between the species only if the quality of the database is high enough, i.e. it contains a high number of reliable sequence entries taken from a comprehensive range of species.

The present data suggest that an integrated genetic and phenotypic approach for taxonomic classification, a so-called “polyphasic approach” (Vandamme et al. 1996), provides more specific and accurate species definition.

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Chapter 4. Enumeration of two diazotrophic bacteriall strains in plant samples using 16S-23S ISR species specific regions and real-time PCR

4 ENUMERATION OF TWO DIAZOTROPHIC BACTERIAL STRAINS IN PLANT SAMPLES USING 16S-23S ISR SPECIES SPECIFIC REGIONS AND REAL-TIME PCR

4.1 Abstract

To evaluate the effect of inoculated bacteria, its colonisation ability must be accurately measured. For two diazotrophic bacteria, which have shown PGP effect on different agricultural crops, strain specific primers were developed from the highly variable regions of 16S-23S ISR, amplifying a 109-bp fragment, for *Bacillus licheniformis* BL43 and yielding a 89-bp product for *Xanthomonas sp.* Xs148, respectively. The strain specificity of the primer sets was checked by comparison with available ISR sequences in databases and by PCR using DNA from target and reference strains as well as some plant-inhabiting bacterial isolates. The primer system used produced only the single expected fragment for only target bacteria. The detection limits of developed method to quantify *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148 were 9.17E+03 and 1.11E+05 gene copies/µl, respectively. In this paper, we reported on the preliminary application of the designed specific primers to the study of the response of inoculated diazotrophic bacteria populations to the N availability.

Key words
strain-specific primers – 16S-23S ISR – colonisation – *Bacillus licheniformis*– *Xanthomonas* – cucumber

4.2 Introduction

The diazotrophic bacterial strains *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 were previously shown to promote the growth of some cereals and vegetables (Juraeva et al. 2003, Juraeva and Ruppel 2005a). Plant growth promoting effect of inoculated bacteria take place if the inoculated strains are able to colonize in the rhizosphere, on or inside of host plant root. When redetection of introduced bacteria proves the colonization ability of potential inoculant strain (Tan et al. 2001), accurate quantification analysis of strain colonization after inoculation is of critical importance to verify the association of inoculated bacteria with plant growth promotion quantitatively and to study the response of these bacterial population to some environmental factors, particularly under competitive conditions in non-sterile substrates.

Assessments of plant growth promoting bacteria in environmental samples have been mainly based on PCR performed with primers with different degrees of specificity (Bauernfeind et al. 1999; Coenye et al. 1999; Vandamme et al. 1997). There is a high possibility for the failure of the species-specific primer based quantification, because (i) DNA sequence composition may vary within and between strains of the same species (Bosshard et al. 2002, Clayton et al. 1995), or (ii) on the contrary, the composition of primer targeted genes may be too conserved in a bacterial group (e.g. *Bacillus subtilis* group) (Nakamura et al. 1999), or (iii) the species-specific primers which have been designed based on the sequences deposited in databases may target the point mutated regions of the studied bacteria DNA. Therefore, for reliable and specific redetection and/or quantification of the bacteria after application to plant, strain-specific primer design is particularly important.

The high variability of the 16S-23S ISR allowed the designed of genus-, species-, and strain-specific primers (Tyler et al. 1995, Moreira and Amils, 1996, Tilsala-Timisjarvi and Alatossava 1997, Chun et al. 1999, de Olivera et al. 1999, Tan et al. 2001). Our previous studies showed that 16S rRNA sequences of the investigated bacteria in this study showed that this gene in both bacteria was highly conserved (Juraeva and Ruppel, 2005b). Therefore, the development of specific primers designed from a consensus sequence obtained from 16S-23S ISR of used bacteria was aimed.

In this study, a quantitative real – time PCR technique with newly designed specific primers allowing detection and quantification of *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 in both pure cultures and environmental plant material were developed and the preliminary application of this method to quantify both diazotrophic bacteria after inoculation to plants was performed with cucumber root samples. Moreover, the utility of the developed method in determination of environmental factor effect, such as N application level on inoculated diazotrophic bacteria colonization ability when grown under greenhouse conditions were tested.

4.3 Material and methods

4.3.1 Bacterial strains

Diazotrophic bacteria originally isolated from maize rhizosphere (provided by Microorganism collection, Institute of Microbiology, Uzbekistan Academy of Sciences) and wheat root (Juraeva and Ruppel, 2005b) identified as *Bacillus licheniformis* and *Xanthomonas spp.*, respectively, using polyphasic approach (see Chapter 2). Inoculation using these diazotrophic

bacteria affected the early plant growth of some agricultural crops grown in loamy sand (Egamberdiyeva et al. 2002, Egamberdiyeva et al. 2003, Egamberdiyeva et al. 2004a, 2004b).

4.3.2 Design of target bacteria-specific primers

To design strain-specific primers, we used the 16S-23S ISR partial sequence data for *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 as determined in our previous study (see Chapter 2). Among the 16S-23S ISR sequences deposited in EMBL-Bank, the entries, which showed the highest similarity to the target bacteria sequences in BLAST search, were selected. These entries with the target bacteria sequences were applied to determine the strain-specific region for primer design. The specific primers were designed using the software Beacon Designer 3.0 (PREMIER Biosoft International).

4.3.3 4.3.3 Greenhouse experiment and plant DNA extraction

The greenhouse experiment was performed as described in our previous study (Juraeva et al. 2006). The bacterial inoculation of plants was performed as following: pure culture of *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 were grown in Standard I (Merck, Darmstadt, Germany) broths for 48h. The bacterial suspensions were centrifuged at 7000 rpm for 10 min. Growth medium was discarded and the bacterial pellet was resuspended in 0.05% NaCl buffer. Seedlings were divided into three treatment groups and were immersed in an appropriate suspension for 2 min prior to placement in pots. The control group seedlings were immersed in sterile 0.05% NaCl. The inoculation treatments were set-up in a randomized design with 12 replicates. Cell densities of bacterial suspensions used for the inoculation were counted by dilution plating and CFU counts and were 10^{10} in the inoculant material for both bacteria.
Plants harvested (three replicates for each nitrogen treatment) on Days 7 and 42 after planting and plant DNA extraction was performed as described in Juraeva et al. (2006).

4.3.4 Quantitative real-time PCR assay and quantification

4.3.4.1 Method optimization

Primer concentrations were optimised for each assay testing different range concentrations, from 50 to 500 nM. The optimised quantitative real-time PCR assay for both bacteria was 300 µM for each forward and reverse primer, 12.5 µl of QuantiTect SYBR® Green 2x mastermix (Qiagen, Hilden GmbH, Germany), 2.5 µl of template, and H2O to a final volume 25 µl. Samples were tested in triplicate. This assay started with 15 min at 95°C followed by 50 cycles

of amplification, 30s at 94°C, 30s at 59°C and 1 min 15s at 72°C. A final elongation step consisted of 72°C for 5 min was followed by DNA melting protocol in which 85 cycles of half a degree increase in temperature every 30s beginning at 44°C.

4.3.4.2 Real-time PCR examination of primers

Amplification of target gene from positive and negative control strains was used to confirm primer sensitivity and specificity. The dilutions of a pure culture of appropriate bacteria (*B. licheniformis* BL43 or *Xanthomonas sp.* Xs148) were used to determine the sensitivity of the real - time PCR. The assay specificity was tested with approximately 50 ng of genomic DNA extracted from different bacteria species (data not shown) which have been shown to have the closest sequence similarity in CLUSTALW analyses for 16S-23S ISR partly sequence of *B. licheniformis* BL43 and of *Xanthomonas sp.* Xs148, respectively. DNA extracted from inoculated cucumber root samples were also tested further to prove the assay with plant samples. PCR products were examined by 2.5 % agarose gel electrophoresis using Marker 5, 100bp: pBR322 DNA/BsuRI (HaeIII)(MBI Fermentas GmbH, St.Leon-Rot, Germany) as size standard.

4.3.4.3 Standard curve generation

In order to obtain standard samples to use for the real-time PCR standard curve, DNA from target bacteria (*Bacillus licheniformis* BL43 or *Xanthomonas sp.* Xs148) was extracted using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Hamburg, Germany) and target gene was amplified with the newly developed specific primer pair (Tab. 13).

PCR cycling parameters and reaction mixtures were the same as described above except that melting protocol was not performed and that Probe master mix (Qiagen, Hilden GmbH, Germany) was used instead of QuantiTect SYBR® Green 2x mastermix (Qiagen, Hilden GmbH, Germany) The PCR product was cleaned using MiniElute™ PCR Purification Kit (Qiagen, Hilden GmbH, Germany) and the DNA concentration photometrically measured at the absorbance of 260 nm. Copy numbers of the standard were calculated using the known DNA concentration and the template length.

The real - time PCR standard curve was generated from the those standards and was adjusted within a range of nine 10-fold dilutions from 9.17E+09 to 9.17E+00 (for *B. licheniformis* quanti-PCR, Fig. 7a) and from 1.11E+09 to 1.11E+00 (for *Xanthomonas sp.* quanti – PCR, Fig.

7b) copies per µl DNA and by this way the number of DNA copies could be calculated for each sample.

Tab. 13: Specificity and nucleotide sequences of PCR primers used in this study.

Target Taxon	Primer sequences	Target gene	Product length	Reference
Plant				
	Teff: 5'-ACT GTG CAG TAG TAC TTG GTG -3'	*TEF*	155bp	Wulf et al.
	Tefr: 5'-AAG CTA GGA GGT ATT GAC AAG-3'			2003
Eubacteria	1) 785: 5'-GGATTAGATACCCTGGTAGTC-3'	16S rRNA	~2000bp	Rumf et al.
	317R: 5'-GGCTGGATCACCTCCTT-3'	16S rRNA		1999
	2) EricM:5'-GCCAAGGCATCCACCG-3'	23S rRNA	~830bp	
	422: 5'-GGAGTATTTAGCCTT-3'	23S rRNA		
Bacillus licheniformis	BL43F1: 5'-ACCCACCAAGTCTACTGAACAC-3'	16S-23S ISR	108 bp	In this study
BL43	BL43R1: 5'-CGAACCGTTGACCTCCTGC-3'			
Xanthomonas sp.	Xs148F1: 5'-GCCGATATGAGAGTCCCTTTTG-3'	16S-23S ISR	89bp	In this study
Xs148	Xs148R1: 5'-GCCTGTCGGGATCGAACC-3'			

4.3.4.4 TEF-gene quantification

In order to avoid of the misquantification due to the presumable different DNA extraction efficiencies from plant samples, as a housekeeping gene, *TEF* gene quantification from plant DNA was performed using the *TEF*f and *TEF*r primers (Tab. 13) as described in Juraeva et al. (2006). PCR reaction mixture and PCR program were the same as used in real – time PCR examination of specific primers. All quantified bacteria gene copy numbers were calculated as relative values to the housekeeping *TEF* gene copy numbers (relative bacteria gene copy number = (absolute gene copy number *100)/ *TEF* gene copy number) to compensate for any differences in initial template DNA amounts due to variations in different plant sample DNA extraction efficiencies. Since, the calculation of number of bacteria cells is not practically possible from that calculated 16S-23S ISR gene copies, because of not known rrn operons for *B. licheniformis* bacteria abundance was defined in gene copy numbers level.

In this study, all PCRs were performed using the iCycler (Bio-Rad, Inc. München, Germany). The quality of the SYBR Green® I quantification method was further verified for each measurement to avoid the possibility of false positive signals induced by primer dimers or other non-specific PCR products. First, a melting profile was recorded after each run, which resulted

in one melting peak of the first deviation with the specific melting temperature of the PCR product. Second, PCR products were run on an agarose gel.

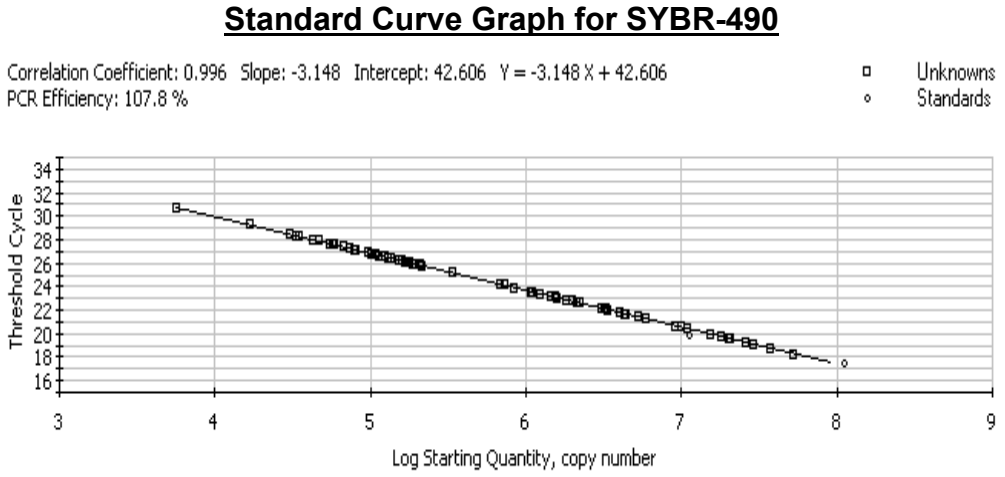

Fig. 7a: Standard curve graph (cycles = standard samples) for *Bacillus licheniformis* BL43 16S-23S ISR copy number calculation of unknown samples from control and inoculated (with *B. licheniformis* BL43) cucumber plant DNA (squares).

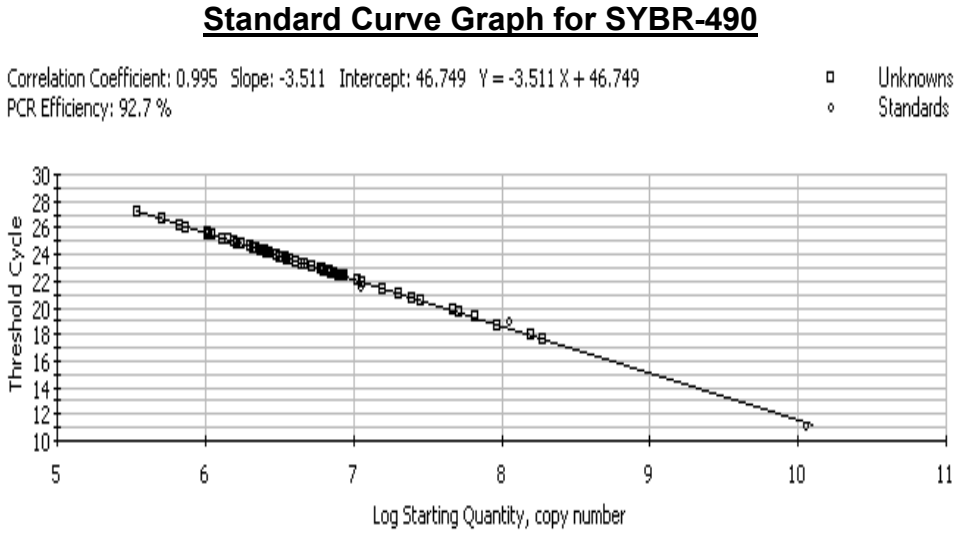

Fig. 7b: Standard curve graph (cycles = standard samples) for *Xanthomonas* sp. Xs148 16S-23S ISR copy number calculation of unknown samples from control and inoculated (with *Xanthomonas sp.* Xs148) cucumber plant DNA (squares). The T_m was emperically determined by plotting the change in fluorescence with temperature (dRFU/dT) versus temperature (T). RFU, relative fluorescent units.

4.3.5 Statistical analyses

Comparison of mean values of three or six replicates for molecular or plant growth measurements, respectively, was performed using Student's *t*-test at a *P*-level of ≤ 5 %. Pearson-type correlations were calculated at a *P*-level of ≤ 5 %. Where necessary, log transformations were applied to data sets in order to establish homogeneity of variances. All statistical analyses were performed using STATISTICA 6.0 (StatSoft 2001).

4.4 Results and Discussion

In previous studies used bacteria in this study influenced positively on the plant growth promotion of wheat, cotton and maize, tomato. In this work, their growth promoting activities were tested with cucumber and significantly increasing of shoot and root growth was observed in plants inoculated with both bacteria (data not shown). The specific primers for both bacteria *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148 were designed and plant samples from this pot experiments were used to test the specific primers to quantify the copy numbers of inoculated bacteria after inoculation to evaluate colonisation ability of the used bacteria.

4.4.1 16S-23S ISR sequences as a means of developing strain-specific primers

Three primer pair sets for *B. licheniformis* BL43 and two primer pair sets for *Xanthomonas sp.* Xs148 were designed based on 16S-23S ISR sequence data (Juraeva and Ruppel 2005b, detailed in Chapter 3). Using pure culture DNA extracted from several bacteria species non-related to the bacteria in query, which produced hits in the BLAST assay were included in the real-time PCR test of primer specificity as the templates showed that the specificities and sensitivities of the primer sets tested varied slightly. Primers BL43aF1 and BL43aR1 appeared to be the most specific while primers BL43aF2 -BL43aR2 and BL43aF3 -BL43aR3 appeared to be the most sensitive (data not shown). Since specificity is more important, the BL43aF1-BL43aR1 primer pair was used for further tests (Tab. 13). Among *Xanthomonas sp.* Xs148 specific primers, Xs148aF1-Xs148aR1 was selected for its best specificity (Tab. 13) (data not shown).

In addition, the specificity of the selected forward primers were analysed in BLAST search using 16S-23S ISR sequences deposited in the EMBL-Bank database. BLAST searches with *B. licheniformis* BL43 targeted forward primer BL43aF1 sequence showed only 10 sequence similarities in GenBank database that 2 of obtained 10 to the members of the genus *Acinetobacter genomosp*. and the rest 8 belonged to non-microbial genera such as Mouse DNA sequences (4), *Homo sapiens* chromosome (2), and *Mus. musculus* chromosome (2). The same

analysis with *Xanthomonas sp.* Xs148 targeted forward primer also showed only 10 similar sequences and target organisms were *Xanthomonas maltophilia* (1), *Pseudomonas sp.* (1), *Stenotrophomonas* (2), as-yet-unidentified bacteria (1), *Thermococcus kodakarensius* (1), *Methanosarcina marzei* (1), and *Homo sapiens* (3).

For accurately quantification of the bacteria, the approach based on direct quantification is important. Tan et al. (2001) reported that developed 16S-23S ISR sequence based strain specific primers did not allow detecting target bacteria directly resulting in misamplification in un-nested PCR. In this study, using pure culture DNA and inoculated plant DNA, as a complex DNA mixture, in direct real – time PCR quantification analysis confirmed the excellent specificity and accuracy of approach with melting curve (Fig. 8) analysis followed by agarose gel electrophoresis showing primer pairs gave a single specific product at the expected length (108 bp for *B. licheniformis* BL43 and 89 bp for *Xanthomonas sp.* Xs148), but not with DNA samples of non – target bacteria species and produced no PCR product with a blank control (H_2O) (data not shown).

4.4.2 Optimisation and performance of quantitative real- time PCR protocol

The optimal primer concentrations and annealing temperatures to obtain a sensitive, repetitive and specific test using specific primers was 300 nM and 57°C of each primer set. Under these conditions, the standard curve had a slope in the range -3.75 to -3.00 and a correlation coefficient greater than 0.97 (Fig. 7a and Fig. 7b) and could therefore be accepted for quantification (Boeckman et al. 2001). The assessment of detection limit of the assay using the same dilution series of external standard in quantitative real time PCR with standard curve analyses showed that up to 9.17E+03 and 1.11E+05 gene copies/µl of *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148, respectively, could be detected. In order to test for competitive or inhibitory effects of plant DNA on PCR amplification performed experiments using original DNA of some plant samples, the inhibition of PCR amplification with original plant DNA was observed and the amplification with 1:10 diluted and 1:100 diluted plants DNA showed that PCR with 1:10 dilutions was not inhibited. Therefore, 1:10 diluted plants DNA was used for quantification analysis.

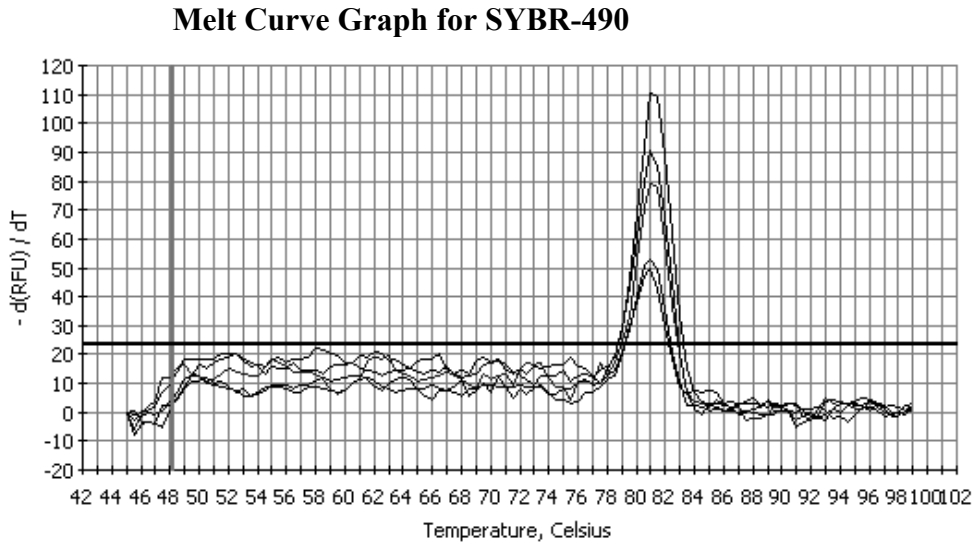

Fig. 8: Melting profile analyses to confirm only single PCR product amplification from tomato plant DNA during the PCR: bacteria (*Bacillus licheniformis* BL43 as an example) specific gene PCR product amplified from the standard sample (line with highest curve) and DNA extracted from non-inoculated and inoculated tomato plant. Melting temperatures for all PCR products are the same (81.0°C). The T_m was empirically determined by plotting the change in fluorescence with temperature (dRFU/dT) versus temperature (T). RFU, relative fluorescent units.

4.4.3 Utility of the developed tool in ecological studies

Real-time PCR based bacteria specific quantification enabled us to re-detect and accurately quantify introduced bacteria abundance in plant root 7 and 42 days after inoculation. The quantification of significantly higher copy numbers of target bacteria from plant root sampled 7 days after inoculation showed that both *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148 were able to colonise in cucumber root (Fig. 9).

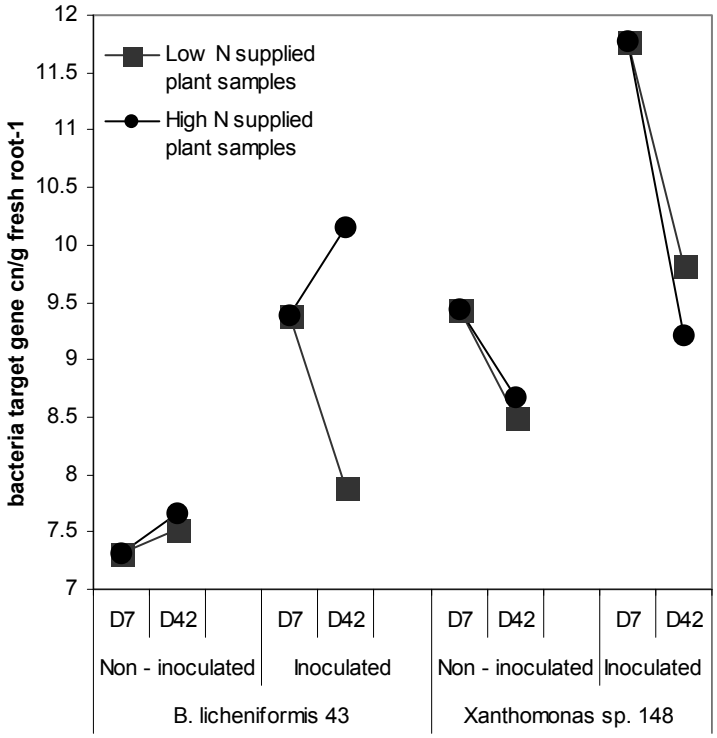

Fig. 9: The effect of N availability on colonisation of the introduced bacteria like population in both inoculated and non – inoculated plant root samples 2 days (Day 7) and 37 days (Day 42) after inoculation. Bacteria 16S-23S ISR copy numbers are calculated relative to housekeeping *TEF* gene copy numbers.

Evaluation of the factor effects, which may alter the inoculated bacteria abundance (or colonisation ability) is critical to achieve successful inoculation experiements and to assess the effect of inoculated bacteria on plant growth parameters. It is well recognized that N availability is mostly negatively correlated with diazotrophic bacteria abundance (Cejudo and Paneque 1986, Limmer and Drake 1998; Tan et al. 2003). Quantification results showed that N availability was negatively correlated to *Xanthomonas sp.* Xs148 abundance showing relatively less bacteria abundance in high N supplied plants (Fig. 3), while *B. licheniformis* BL43 abundance in high N supplied plants were significantly higher than low N supplied plants. Unexpectedly, *B. licheniformis* BL43 abundance in high N supplied plants were significantly higher even in comparison to 2 days after inoculation suggesting that the response of diazotrophs to N availability is different (Juraeva et al. 2006) and N stimulated *B. licheniformis* BL43 abundance in cucumber plants root. While this technique can provide accurate measurements of gene copy numbers per unit of plant DNA, to extrapolate those values to cell density, the knowledge in operons contained in considered species is required. At the present time, rRNA database of microorganisms (Klappenbach et al. 2000) has very limited information

about rrn operons. Of *Xanthomonas* genus, it has been reported for only *Xanthomonas* that rRNA consists 6 operons and the number of operons contained in *B. licheniformis* species are unknown. However, this approach is still powerful tool in quantification analysis.

4.5 Conclusion

One of the remarkable conclusion of this study is that if re-detection or/and quantification of inoculated bacteria has been aimed, the specific primer designing in either genus or species level based on sequence alighments available in EMBL-Bank database could be unsuccessful due to one or another level of misidentification or highly different sequence composition in the species in one genus.

Our study shows that rRNA ISR of bacteria has significantly high variability to design specific primers in comparison to 16S rRNA gene sequences.

In addition, amplification and sequencing of the considered bacteria can allow finding DNA regions which are unique to the considered bacteria which make the PCR enable for more reliable quantification of the inoculated bacteria in strain level.

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Chapter 5. Detection and quantification of the *nif*H gene in shoot and root of cucumber plants

5 DETECTION AND QUANTIFICATION OF THE nifH GENE IN SHOOT AND ROOT OF CUCUMBER PLANTS

5.1 Abstract

A real-time polymerase chain reaction method (PCR) method was applied to quantify the *nif*H gene pool in cucumber shoot and root and to evaluate how N supply and plant age affect the *nif*H gene pool. In shoots, the relative abundance of the *nif*H gene was neither affected by different stages of plant growth nor by N supply. In roots, higher numbers of diazotrophic bacteria were found compared to the shoot. The *nif*H gene pool in roots significantly increased with plant age and, unexpectedly, the pool size was positively correlated with N supply. The relative abundance of *nif*H gene copy numbers in roots was also positively correlated (r = 0.96) with total N uptake of the plant. The data suggest that real-time PCR-based *nif*H gene quantification in combination with N-content analysis can be used as an efficient way to perform further studies to evaluate the direct contribution of the N_2–fixing plant-colonising plant growth promoting bacteria to plant N nutrition.

Key words

Real-time PCR – Biological nitrogen fixation – cucumber – N-nutrition, plant growth promoting bacteria

5.2 Introduction

One of the current challenges faced by microbial ecologists is the difficulty to relate information on the abundance of the specific bacterial populations in the environment with their functional activity (Gray and Head 2001). Molecular measurements of the functional gene abundance, as a potential of activity, can link structural and functional data. Most molecular studies on diazotrophic organisms are primarily based on PCR amplification of the *nif*H gene, a marker gene for biological nitrogen (N_2) fixation. In those studies, the abundance and diversity of the *nif*H gene were investigated using a variety of approaches including PCR amplification followed by denaturing-gradient gel electrophoresis (Nicolaisen and Ramsing 2002, Freitag and Prosser 2003), restriction fragment analysis (Mintie et al. 2003, Stres et al. 2004), DNA hybridisation analysis (Mergel et al. 2001, Neufeld et al. 2001) and cloning-sequencing approaches (Kirshtein et al. 1991, Hamelin et al. 2002). However, despite this technical

progress, the relationship between microbial community structure and nitrogen cycling processes in soils or ecosystems is still relatively poorly understood (Wallenstein 2004).

Several approaches based on quantitative or semi–quantitative PCR, including reverse–transcriptase PCR (Bürgmann et al. 2003), gene hybridisation (Neufeld et al., 2001) and real–time PCR with SYBR® Green I (Wallenstein 2004), have been suggested for the quantification of *nif*H gene abundance in environmental samples or pure culture DNA. All these approaches have their advantages and disadvantages.

To investigate the relationship between the abundance of *nifSKH* genes and the corresponding biochemical reaction rates, Neufeld et al. (2001) used a gene hybridisation method based on quantitative gene probing. This method, however, probably underestimated the true number of *nif* genes present in the samples, because the *nif* genes may have shown reduced DNA-sequence similarity with the probes used in that study (Neufeld et al. 2001). Moreover, although the reverse–transcriptase PCR approach followed by image analysis of electrophoresis gels used by Bürgmann et al. (2003) was sufficient to detect the *nif*H gene abundance, the amount of product at the end of the reaction, quantified by end-point analysis (also called semi-quantitative analysis), does not truly represent the initial amount of starting material. In addition to poor precision, end-point PCR analysis produces narrower dynamic ranges than real-time PCR methods (2 to 3 orders of magnitude versus 5 to 8), and has a lower sensitivity and resolution (Schmittgen et al. 2000). For a more reliable quantification of template concentrations, real-time PCR approaches based on direct determination of the amount of product offer an attractive alternative (Boeckman et al. 2000).

Real-time PCR-based methods as developed by Wallenstein (2004) have been shown to be a powerful tool to quantify N_2-fixing genes in soil. However, these methods require implementation of an internal control that prevents the miscalculation of the quantified gene pool due to the presumed different DNA extraction efficiencies from environmental samples. To overcome this difficulty, *nif*H gene copy numbers could be calculated relative to plant housekeeping gene copy numbers. This approach would be more accurate than using adjusted concentrations of DNA extracted from environmental samples.

In this study, we developed a method to quantify *nif*H gene copy numbers in plant DNA with a quantitative real-time PCR approach using SYBR® Green I as an intercalating dye. In addition, the preliminary application of this newly developed quantification method in combination with plant N content analyses was used to evaluate the direct contribution of the N_2-fixing plant-inhabiting diazotrophic community to plant N nutrition. Finally, we address whether the mineral N availability and/or plant age affect the abundance of the *nif*H gene pool in cucumber.

5.3 Materials and methods

5.3.1 Greenhouse experiments

Cucumber (*Cucumis sativus L.* 'Corona F1') seeds were germinated for 10 days on trays containing vermiculite under greenhouse conditions (latitude 52° 21`N, longitude 13° 18`E at an altitude of 50 m), close to Berlin, Germany. At the 2-leaf stage, plants were transplanted into pots (one seedling per pot) filled with 800 g quartz sand. 12 replicates were grown for 6 wk (in May-June 2004) at temperatures of 26°C to 28°C during the day and 16°C to 18°C at night without artificial illumination. The substrate water content after planting was approximately 60% water holding capacity and was kept nearly constant throughout the experiment. Percolating water was recycled for each pot. Plants were supplied with half-strength Hoaglands' nutrient solution lacking nitrogen (Hoagland and Arnon 1950). N was supplied separately at two different concentrations with six replications for each treatment: (1) a high N supply, calculated to fully meet the plant's nitrogen demand for a 6 week growth period; and (2) a low (N-deficient) treatment, equivalent to approximately 40% of the high level. Two days after planting, 100 mg N were supplied to each plant by injecting NH_4NO_3 solution into the root zone. Half the plants, selected at random, also received an additional 150 mg N on two further occasions (75 mg N each at 21 and 28 d after planting) and are referred to here as high N supplied plants. As total mineral fertiliser during growth, plants received potassium/nitrogen/phosphorus in a ratio of 10:0.8:1 and 10:2:1 for low and high N supplied plants, respectively.

5.3.2 Harvesting of plant samples and DNA extraction from plant samples

For *nif*H gene abundance measurements, plants (three replicates for each nitrogen treatment) were harvested on Days 7 and 42 after planting. Roots and shoots were separated and quartz sand particles were carefully removed under a gentle stream of tap water. About 0.1 g (fresh mass) samples from the middle part of roots and youngest leaves were cut, transferred to separate Eppendorf tubes containing 0.1 g of sterile glass beads (0.5 mm diameter), and then frozen at –20°C and lyophilised.

DNA was extracted from the lyophilised plant samples using the DNeasy Plant Mini Kit (Qiagen, Hilden GmbH, Germany) according to the manufacturer's instructions. First, the lysis buffer was added to the lyophilised samples, which were then shaken vigorously using the wrist action shaker MM 200 (Retsch, Haan, Germany) at a frequency of 30 shakes per second for 5

min. DNA concentrations were photometrically measured at λ=260 nm using an Eppendorf spectrophotometer.

The additional observations undertaken at harvest d 42 were shoot and root dry mass and total plant N analyses. Six plant samples (for each N supply) were prepared to determine the effect of N supply on plant growth and total N analysis. Plants were separated from substrate as described above for molecular measurements and dried at 80°C for 48 hours. After measurements of shoot and root dry mass, total N was analysed using a CHN-O Rapid elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

5.3.3 Real-time PCR assays

To measure PCR products in real-time PCR, the non-specific intercalating dye, SYBR Green I, was used. In this method, the signal is quenched when the dye is not bound. Thus, as PCR products accumulate, the fluorescent signal increases proportionately, thereby allowing quantification of PCR amplification.

Quantitative real-time PCR was conducted using an iCycler™ detection system (Bio-Rad, Laboratories, München, Germany). The fluorescence of the reporter molecule was measured at 520 nm after excitation at 490 nm. Each assay was conducted in a 96-well plate with two replicates for each standard and negative control, and sample in triplicate. Amplification was performed with a reaction mixture containing 12.5 µl of QuantiTect SYBR® Green 2 x Master Mix (Qiagen, Hilden GmbH, Germany), 2.5 µl each 300 $nmol \cdot L^{-1}$ primer and 2.5 µl DNA template, which was then brought to a final volume of 25 µl by the addition of 5 µl of DNA-free H_2O. In order to avoid of the failure in quantification of *nif*H gene due to too high or low plant DNA concentration used as a template, PCR was performed using undiluted, 1:10 diluted and 1:100 diluted plant DNA. PCR was performed with an initial step at 95°C for 15 min (required to activate the polymerase enzyme) followed by 50 cycles each of 0.5 min at 94°C, 1 min at 50°C and 1.15 min at 72°C.

The cycle at which the fluorescence of the target amplicon exceeded the background fluorescence (threshold cycle) was calculated using the iCycler iQ Optical System Software (version 3.1, Bio-Rad, Inc). A melting curve analysis was performed following each assay by measuring fluorescence continuously as the temperature was increased by 0.5°C from 50°C to 97.5°C. Different diazotrophic bacterial pure cultures (Tab. 14) were used to evaluate this method (data not shown). The specificity of amplification was confirmed by running samples on a 1.7 % agarose gel and by checking the melting profile of the PCR product. The lengths of the PCR products from plant samples were compared with that of positive controls of

diazotrophic bacterial pure cultures and were estimated using 100 bp DNA Plus Ladder (peqGold, peqLab, Erlangen, Germany).

Tab. 14: Melting temperatures of PCR products amplified using the *nif*H gene primer pairs for DNA extracted from diazotrophic bacterial pure cultures and from cucumber root and shoot samples.

DNA extracted from	Strain number	Melting T° (°C)
Citrobacter spp.[1]	CC322	92.5
Azospirillum sp. [1]	D19/1	90.5
Isolate from winter wheat[1]	K27	89.5
Citrobacter sp. [1]	CC321	87.0
Klebsiella pneumoniae[1]	CC2/17	90.5
Serratia rubidea[1]	CC12/12	90.0
Pseudomonas aeruginosa[1]	CC312	90.0
Azospirillum sp. [1]	K22	88.5
Isolate from winter wheat[1]	CC307	92.0
Enterobacter radicincitans[1]	D5/23	89.0
Arthrobacter simplex[2]	43	90.5
Burkholderia spp.[3]	148	91.5
Shoot samples of cucumber[4]	-	89.5 ±0.5
Root samples of cucumber[4]	-	91.5 ±0.9
Shoot samples of cucumber[5]	-	89.0 ±0.5
Root samples of cucumber[5]	-	90.0 ±1.0

1 – isolated from *Triticum aestivum* and *Ammophila arenaria* (Ruppel and Wache, 1990)
2 – isolated from maize (Microorganism culture collection, Institute of Microbiology, Uzbekistan Academy of Sciences)
3 – isolated from wheat rhizosphere (Juraeva D., Institute of Microbiology, Uzbekistan Academy of Sciences, unpublished data)
4 – low N supplied plants; 5 – high N supplied plants

5.3.4 Preparation of the nifH gene standard

To quantify *nif*H gene copy numbers in unknown samples, real-time PCR was performed with defined standard samples of the diazotrophic bacterial strains *Enterobacter radicincitans* sp. Nov. (D5/23) (Kämpfer et al. 2005) and *Klebsiella pneumoniae* CC 2/17. Cultures were grown in Standard I (Merck, Darmstadt, Germany) broths for 48 h at 28°C and bacterial DNA was extracted using MO BIO Ultra Clean™ Microbial DNA isolation kit (MO BIO laboratories, Inc. Hamburg, Germany). *nif*H gene amplification from these bacteria was performed with the

universal *nif*H gene primers 19F (5'-GCIWTYTAYGGIAARGGIGG-3') and 388R (5'–AAICCRCCRCAIACIACRTC-3') (Ueda et al. 1995). The PCR protocol was the same as described for the real-time PCR assays with the exception of using the QuantiTectTM probe PCR Kit (Qiagen, Hilden GmbH, Germany). The amplified PCR products were analysed on 1.7 % agarose gels and their single products were purified using the QIAquick PCR purification kit (Qiagen, Hilden GmbH, Germany). Purified PCR products of the two bacteria were pooled and mixed, and the standard DNA concentration (µg ml^{-1}) was measured at 260 nmusing an Eppendorf® spectrophotometer. The *nif*H gene copy numbers per µl were then calculated using the known DNA concentration and the template length of 390 bp. The standard range was set within 2.54E+09 to 2.54E+00 copies per µl as a 10-fold dilution series. The number of *nif*H gene copies μl^{-1} of plant DNA was calculated by comparing unknown samples with the *nif*H gene standard samples of defined gene copy numbers.

5.3.5 Spiking of plant DNA samples

In order to test for competitive or inhibitory effects of plant DNA on *nif*H gene PCR amplification, an experiment using *nif*H gene standard samples mixed with plant DNA was performed. Plant DNA extracted from cucumber shoot and root samples (three replicates for each plant part) were used. The recovery of *nif*H genes from plant DNA samples was measured using the real-time PCR approach specific for *nif*H genes. Mixture templates contained *nif*H gene copies ranging from 1.25E+00 to 1.25E+09. Real-time PCR was conducted and the *nif*H gene amplification of mixed DNA and pure *nif*H genes were quantified and correlated.

5.3.6 TEF gene quantification

It is likely that DNA extraction efficiency differs among different plant samples. Therefore, it is not feasible to use absolute quantified copy numbers of the *nif*H gene. The *TEF* gene (Transcriptional Enhancer Factor), a housekeeping gene for plant DNA analysis (Wulf et al. 2003), was therefore used as an internal control as its expression is not affected by different plant growth stages or treatments. Plant *TEF* gene was quantified using real-time PCR in conjunction with the SYBR Green I approach with primers *TEF*f (5'-ACTGTGCAGTAGTACTTGGTG-3') and *TEF*r (5'-AAGCTAGGAGGTATTGACAAG-3') (Wulf et al. 2003). For *TEF* gene quantification, standard samples for cucumber were prepared from DNA isolated from shoot and root parts using the same procedure as described above for the *nif*H gene.

The *nif*H gene copies quantified from all sampling dates and treatments were calculated as relative values to the housekeeping *TEF* gene copy numbers (relative *nif*H gene copy number = (absolute *nif*H gene copy number *100)/ *TEF* gene copy number) to compensate for any differences in initial template DNA amounts due to variations in different plant sample DNA extraction efficiencies.

5.3.7 Statistical analyses

Comparison of mean values of three or six replicates for molecular or plant growth measurements, respectively, was performed using Student's *t*-test at a *P*-level of ≤ 5 %. Pearson-type correlations were calculated at a *P*-level of ≤ 5 %. Where necessary, log transformations were applied to data sets in order to establish homogeneity of variances. All statistical analyses were performed using STATISTICA 6.0 (StatSoft 2001).

5.4 Results

5.4.1 Effect of mineral N supply on plant growth

Our previous experiments revealed that cucumber could not survive during the 6 weeks of growth without supplying approximately 40% of total N application (Juraeva and Ruppel, Institute for Vegetable and Ornamental Crops, Germany, unpublished data). In this study, although, effects of N supply on plant shoot growth were not significant, strong N deficiency was observed at the end of the experiment for low N supplied cucumber plants. The higher nitrogen supply significantly increased total plant N content and plant root dry mass compared to the low N supply (Tab.15).

Tab. 15: Effect of nitrogen fertilizer application on dry mass and N content of 42 days old cucumber plants fertilised with low and high nitrogen supply.

N supply	Total N uptake $(g\ plant)^{-1}$	Shoot dry mass $(g\ plant)^{-1}$	Root dry mass $(g\ plant)^{-1}$
N1	0.08 ± 0.01	5.09 ± 0.30	0.64 ± 0.12
N2	0.12 ± 0.01*	5.23 ± 0.98	0.86 ± 0.09*

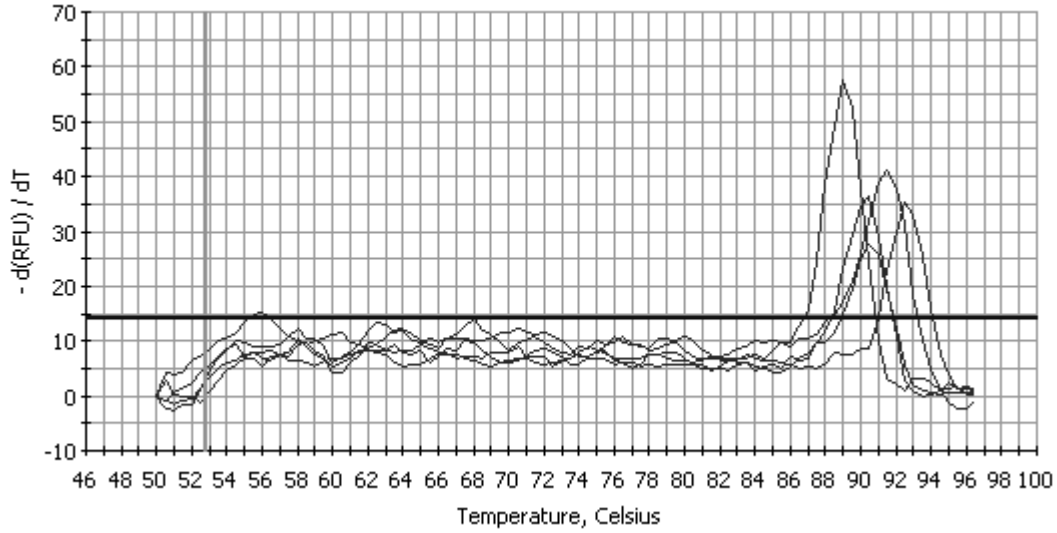
Note: Values are mean ± SE. Values obtained for plants cultivated in the different N availability were compared by a Student's *t*-test. N1, low N treatment (100 $mgN \cdot plant^{-1}$); N2, high N treatment (250 $mgN \cdot plant^{-1}$). Asterisk (*) indicates significant differences ($P < 0.05$) in the effect of N fertilization level on plant growth and N nutrition.

5.4.2 New method developed to quantify the nifH gene in plant tissue using quantitative real-time PCR

The universality of primers 19F and 388R (Ueda et al. 1995) was confirmed by amplification of the *nif*H gene from a range of nitrogenase-positive bacterial strains. Primer concentrations were optimised for the present experimental conditions. In all cases, one specific product was amplified and verified with the melting profile (Fig. 10A) and gel electrophoresis analysis (data not shown).

Using the *nif*H gene standard, target genes within a range of 2.54E+00 – 2.54E+09 copies μl^{-1} were detected. The standard curve documents the detection limit of 2.54 *nif*H gene copies μl^{-1}, measured at the threshold after 50 cycles. It is noteworthy that the quality of the standard curve with a correlation coefficient of 0.997, a curve slope of –3.544 and PCR efficiency of 91.5 % over a range of 9 orders of magnitude is ideal to quantify *nif*H gene copy numbers within unknown samples (Boeckman et al. 2000).

A



B

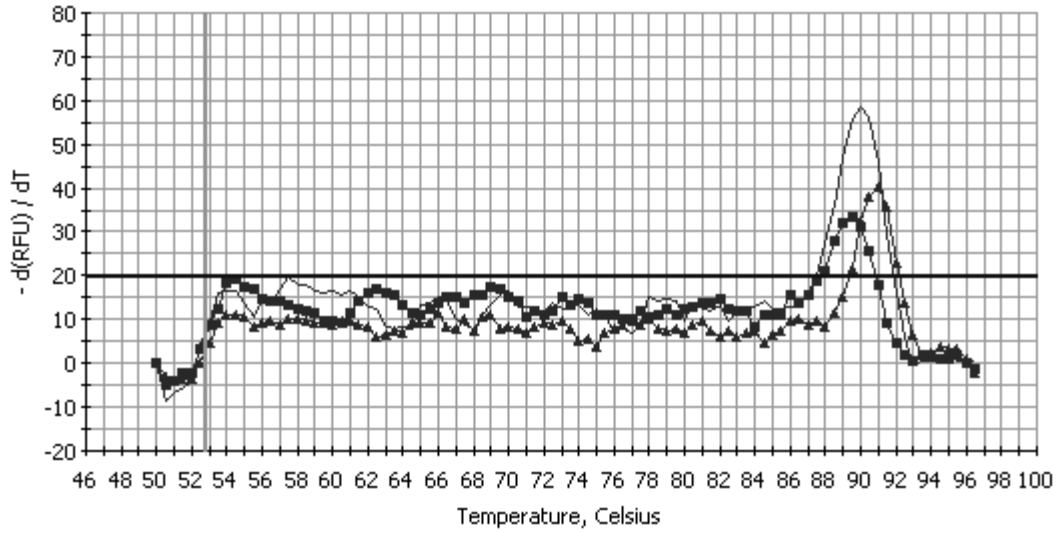


Fig. 10: Examples of melting profile analyses of the *nif*H gene amplified from (A) pure cultures of diazotrophic bacteria belonging to the different genera (see Tab. 1) and (B) cucumber plant DNA to confirm PCR product specificity. (B) The PCR product was amplified from the *nif*H gene standard sample (melting temperature (T_m) = 90.0°C; black line), plant shoot sample (T_m = 89.5°C; ■) and root sample (T_m = 91.0°C; ▲). The T_m was emperically determined by plotting the change in fluorescence with temperature (dRFU/dT) versus temperature (T). RFU, relative fluorescent units.

5.4.3 Plant DNA spiking

According to plant DNA spiking experiments, the concentration of *nif*H genes mixed with plant DNA samples was positively correlated with pure *nif*H genes at a slope of nearly 1 over a range of 7 orders of magnitude (r = 0.992). This indicates that the plant DNA did not inhibit the *nif*H gene specific real-time PCR within a range of 10E+02 to 10E+08 *nif*H gene copy numbers μL^{-1} DNA and that there is a nearly 100 % recovery of added *nif*H gene copies from plant samples (Fig. 11). The highest concentration of 10E+09 *nif*H gene copies μL^{-1} inhibited the PCR reaction. If we transfer these measurements to dimensions per g plant material, then between 10E+04 and 10E+10 *nif*H gene copies (plant fresh matter)$^{-1}$ were detectable.

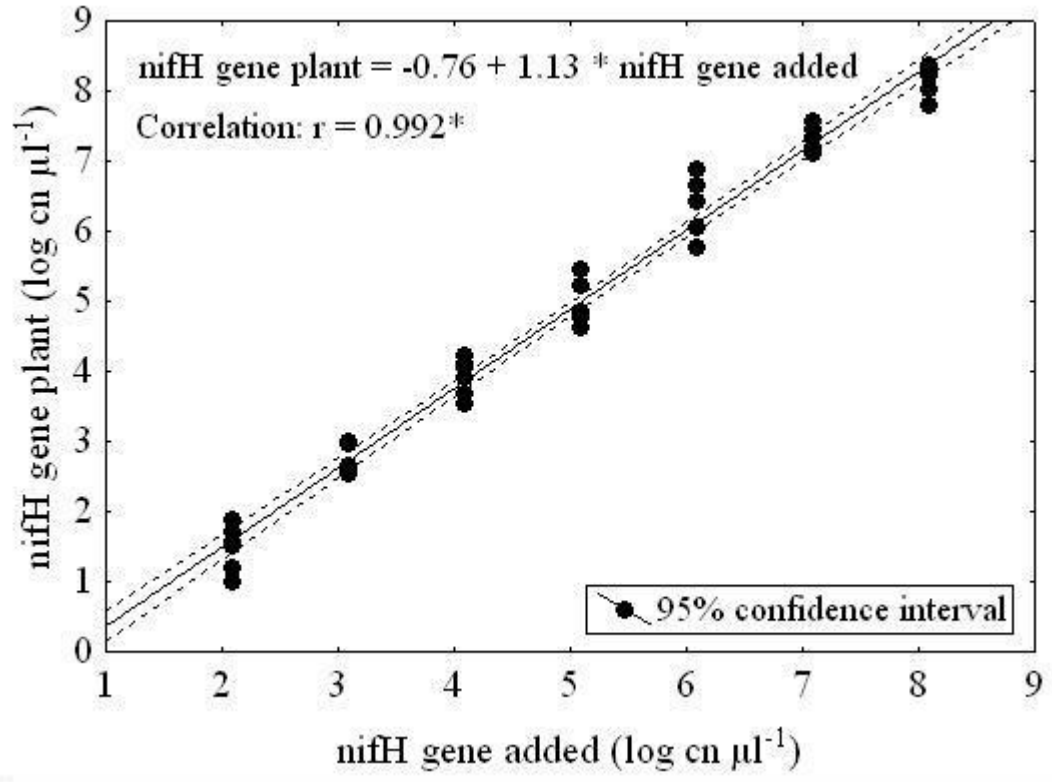


Fig. 11: Result of regression analysis between predefined *nif*H gene copy numbers (*nif*H gene added) and the cucumber plant DNA – *nif*H genomic DNA mixture (*nif*H-gene-plant). The asterisks (*) indicates significant correlation.

5.4.4 Specificity of the quantitative real-time PCR approach to quantify the nifH gene from plant samples

In this study, the detection and quantification of *nif*H gene abundance in cucumber indicated the high sensitivity and reproducibility of the newly developed method based on real–time PCR. In addition, a similar study using tomato plants instead of cucumber further confirmed the ability of this new method to quantify target genes in different plant species (data not shown). PCR amplification with undiluted plant DNA was inhibited in some plant samples. In contrast, for the 1:10 diluted and 1:100 diluted DNA, no inhibition was observed and the 1:10 dilution was quantified precisely. Therefore, 1:10 diluted plant DNA was used for the quantification analysis.

The main disadvantage of the real–time PCR-based quantification approach using SYBR Green I dye is that non-selective detection of any double-stranded DNA molecule could occur and this would lead to a misquantification. However, in this study, this possibility was checked by both melting profiles (Fig. 10B) and gel electrophoresis analysis (Fig. 12). The length of the PCR products amplified from both bacterial pure culture (data not shown) and plant sample DNA was 390 bp (Fig. 12).

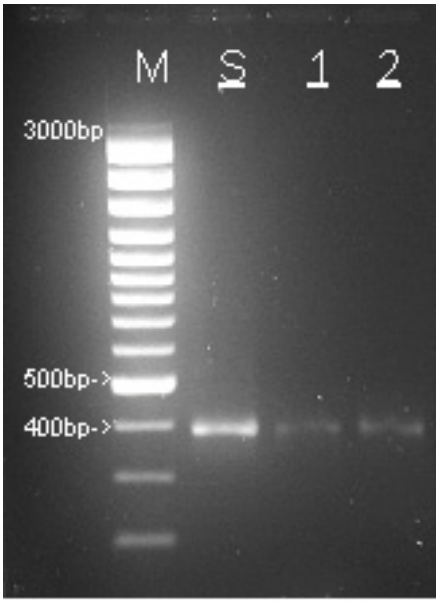


Fig. 12: Agarose gel analyses of the *nif*H gene PCR product (~390 bp) amplified from plant DNA using universal *nif*H gene primers 19F and 388R. M: size marker peqGOLD 100 bp DNA Ladder Plus. S: standard samples' amplified *nif*H gene products from cucumber DNA (1 - shoot; 2 - root).

Melting profiles of the *nif*H gene amplicons of various diazotrophic bacterial species differed within a range of 87°C to 92.5°C (Tab. 13, Fig. 10A). In shoot and root samples of cucumber grown under either low or high N availability conditions, the melting point of *nif*H gene amplicons was detected within the same range as that of bacterial pure cultures of 89.0°C to 91.5°C (Tab. 13, Fig. 10B). The observed different melting temperatures of same-size PCR products could be related to differences in the sequence composition of amplified target DNA.

5.4.5 nifH gene quantification

The quantitative PCR protocol was used to determine the total copy number of amplifiable *nif*H sequences in DNA extracted from cucumber shoot and root samples at early and late plant growth stages. *nif*H gene abundance in plant shoot was found to be relatively stable over time and not affected by mineral N fertilisation ranging between 1.57E+06 – 4.63E+06 copies in 1 g fresh shoot. The results of the statistical analyses for the N supply and plant age effects on relative *nif*H gene abundance within the plant root as determined by Student's *t*-test are summarised in Tab. 16. Relative *nif*H gene abundance in root increased with increasing plant age (Tab. 16C). Seven days after planting, the *nif*H gene pool consisted of 1.24E+07 copies (g fresh root)$^{-1}$, while in 42 d old plant roots, this figure increased to 5.25E+07 and 1.59E+09

Tab. 16: Effects of different factors on *nif*H gene distribution. Mean values from three replicates were compared by a Student's *t*-test. Relative log *nif*H gene copy numbers per *TEF* gene copy number (*nif*H cn) ± standard deviation are shown. Asterisk (*) indicates significant differences ($P < 0.05$). N1, low N treatment (100 mgN·plant^{-1}); N2, high N treatment (250 mgN·plant^{-1}). [a]Copy no. was calculated commonly from harvest 1 (at day 7) and harvest 2 (at day 42).

Factor	*nif*H cn
(A) Plant part[a]	
Shoot	5.83 ± 0.42
Root	8.18 ± 1.05*
(B) N supply	
N1	6.96 ± 0.46
N2	8.61 ± 0.87*
(C) Plant age	
7 days old	6.39 ± 0.20
42 days old	8.62 ± 0.67

in both low and high N-supplied plants, respectively. Interestingly, at the end of the experiment, *nif*H gene abundance in high N supplied plants was significantly higher compared to low N supplied plant roots. According to the regression analysis, a strong correlation (r = 0.96) was observed between the relative *nif*H gene copy numbers measured for the plant and the plant N content (Fig. 13).

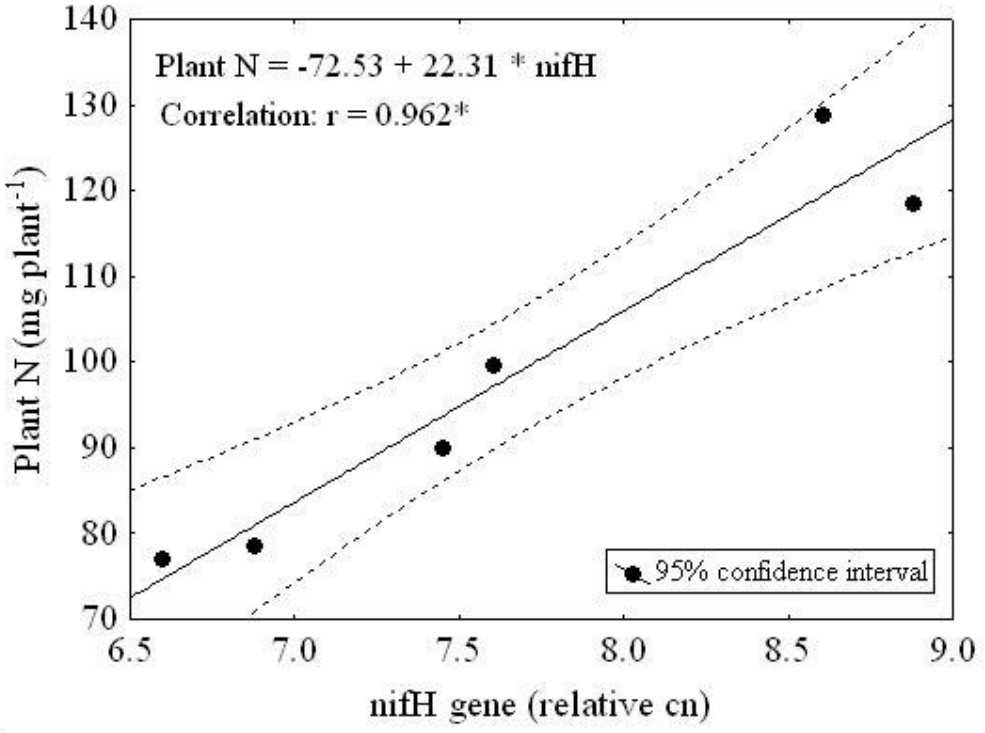


Fig. 13: Result of regression analysis between the *nif*H gene copy numbers relative to housekeeping *TEF* gene copy numbers (*nif*H gene, measured as relative cn) and the plant N content (Plant N, measured as mg N plant^{-1}) of cucumber plants.

5.5 Discussion

5.5.1 Bacterial DNA extraction from plant samples

From cucumber plant samples, DNA could be obtained in the range of 55.08-140.50 ng/ mg fresh shoot and 12.58-52.08 ng/ mg fresh root. Efficiency of DNA isolation with DNeasy Plant mini kit was different for plants grown in different level of N availability and of different age. Our previous study performed to quantify inoculated bacteria abundance in tomato plant shoot and roots using a real-time PCR approach revealed that DNeasy Plant mini kit enabled us to recover bacterial DNA within plant samples precisely (Juraeva and Ruppel 2005).

5.5.2 Productivity and specificity of the quantitative real-time PCR approach to quantify the nifH gene from plant samples

Ueda et al. (1995) reported that *nif*H gene amplification from dryland plant DNA (maize and soybean) failed; whereas using the same protocol, the *nif*H genes from rice root DNA could be amplified. The lack of amplification could result from either a target gene concentration below the detection limits of the employed assay or from inhibition of PCR due to PCR inhibitors in template DNA. In this study, the detection and quantification of *nif*H gene abundance in cucumber indicated the high sensitivity and reproducibility of the newly developed method based on real–time PCR. In addition, a similar study using tomato plants instead of cucumber further confirmed the ability of this new method to quantify target genes in different plant species (data not shown).

In this study, the advantages and disadvantages of employed approach for gene quantification purposes were carefully taken into consideration. The main disadvantage of the real–time PCR-based quantification approach using SYBR® Green I is that any double-stranded DNA molecule is measured and this would lead to misquantification if primer dimers occurred. However, in this study, product specificity was checked by both melting profiles (Fig. 10B) and gel electrophoresis analysis (Fig. 13). The length of the PCR products amplified from bacterial pure culture (data not shown) and plant DNA were within the same range of previously published *nif*H gene sequences amplified using the same primer pair (Ueda et al. 1995) and thus, confirmed the accuracy of the method to amplify the target gene.

One advantage of the real–time PCR-based approach used in combination with SYBR Green I is that melting profile analyses allows checking of the specificity of PCR products and discovery of unspecific primer dimers by generating one single melting peak with the known specific melting temperature of the product, thereby testing correct gene quantification. Additionally, shifts in specific melting temperatures may be indicative of differences in amplified PCR product sequence composition. The observed different melting temperatures of same-size PCR products amplified from both pure bacterial culture DNA and plant DNA could be related to differences in the sequence composition of amplified target DNA. Moreover, different melting temperatures of *nif*H gene sequences amplified from the shoot and from root samples probably indicate that different dominating diazotrophic bacterial communities colonise the shoot and root parts of the plant. However, whether or not the *nif*H-gene-specific real-time PCR approach with SYBR Green I can be used to measure *nif*H gene diversity in environmental samples has to be proven in further experiments.

5.5.3 *nif*H gene quantification

Quantified *nif*H gene copies were calculated relative to the quantified copy numbers of the housekeeping *TEF* gene. Although the DNA-based PCR method is not directly related to *nif*H gene expression, it does allow comparison of the effects of different treatments on the N_2–fixing potential of the microbial community in plant samples by quantification of *nif*H gene abundance in plant. These *nif*H gene values reflect the abundance of diazotrophic populations associated with the plant.

For the first time, the *nif*H gene pool in the plant shoot has been quantified and found to be lower compared to root samples. Various studies indicated that the abundance of total diazotrophs or of specific populations in both pure culture and environmental samples can be influenced by the amounts of inorganic N applied (Cejudo and Paneque 1986, Herridge and Brockwell 1988, Limmer and Drake 1998, Fuentes-Ramirez et al. 1999). We found that although, high N supply did significantly increased N amount in both plant shoot and root (data not shown), it did not affect the gene abundance in plant shoot. Therefore, we suggest that plant N status did not influence the abundance of diazotrophic bacteria inhabiting plant tissue. Moreover, the stability of the *nif*H gene in plant shoot over time suggests that in contrast to plant roots, physiological changes occurring during the plant shoot development did not significantly affect diazotrophic population abundance in plant shoot.

5.5.4 The effect of N amount supplied on nifH-gene abundance in plant root

The significantly higher *nif*H gene copy numbers in high N- compared to low N-supplied cucumber plant roots (Tab. 16B) run contrary to previous reports (Tan et al. 2003; D. Juraeva and S. Ruppel, Institute for Vegetable and Ornamental Crops, Germany, unpublished data). Our data suggest that (1) higher N supply stimulated the growth of diazotrophic bacteria in cucumber roots and (2) the effect of N availability on diazotrophs may be plant-species dependent, as in rice (Tan et al. 2003) and tomato roots (D. Juraeva and S. Ruppel, Institute for Vegetable and Ornamental Crops, Germany, unpublished data) where the diazotrophic communities were suppressed by higher mineral N fertilisation. In our experiment, high N supplied cucumber plants developed a significantly larger root system (Tab. 15) than the low N supplied plants, which provides favorable conditions for microorganism growth, including those for diazotrophic bacteria. Seldin et al. (1984) reported no repression of nitrogenase genes in some diazotrophs and Piceno and Lovell (2000) even documented increased nitrogenase gene expression with increasing N availability. Therewith, it seems imaginable that an increased abundance of certain diazotrophic groups in higher N supplied plants lead to an increased N

nutrition of plants due to biological N_2 fixation. Since the number of *nif*H gene operons contained in various organisms may vary and is usually unknown, it is not possible to extrapolate quantified *nif*H gene copy numbers to cell density values exactly. However, a rough estimation of the population size of diazotrophic bacteria in cucumber plant shoot and roots at early and late plant growth stages can be calculated. Assuming the DNA extraction efficiency of 100% from plant material and the presence of two *nif*H gene copies per bacterial cell (Yeager et al. 2004), diazotrophic bacterial numbers exceeded between 1.57E+06 – 4.63E+06 in cucumber shoot parts, and 1.24E+07 – 1.59E+09 in root parts. From day 42, diazotrophic bacterial numbers inhabiting high N supplied cucumber roots were 15-fold higher than those in low N supplied plants. Comparative analysis of previous reports on diazotrophic bacteria abundance in plant rhizoplane and inside of roots (Reinhold et al. 1986, Barraquio et al. 1997), and approximate calculation of diazotrophic bacteria cells in cucumber shoot and root based on the present study's results, demonstrated that our results in *nif*H gene copy numbers quantified from the plant DNA using real – time PCR approach was reasonable. Reinhold et al. (1986) reported 2.0E+07 diazotrophs (g dry weight)$^{-1}$ of rice roots, while Barraquio et al. (1997) enumerated the size of populations of diazotrophic bacteria in different rice genotypes ranged from 10E+03 and 10E+07 per g roots.

5.5.5 Correlation of nifH-gene abundance to plant N nutrition

In this study, the combination of *nif*H gene quantification and plant N-uptake measurements was shown to be a possible tool to evaluate the contribution of the N_2–fixing plant-inhabiting diazotrophic community to plant N nutrition. The positive correlation between *nif*H gene abundance and plant N nutrition highlights the potential value of studying functional genes in the context of ecosystem processes. However, these results are only suggestive of this relationship, and future studies should focus on measuring the relationships of gene abundance to the target gene expression and activity simultaneously. Additionally, the relationship between the diversity of special diazotrophic bacterial populations and their sensitivity to environmental changes should be examined.

New technologies and methods to investigate microbial communities are being developed at a rapid pace and provide new opportunities to link community structure to ecosystem processes. The herein described DNA-based real-time PCR quantification of *nif*H gene abundance in plant tissues can be extended to RNA-based approaches as DNA is more likely to reflect the standing biomass of a particular community and mRNA should be more closely related to activity rates

(Bürgmann et al 2003). Therefore, these real-time PCR approaches offer an additional promising avenue for linking microbial communities to environmental processes.

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Chapter 6. Quantitative real – time PCR based evaluation of the direct potential of diazotrophic bacteria to the plant N nutrition

6 QUANTITATIVE REAL-TIME PCR BASED EVALUATION OF THE DIRECT POTENTIAL OF DIAZOTROPHIC BACTERIA TO THE PLANT NITROGEN NUTRITION

6.1 Abstract

Understanding the factors involved in controlling the colonisation/distribution of *nif*H gene containing microorganisms in the environment may allow identifying the factors controlling N_2 fixation in the environment. Two diazotrophic bacteria were tested for responses in root colonization, growth stimulation, and nitrogen supply to the plant in the presence of N in different levels. We proposed that in the low level of N availability, the diazotrophic bacteria are more abundant than in high N supplied conditions, and that even in high N availability, the application of diazotrophic plant growth promoting bacteria (PGPB) strains can increase the diazotrophic population allowing increased potential for plant N nutrition. The results of performed plant experiments suggested that *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148 had the potential to improve the N nutrition of tomato in low level of N availability. *B. licheniformis* BL43 showed the significant effect on plant N uptake in the presence of high N as well. Furthermore, it was hypothesized that the improved plant N nutrition is due to N_2-fixing ability of inoculated bacteria, and that the correlations between plant N content and applied bacteria cell numbers and quantified *nif*H gene abundance in plant tissue indicate/evaluate the capacity of the applied diazotrophic bacteria to fix atmospheric nitrogen. Quantitative evaluation of *nif*H gene abundance in the inoculation experiment using real – time PCR based direct quantification approach indicated a high potential for plant-associated N_2-fixation in both BL43 and Xs148 strains. Both bacteria showed significantly high abundance in low N availability and were positively correlated to *nif*H gene abundance in the plant root. We found significant positive correlation between quantified target bacteria abundance, *nif*H gene copies and plant N nutrition. Relationship of quantified *nif*H gene to total N nutrition of plant was less close in inoculated plant compared to non – inoculated plant. Those correlation coefficients were affected strongly by N availability.

Key words

Plant growth promoting bacteria – inoculation - biological nitrogen fixation – *nif*H gene – 16S-23S ISR – quantification – real time PCR

6.2 Introduction

Asymbiotic biological nitrogen fixation (ANF) can help to meet plant N requirements even in intensive agriculture (Tchan et al. 1988). At present, ANF can only partly meet the N demand of plants since cereals and other non-legumes usually require high N fertilizer amounts for reaching high yields. This is at least partly due to the factor that soil N is mostly negatively correlated with diazotroph abundance, that means the occurrence of bacteria which are able to bind atmospheric nitrogen (Cejudo and Paneque 1986, Limmer and Drake 1998). High soil N availability strongly down regulates the expression the *nif*H gene which is responsible for the biological N_2 fixation in diazotrophs (Triplett et al. 1989, Merrick 1992, Bürgmann et al. 2003). For a more efficiently exploitation of ANF in agriculture, the factors affecting the N_2 – fixing ability of diazotrophic bacteria shall be better understood. It is important to gain more detailed knowledge about the colonization ability and stability of diazotrophic populations in the presence or absence of high mineral N levels. In particular, the effect of soil N availability on the potential contribution of different diazotrophic populations should be studied, to select, bacteria that are specifically tolerant to high soil N levels (Ruppel and Merbach 1995, Ruppel and Merbach 1997).

Several crop-inoculation studies have been performed using acetylene reduction, N balance and ^{15}N isotope dilution methods to quantify the contribution of diazotrophic bacteria to the plant N nutrition. The acetylene reduction assay is widely used because of its simplicity and low cost, but measures only nitrogenase activity and reveals no information on whether the fixed N is incorporated into the plant (Boddey et al. 1995). N balance experiments contain two unknown values N-losses and biological N_2-fixation and can therefore underestimate the latter one. The ^{15}N isotope dilution and ^{15}N natural abundance techniques are currently accepted as the most useful methods for examining ANF in in vivo conditions (James 2000). However the ^{15}N isotope dilution method is based on the addition of mineral N to the system, which significantly affects the bacterial gross N-transfer rates and biological N_2 fixation (Ruppel et al. 2006 a, b). In recent years, a molecular method was employed to detect and quantify the functional genes which are regulation ANF (Buergmann et al. 2003, Juraeva et al. 2006).

However, many studies show that ANF is one of the primary mechanisms responsible for improved plant N nutrition after inoculation with PGPB (Boddey and Döbereiner 1995, Garcia de Salamone et al. 1996, Malik et al. 1997, Boddey et al. 1995, James 2000), diazotrophic PGPBs can also enhance plant N uptake by several other mechanisms, as for example, an increased uptake of mineral N by enhanced root N uptake mechanisms (Bashan 1990, Bashan and Levanony 1991), or by increasing the plant root system as the root branching, root number,

thickness, and length (Guanarto et al. 1999, Biswas et al. 2000). There may also exist an antagonistic effect of the bacteria against pathogens, protecting the root from diseases (Bashan and de-Bashan 2002). Therefore, when the role of diazotrophic bacteria for plant growth is studied, it is important to estimate the ANF activity of these bacteria. Although, the presence of *nif*H genes themself can not quantify the value of ANF, the abundance of this gene indicates the potential of diazotrophic N_2 fixation (Juraeva et al. 2006).

In the present study, the relationship between the numbers of introduced diazotrophic bacteria, the numbers of *nif*H genes and the plant N nutrition is estimated. A tomato greenhouse experiment was established using two different N fertilization treatments and the inoculation with two diazotrophic bacterial strains. Bacterial colonization and *nif*H gene quantification was monitored using quantitative real-time PCR and N-balances were calculated.

6.3 Materials and methods

6.3.1 Experimental setup

A two-factorial pot experiment was established with tomato (*Lycopersicon esculentum* [Mill] L. 'Counter F1') in greenhouse conditions (latitude of 52° 21`N, longitude of 13° 18`E at an altitude of 50 m), close to Berlin, Germany. The two factors were mineral N-fertilization values (low nitrogen supply (N1) and high nitrogen supply (N2)) and the application of diazotrophic bacterial strains (control without bacterial application, *Bacillus licheniformis* BL43, *Xanthomonas sp.* Xs148), all treatments were 6 times replicated and completely randomized. Tomato seeds were germinated on trays containing vermiculite under greenhouse conditions. Ten days after germination, tomato plants were removed from the trays and vermiculite was gently washed from root system, plants were inoculated with the bacterial strains (see later in this chapter) and transferred into pots (one seedling per pot) filled with 800 g quartz sand. Plants were grown for 6 weeks (in May-June 2004) at temperatures of 26°C to 28°C during the day and 16°C to 18°C at night in a greenhouse without artificial illumination. The water content was adjusted to 60% of maximal water holding capacity and kept during the entire experiment. Plants were supplied with half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950) lacking N. Nitrogen was supplied separately according the N treatments: N1- low N fertilization, equivalent to approximately 35% of plants N demand and N2 - higher N fertilization, calculated to fully meet the plant N demand for a six week growth period (Tab. 17) at three occasions (2, 21 and 28 days after planting) by injecting NH_4NO_3 solution

homogeneously into the root zone. Plants received potassium/nitrogen/phosphorus in a ratio of 1:0.6:1 and 1:1.8:1 for low and high N supplied plants, respectively.

Tab. 17: Amounts of N fertilized per plant for low (N1) and high N (N2) fertilization treatments, in mg N supplied per plant. The total amount of N was applied at three occasions (2, 21 and 28 days after planting).

N application (days after planting)	N1 (mg)	N2 (mg)
First (Day 2)	25	50
Second (Day 21)	25	60
Third (Day 28)	25	60
Total	75	170

6.3.2 Bacterial strains

Two diazotrophic bacterial strains were used: *Xanthomonas sp.* Xs148 (Xs148) (former *Burkholderia* sp. 148 (Juraeva and Ruppel 2005a)) isolated from the root of wheat grown on Calcisol (loamy sand) in Uzbekistan (Juraeva and Ruppel 2005b, Juraeva and Ruppel, unpublished data) and *Bacillus licheniformis* BL43 (BL43) isolated from maize rhizosphere (Culture collection, Institute of Microbiology, Uzbekistan Academy of Sciences). These strains are proven to promote plant growth significantly (Juraeva and Ruppel, 2005a) and to express antagonistic activity against some pathogen *Fusarium* species.

6.3.3 Inoculation

Pure cultures of bacteria, BL43 and XS148, were grown in Standard I (Merck, Darmstadt, Germany) nutrient solution on a rotary shaker at 28°C for 48h. The bacterial suspensions were centrifuged at 7.000 rpm for 10 min. Growth medium was discarded and the bacterial pellet was resuspended in 0.05M NaCl buffer to a cell density of 10^9-10^{10} cells ml^{-1}, checked by plate dilution technique on Standard I nutrient agar (Merck, Darmstadt, Germany) and MPN method. Seedlings were divided into three treatments: control without bacterial inoculation (-B); bacterial strain BL43 ($+B^{BL}$); and bacterial strain Xs148 ($+B^{Xs}$). At transplanting tomato roots

were dipped into the bacterial suspension for 2 min. Control plants were dipped into sterile 0.05M NaCl buffer.

6.3.4 Plant sampling

Roots and shoots of the plants were sampled at two dates: 7 and 42 days after planting. At day 7 after planting, three replications were harvested, roots and shoots were separated and quartz sand particles were carefully removed from the roots under a gentle stream of tap water.

For molecular measurements 0.1 g fresh samples from the middle part of roots were cut, transferred to separate Eppendorf tubes containing 0.1 g of sterile glass beads (0.5 mm diameter), were frozen at –20°C and lyophilised. Plant DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden GmbH, Germany) as described previously (Juraeva et al. 2006). The rest of plant was used to measure plant fresh mass, total root length and plant dry mass. 42 days after planting, additionally total N content of plants was measured (six replications). Total N concentration in plant tissues was analyzed using a CHN-O rapid elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). At sampling time 42 days after planting a random sub sample: 1 g fresh mass from different parts of root were collected and root length was determined using optical picture analysis system and the KS400 4.0 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany).

6.3.5 Target bacteria, nifH- and TEF gene quantification

Target bacteria 16S-23S ISR of both inoculated bacterial strains (Juraeva and Ruppel 2005a), *nif*H gene (Juraeva et al. 2006) and *TEF* gene copy numbers (Juraeva et al. 2006) in plant samples were quantified using real-time PCR and SYBR® Green I approach, as described previously. The primers used are listed in Tab. 18. Quantitative real-time PCR was performed using an iCycler iQ system (Bio-Rad laboratories, München, Germany) associated with the Icycler iQ Optical System Software (version 3.1; Bio-Rad laboratories, München, Germany). All PCRs were performed in triplicate in a volume of 25 µl. To compensate for any differences in initial template DNA amounts due to variations in different plant sample DNA extraction efficiencies, the 16S-23S ISR and *nif*H gene copy number were calculated as relative values to the housekeeping *TEF* gene copy number using the following equation: relative *nif*H gene copy number = (absolute *nif*H gene copy number *100)/ *TEF* gene copy number; relative 16S-23S ISR copy number = absolute 16S-23S ISR copy number *100/ *TEF* gene copy number.

6.3.6 Statistical analyses

A two-way factorial analysis of variance was used to gauge the significance of differences in mean values between all factor pairs, such as supplied N level versus vegetation stage, supplied N level versus inoculation, vegetation stage versus inoculation. Comparison of mean values of three or six replicates for molecular or plant growth measurements, respectively, was performed using Student's *t* - test at a *P*-level of ≤ 5 %. Linear regression analysis was performed and correlation coefficients were calculated at a *P*-level of ≤ 5 %. Where necessary, log transformations were applied to data sets in order to establish homogeneity of variances. All statistical analyses were performed using STATISTICA 6.0 (StatSoft 2001)

Tab. 18: Specificity and nucleotide sequences of PCR primers used in this study. * I= inosine, R= A or G, W= A or T, Y= C or T

Target Taxon	Primer sequences	Target gene	Product length	Reference
Plant	Teff: 5'-ACT GTG CAG TAG TAC TTG GTG -3' Tefr: 5'-AAG CTA GGA GGT ATT GAC AAG-3'	*TEF*	155bp	Wulf et al. 2003
Bacillus licheniformis BL43	BL43F1: 5'-ACCCACCAAGTCTACTGAACAC-3' BL43R1: 5'-CGAACCGTTGACCTCCTGC-3'	16S-23S *ISR*	108 bp	Juraeva and Ruppel 2005a
Xanthomonas sp. Xs148	Xs148aF1: 5' GCCGATATGAGAGTCCCTTTTG-3' Xs148aR1: 5'-GCCTGTCGGGATCGAACC-3'	16S-23S *ISR*	89bp	Juraeva and Ruppel 2005a
Bacteria	19F*: 5'-GCIWTYTAYGGIAARGGIGG-3' 407R*: 5'-AAICCRCCRCAIACIACRTC-3'	*nifH* gene	~390bp	Ueda et al. 1995

6.4 Results

6.4.1 Plant growth responses

Shoot N concentrations were in range of 0.65-0.75% in low N supplied plants and 1.36-1.46% in high N supplied plants that is below than threshold for N deficiency (<2%; all expressed on a dry matter basis), indicating that even the high N supplied plants were slightly N-starved (World Fertilizer Use Manual, (Online source)). Thus, in all plants, N was a growth-limiting factor. However, inoculated plants appeared more robust and greener than the non-inoculated. Increased N fertilization of the tomato plants induced significant plant growth responses in shoot and root dry weight and N content in both non inoculated and with diazotrophic bacteria inoculated treatments (Tab. 19). High N supplied control plants resulted in significantly increased root length compared with low N supplied control plants (Tab. 19). Bacterial inoculation induced similar high root growth enhancement in low N1 and in high N2 fertilized treatments.

The known plant growth promoting activity of the selected diazotrophic bacterial strains *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148 was established in the tomato experiment. All parameters measured were significantly increased after bacterial inoculation compared to the non inoculated control in the low N1 fertilized treatment. With higher N2 fertilization, the PGPR effect was still detecTab. in total plant growth, N content and root length, however, BL43 did not significantly increase the shoot dry weight and Xs148 failed to significantly increase root dry weight and root N content (Tab. 19).

6.4.2 Plant root colonization and persistence of inoculated bacteria

Inoculated BL43 cells were shown to colonize the young tomato roots (7 days after planting) independently of the N fertilization level confirming the colonization ability of both inoculated bacterial strains on roots (Tab. 20). The inoculation increased the species specific gene copy numbers significantly over the native population at the non-inoculated plants. However, at the end of the experiment, these bacteria were only able to persist at the roots at low N fertilized plants (N1 42 days after inoculation), where significantly higher BL43 target genes were detected compared to the non-inoculated plants. The lower abundance of BL43 in high N supplied $+B^{BL}$

Tab. 19: Effect of N fertilization and diazotrophic bacterial inoculation on plants shoot and root dry wight (d.wt), N content and root length of 42 days old tomato plants. Mean values of 6 replicates ± SE. Asterisk (*) indicates significant bacterial inoculation effects ($P < 0.05$). Bold numbers indicate significant N fertilization effects ($P < 0.05$; bold) N1, low N fertilization (75 mg N·plant^{-1}); N2, high N fertilization (170 mg N·plant^{-1}). -B – non-inoculated, +B^{BL} and +B^{Xs} – plants inoculated with *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148, respectively.

	N1			N2		
	-B	+B^{BL}	+B^{Xs}	-B	+B^{BL}	+B^{Xs}
Total plant d. wt (g plant^{-1})	2.07 ± 0.16	3.15 ± 0.16*	2.95 ± 0.18*	**4.86 ± 0.16**	**5.24 ± 0.12***	**5.35 ± 0.14***
Shoot d. wt (g plant^{-1})	1.82 ± 0.13	2.59 ± 0.17*	2.48 ± 0.14*	**4.12 ± 0.15**	**4.31 ± 0.11**	**4.49 ± 0.14***
Root d. wt (g plant^{-1})	0.35 ± 0.02	0.56 ± 0.04*	0.47 ± 0.04*	**0.83 ± 0.03**	**0.93 ± 0.02***	**0.86 ± 0.03**
Shoot N (mg plant^{-1})	13.05 ± 0.96	16.77 ± 1.45*	18.63 ± 3.71*	**56.13 ± 2.98**	**63.00 ± 1.95***	**61.20± 1.87***
Root N (mg plant^{-1})	5.21 ± 0.39	7.27 ± 0.48*	6.73 ± 0.47*	**21.62 ± 0.76**	**23.56 ± 0.64***	**22.07 ± 0.75**
Total plant N (mg plant^{-1})	18.26 ± 0.65	24.03 ± 1.24*	25.35 ± 1.68*	**77.75 ± 3.46**	**86.56 ± 2.28***	**83.90 ± 2.19**
Root length (m plant^{-1})	29.38 ± 6.60	69.92 ±14.22*	57.34 ± 17.18*	**51.06 ± 2.50**	61.54 ± 5.44*	67.97 ± 5.68*

Tab. 20: Abundance of inoculated bacterial genes and *nif*H genes in tomato roots of non inoculated (-B) and with *B. licheniformis* (BL43) and *Xanthomonas sp.* (Xs148) (+B) inoculated plants in treatments with low N1 and high N2 fertilization levels, all investigations carried out 7 and 42 days after planting. Gene abundance is expressed relative to the housekeeping *TEF* gene. Mean values of three replicates ± SE. Asterisk (*) indicates significant bacterial inoculation effects ($P < 0.05$) Bold numbers indicate significant N fertilization effects ($P < 0.05$; bold) N1, low N fertilization (75 mg $N \cdot plant^{-1}$); N2, high N fertilization (170 mg $N \cdot plant^{-1}$) separately at each sampling time. -B – non-inoculated, $+B^{BL}$ and $+B^{Xs}$ – plants inoculated with *B. licheniformis* BL43 and *Xanthomonas sp.* Xs148, respectively.

		Target bacterial abundance		*nif*H gene abundance	
Sampling time (days after planting)	N level	BL43	Xs148	BL43	Xs148
7 (-B)	N1	8.73 ± 0.86	8.58 ± 0.26	9.95 ± 0.76	9.95 ± 0.76
	N2	8.27 ± 0.20	**10.55 ± 0.08**	9.33 ± 0.51	9.33 ± 0.51
7 (+B)	N1	**12.30*** ± 0.70	**10.83* ± 0.26**	11.42* ± 0.17	11.66* ± 0.60
	N2	11.07* ± 0.10	10.41 ± 0.07	10.07* ± 0.14	10.65* ± 0.81
42 (-B)	N1	9.43 ± 0.30	**9.85 ± 0.00**	8.84* ± 0.25	8.84* ± 0.25
	N2	9.88 ± 0.22	9.19 ± 0.33	8.15 ± 0.45	8.15 ± 0.45
42 (+B)	N1	9.67* ± 0.39	9.65 ± 0.23	**8.58 ± 0.30**	7.05 ± 0.18
	N2	8.30 ± 0.22	**10.73* ± 0.23**	7.20 ± 0.07	**7.90 ± 0.61**

plant roots in comparison to low N supplied $+B^{BL}$ plant roots sampled on both sampling times suggested that N availability affected BL43 population in tomato plant root.

The effect of N availability was shown to be stronger on Xs148 abundance. Due to the high native population density of *Xanthomonas sp.* the persistence of inoculated Xs148 bacterial cells was hardly to show. In early growth stage, with increased N fertilization the native *Xanthomonas sp.* population was raised (Tab. 20) Therefore, the measurements showed that the inoculated Xs148 cells only increased the cell numbers of the *Xanthomonas* species at the low N fertilized tomato roots, while this significance was lost in inoculated plants fertilized with high N (Tab. 20). These data indicate a better colonization rate of native *Xanthomonas sp.* cells at tomato plant roots grown in higher N fertilization level.

6.4.3 *nif*H gene abundance in inoculated and non-inoculated plant root

Inoculation with both diazotrophic bacterial strains BL43 and Xs148 increased the *nif*H gene abundance at inoculated plant roots in low and high N fertilized treatments over the *nif*H gene abundance at the non-inoculated control roots 7 days after planting (Tab. 20). That increased

*nif*H gene level, which was established 7 days after the inoculation of diazotrophic bacterial cells, did not remain stable during the plant growth. In contrast, at 42-day-old tomato plant roots *nif*H gene abundance was higher at non-inoculated plant roots than at inoculated ones (Tab. 20). Data from both sampling times of non-inoculated plants revealed that the *nif*H gene abundance in high N supplied plants was relatively lower when compared with low N supplied plants (Tab. 20). In younger plant roots, gene abundance was relatively higher than older plants. However, those values did not reach the significant level.

6.4.4 Interrelationship between diazotrophic bacterial inoculation, nifH gene abundance and plant N nutrition

The inoculation of tomato plants with the diazotrophic bacterial strains BL43 and Xs148 increased the *nif*H gene abundance significantly at low N fertilization level at both investigation times 7 and 42 days after planting and inoculation (positive correlation *nif*H cn vs. ISR cn Tab. 21). Since this positive correlation was observed in most treatments and plant growth stages (Tab. 21), *nif*H gene concentrations in +B plant roots shown to be controlled by the introduced bacterial population. The only exception was the high N supplied $+B^{Xs}$ treatment.

Plants influenced by the inoculum during early stages of development may be affected during early stages of development, even if the inoculum does not persist as a dominant rhizosphere inhabitant throughout plant growth. This effect, for example, been shown in wheat inoculated with Azotobacter, *Bacillus* and Clostridium (Rovira 1965).

The response of inoculated bacteria to N availability indicating that both bacteria were suppressed by higher mineral N fertilization (Tab. 20), reflected in *nif*H gene quantification from +B plants showing high degree of correlation between introduced bacteria abundance and *nif*H gene pool of both bacterial strains in low N fertilized treatments – however, there was no significant correlation in the high N fertilized treatments at Day 42 after planting (Tab. 21). In the low N fertilized treatments, the plant N content was increased with both increased numbers of inoculated bacterial cells and increased *nif*H gene copy numbers at the end of the experiment, 42 days after inoculation. Such a positive correlation was not detecTab. in the high N fertilized treatments (Tab. 21). In the higher N treatment a positive correlation did only occur between *nif*H gene copy numbers and the BL43 gene copy numbers immediately (Day 7) after the bacterial inoculation. In the older plants this effect was lost.

Tab. 21: The effect of N fertilisation level on the linear regression relationships between *nif*H gene copy number (*nif*H cn) and the BL and Xs bacterial copy numbers (ISR cn), and total plant N content. ND – not determined; BL43, plants inoculated with *B. licheniformis* BL43; Xs148, plants inoculated with *Xanthomonas sp.* Xs148. Asterisk (*) indicates significant correlation.

Sampling time (days after planting	N level	*nif*H cn vs ISR cn		*nif*H cn vs plant N content		ISR cn vs plant N content	
		BL43	Xs148	BL43	Xs148	BL43	Xs148
7	N1	0.96*	0.82*	ND	ND	ND	ND
7	N2	0.89*	-0.23	ND	ND	ND	ND
42	N1	0.86*	0.99*	0.95*	0.99*	0.95*	1.00*
42	N2	0.43	0.80	-0.55	0.55	0.51	-0.06

6.5 Discussion

6.5.1 Plant growth responses

Besides the biological nitrogen fixing activity, a range of other plant growth promoting mechanisms can be induced by bacterial inoculation (Dobbelaere et al. 2003, Bashan et al. 2004) and positive response of the inoculation process in improving plant N nutrition can be related to one of those plant growth mechanisms of the inocula tested. PGPB have a significant impact on nitrogen nutrition by increasing N uptake capacity, indirectly as a consequence of stimulated lateral root development (Okon and Vanderleyden 1997, Bertrand et al. 2000) and possibly directly by stimulating ion uptake systems. In this study, the inoculation process also showed significant effects on plant root resulting in increased root length (Tab. 19). Therefore, the indirect effect of PGPB on nutrient uptake via the increased root surface area due to the stimulation of root development can also be suggested as one possible effect mechanism for the improvement of N uptake (Guanarto et al. 1999, Biswas et al. 2000, Mantelin and Touraine 2004).

In its turn, the availability of NO^{-3} is known to affect root branching (Forde et al. 2002). In this study, root measurement results were consistent with the suggestions of previous reports (Dobbelaere et al. 2002, Mantelin and Touraine, 2004) that the total root length is the highest in inoculated plants grown with low N fertilization. In high N fertilized plants, although, the total root length was significantly higher than control plants, the effect of inoculation on root development was much lower than in low N supplied plants.

6.5.2 Interrelation between the abundance of introduced diazotrophic bacteria and plant N nutrition

Effective colonization of plant roots by PGPB plays an important role in growth promotion, irrespective of the mechanism of action (Bolwerk et al. 2003, Raaijmakers et al. 1995).

There are different reports considering the effect of N availability on diazotroph bacterial abundance. For instance, our observations in this study are in agreement with some reports (Cejudo and Paneque 1986, Limmer and Drake 1998, Muthukumarasamy et al. 2002), showing that N availability is negatively correlated with inoculated diazotrophic bacteria abundance tested in this study (Tab. 3). Since the plants select for functional groups in the rhizosphere to be colonised (Grayston et al. 1998, Grayston et al. 2001, Baudoin et al. 2003), it can be suggested that in N limiting conditions, plants select more N_2 fixers.

In this study, we document that N availability affected different diazotrophic bacterial species in different level. The strong effect of N fertilization on diazotrophic bacteria abundance demonstrates the importance to determine optimal N fertilizer levels for efficient inoculation experiments. Measurements of introduced bacteria like bacteria population in +B and -B plants showed that, although, the abundance of both introduced bacteria in +B plants and introduced bacteria like natural bacteria populations in -B plants was significantly decreased in response to high N fertilization, inoculation of plant with diazotrophic bacteria allowed to provide highly abundance of diazotrophic community in inoculated plant root even in high N fertilization providing high potential of diazotrophic community to fix atmospheric N_2.

The significantly close relationship between introduced bacterial abundance and *nif*H gene copy number in plant roots (Tab. 21) indicated the presence of a high potential of introduced bacteria to fix atmospheric N_2 in low N conditions. The positive correlation between introduced bacterial cells and the *nif*H gene copy numbers and the plant N content strongly suggest a positive impact of inoculated diazotrophic bacteria on plant N nutrition in young plant growth stages under N limited conditions.

Regression analysis to determine the relationships of those introduced diazotrophic bacteria and *nif*H gene abundance, and the significant correlation of their abundance to plant N content (Tab. 21) could be employed to identify the possible contribution of biological N_2-fixation by introduced bacteria to plant N nutrition. Introducing the active members of diazotrophs to plant root increased the amount of this contribution, however, relatively less close relationships of *nif*H gene abundance and plant N nutrition of +B plants in comparison to -B plants was observed (Tab. 21). It may be due to the secondary mechanisms, like root growth promotion by phytohormonal effects (Tab. 19), of introduced bacteria contributed to general N nutrition of plant (Bashan et al. 1989, Hurek et al. 1994).

Regression analysis allowed to evaluate the effect of supplied N to the supposed activity of *nif*H gene existed in plant root. The correlation between *nif*H gene and plant N nutrition for low N supplied plants was stronger in comparison to high N supplied plants (Tab. 21) indicating that an increased N input does not only induce changes in the abundance of *nif*H gene, but also influences the potential activity of this gene suggesting that the proportion of biological N_2-fixation was higher at low N levels.

Plant experiments performed using the non-nitrogen fixing (*Nif*) *Azoarcus* sp. mutant as a negative control, combined with ^{15}N – based balance studies (Hurek et al. 1998, Hurek et al. 2002) can provide the direct evidence whether plant benefits from the N_2 fixed by introduced diazotrophic strains. However, as it is observed in this study that increasing of *nif*H gene pool in plant root was not due to the contribution of introduced bacteria (because, it was not colonized significantly), but it was shown to be the effect of introduced diazotrophic bacteria on the abundance of natural diazotrophic population, such methods which centres on only introduced diazotrophs, would ignore the possible contribution of inoculated bacteria on the native diazotrophic population inhabiting the plant and can led to the wrong conclusion that plant N nutrition was improved by another plant growth promoting effects of introduced bacteria.

6.5.3 Native diazotrophic bacteria abundance in tomato plants

It is often claimed that the presence of *nif*H gene does not directly related to its activity, because *nif*H gene expression is highly regulated (Hoover 2000), at both transcriptional (Chen et al. 1998) and post-translational levels (Kim et al. 1999). However, the assumption that "genes are ultimately not retained by microorganisms, unless they are functional and thus, are selected for in the environment" (Zehr et al. 2003) should be true for nitrogenase, since N_2 fixation can involve around 20 genes. Therefore, it can be suggested that significantly close relationships of *nif*H gene abundance and plant N content (correlation coefficient of 1.00 and 0.71 for low and high N supplied, respectively; data not shown) can indicate evidence for direct contribution of natural diazotrophic population to plant N nutrition.

The population of native diazotrophic bacteria may vary in different plant species, even in different varieties (Hoefsloot et al. 2005). We earlier reported (Juraeva et al. 2006) that natural diazotrophic community abundance inhabiting cucumber plant root was positively correlated with N availability. It is likely that the effect of N availability on diazotrophs may be plant-species dependent, as we have observed in measurements of both inoculated-like-native-diazotrophic bacteria and *nif*H gene abundance, the lower availability of inorganic nitrogen was favorable to tomato-root-inhabiting-native-diazotrophic bacteria.

6.6 Conclusion

The observed significant effect of N availability on the colonization ability of inoculated bacterial strains underlines the importance to optimize the mineral N fertilization level for a successful application of diazotrophic biofertilizers. Factorial experiment studies of *nif*H gene abundance and plant N nutrition can serve to evaluate the contribution of diazotrophic bacteria to the N nutrition of plants. One of the remarkable conclusions of this study is that if three data, (i) considered bacteria, (ii) *nif*H gene abundance and (iii) total plant N nutrition, are significantly positive correlated to each other, a direct contribution of inoculated bacteria to the improved plant N nutrition due to its N_2 - fixing ability can be assumed.

6.7 References

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7 GENERAL DISCUSSION

The previous chapters of this thesis describe results obtained in several separate experiments. In this last chapter, the main results of these experiments are discussed in view of the hypotheses given in the General Introduction. Finally, some perspectives for further research are presented.

7.1 Importance of a polyphasic approach in identifying bacterial isolates

The exact taxonomic affiliation of some microorganisms can only be ascertained by using a polyphasic taxonomic approach. The present study genetically characterised two bacterial isolates isolated from plant endorhizosphere and compared these results against those derived from bacteriological and metabolic identification techniques to assess the need for a polyphasic approach to identify bacteria.

At the first stage of our investigation, we identified the strains at genus level only (chapter 2). Majority were identified as *Bacillus* sp. The remaining isolates were identified as *Pseudomanas*, *Burkholderia, Klebsiella* and others.

For both strains of interest, BL43 and Xs148 which have been selected for further studies for their universal PGP effect on wheat, cauliflower, cucumber, paprika and tomato plants, BIOLOG test based identifications did not agree with the sequence-based identification (chapter 3). Although the BIOLOG system has been evaluated as having the large database (Holmes et al. 1994), this system's identification did not always agree with biochemical criteria belonging to some genera including *Bacillus* (Tang et al. 1998). Therefore, failure of the BIOLOG system to identify isolate BL43 could be explained by the fact that this isolate belongs to the genera *Bacillus* as established using rRNA sequence tests (chapter 3). Also for Xs148, the BIOLOG test resulted in *Burkholderia glumae* being identified, whereas 16S rRNA sequence-based analysis showed the highest matches to *Xanthomonas*. The EMBL-Bank sequences of *Burkholderia* were not even shown on the BLAST match list due to too low similarity. Since, we found that there are more than 600 entries for cultured *Burkholderia* species in the EMBL-Bank (January 2006), such a number should be enough to determine the sequence in question, at least to the genus level, if the target bacteria belonged to this genus.

Because the 16S rDNA gene is highly conserved, determination of species and strain distinctions relies upon the resolution of only small differences between sequences. Moreover, as two distinct species may possess identical 16S rRNA sequences including *Bacillus*, gene sequence identification is not foolproof (Ash et al. 1991, Fox et al. 1992, Clarridge 2004).

Therefore, taken together, we suggest that 16S rRNA-based identification of strains of some genera at the species level is not reliable enough and requires additional tests. Moreover, reliance on a single molecular method for species definition, such as 16S rRNA gene sequencing, cannot take into account small evolutionary changes, such as point mutations (Stackebrandt et al. 2002). Thus, in practice, a polyphasic approach including alternate gene targets performed in parallel with the examination of a number of phenotypic properties is necessary for definitive species identification.

The 16S-23S rRNA ISR proved to be the best alternate target to 16S rRNA because we found a high consensus between results from 16S rRNA and 16S-23S rRNA ISR sequence-based identification for isolate Xs148. Direct sequence determination of 16S-23S ISR fragments represented a highly accurate method for bacterial identification to the species level, even when the species in question was notoriously difficult to identify by 16S rRNA sequence-based identification. This is because 16S-23S rRNA ISR similarities reflect phylogenetic relationships and has more discriminative nucleotide stretches, which allow identifying in species, even to the strain level (Blackwood et al. 2004). Since, the 16S-23S rRNA ISR-based approach failed to identify isolate BL43 producing differing data to both the basic bacteriological and 16S rRNA sequence-based identification results, we suggest that the potential of ISR analysis for bacteria identification is likely to depend on specific nucleotide stretches of the ISR or on the bacteria species studied (Yoon et al. 1997, Yoon et al. 1998, Kuwahara et al. 2001).

In this study, genotypic methods based on rRNA sequence analysis improved the identification of plant-inhabiting diazotrophic bacteria, thereby completing the identification results obtained using conventional biochemical methods and the BIOLOG® test. From our analyses, we conclude that rRNA sequence-based identification should be performed in conjunction with traditional biochemical bacteriological tests that provide basic information about the microorganism in query.

7.2 Potential of different databases and search programs in molecular identification of bacteria

To demonstrate the quality and accuracy of results provided from different programs and databases, searches for sequence similarity analysis were performed using programs such as BLASTN, FASTA comparing the sequence to the databases EMBL-Bank and RDP-II.

The identification of bacteria based on 16S rRNA sequences was complicated due to numerous ambiguous results.

1. Different results were obtained even when the sequence was compared against the same database (EMBL-Bank) by using different programs (FASTA and BLASTN), resulting in the assignment of different identities. For example, for isolate Xs148, using BLAST searches resulted in the highest matches to the *Pseudomonas* genus, while using the FASTA program, isolate Xs148 was closest in identity to the *Xanthomonas* species (chapter 3).

2. 16S rRNA sequence based searching results were not specific enough to differentiate the isolate to the species level. For example, BL43 was 99% identical to many different species of *Bacillus subtilis* group: *B. licheniformis, B. subtilis*, *B. mojavensis* and *B. vallismortis* and also *B. amyloliquefaciens*.

3. 16S rRNA sequence based search result disagreed with the phenotypic characterization of the strain. For BL43, BLAST searches showed 99% identity of the sequence in query to a micro-organism belonging to gram-negative bacteria, *Pseudomonas* sp. As sequences can be deposited in the GenBank-EMBL and RDP-II databases without undergoing any checks, it is not surprising that errors do occur as regards species assignment (Harmsen et al. 2003).

4. RDP-II database based searches showed highly ambiguous results, most probably, due to the limited number of sequences included in the sequence match search (Chapter 3, Tab. 12b).

5. We found, that the RIDOM database is not useful for identification of the bacterial strains investigated in this study, although Blackwood et al. (2004) reported that 16S rRNA gene sequences for 65 (of all 83) type strains of the *Bacillus* genus have been submitted to the RIDOM database at http://rdna.ridom.de/.

From our analyses, we conclude that (i) for similarity scores of different species belonging to the same genus of less than 1 % dissimilarity, the FASTA program displays more accurate values, i.e. to 3 decimal places, than BLAST, and (ii)16S rRNA in conjunction with 16S-23S rRNA ISR sequence-based identification can be used to identify and differentiate between the species only if the quality of the database is high enough, i.e. it contains a high number of reliable sequence entries taken from a comprehensive range of species.

7.3 Effect of supplied nitrogen level on the abundance and activity of natural and introduced diazotrophic bacteria on plant root

Various studies indicated that the abundance of total diazotrophs or of specific populations in both pure culture and environmental samples can be influenced by the amounts of inorganic N applied (Cejudo and Paneque 1986, Herridge and Brockwell 1988, Limmer and Drake 1998, Fuentes-Ramirez et al. 1999, Tan et al. 2003).

We have studied (i) the abundance of natural diazotrophic bacteria in plant tissue by quantifying *nif*H gene copies using a new method developed in this study (chapter 5), and (ii)

the colonization ability of certain bacteria in plant root and shoot by quantifying strain-specific ISR using real-time PCR approaches (chapters 4, 6). The quantified *nif*H gene values reflect the abundance of diazotrophic populations associated with the plant.

Effective colonization of plant roots by PGPB plays an important role in growth promotion, irrespective of the mechanism of action (Bolwerk et al. 2003, Raaijmakers et al. 1995).

It is well recognized that N availability is mostly negatively correlated with inoculated diazotrophic bacteria abundance (Cejudo and Paneque 1986, Limmer and Drake 1998; Tan et al. 2003). We document that N availability affected different diazotrophic bacterial species in different level (chapter 6). Quantification results showed that N availability was negatively correlated to *Xanthomonas sp.* Xs148 abundance showing relatively less bacteria abundance in high N supplied plants (Fig. 3), while *B. licheniformis* BL43 abundance in high N supplied plants were significantly higher than low N supplied plants. The strong effect of N fertilization on diazotrophic bacteria abundance demonstrates the importance to determine optimal N fertilizer levels for efficient inoculation experiments.

Measurements of introduced bacteria like bacteria population in +B and -B plants showed that, although, the abundance of both introduced bacteria in +B plants and introduced bacteria like natural bacteria populations in -B plants was significantly decreased in response to high N fertilization (chapters 2, 6), inoculation of plant with diazotrophic bacteria allowed to provide highly abundance of diazotrophic community in inoculated plant root even in high N fertilization providing high potential of diazotrophic community to fix atmospheric N_2.

Results showed that the effect of N availability on diazotrophs may be plant-species dependent, as in tomato roots (chapter 6), the diazotrophic communities were suppressed by higher mineral N fertilization, while higher N supply stimulated the growth of diazotrophic bacteria population in cucumber roots. We suggest that a significantly higher diazotrophic bacteria abundance in high N supplied cucumber plants can be partly explained by a significantly larger root system developed (chapter 5, Tab. 15) compared to the low N supplied plants, providing favorable conditions for microorganism growth, including those for diazotrophic bacteria.

Our comparative quantified-gene analyses indicated that methods which centre on only introduced diazotrophs, would ignore the possible effect of introduced bacteria on the native diazotrophic population inhabiting the plant and can led to the wrong conclusion that plant N nutrition was improved by another plant growth promoting effects of introduced bacteria. We observed significantly higher *nif*H gene abundance in inoculated plant roots compared to control plant roots, while introduced bacteria was not colonized significantly, indicating that increasing of *nif*H gene pool in plant root was not due to the contribution of introduced bacteria,

but it was shown to be the effect of introduced diazotrophic bacteria on the abundance of natural diazotrophic population (chapter 6).

7.4 Relationship between nifH-gene abundance and plant N nutrition

Although the DNA-based PCR method is not directly related to *nif*H gene expression, it does allow comparison of the effects of different treatments on the N_2–fixing potential of the microbial community in plant samples by quantification of *nif*H gene abundance in plant. Therefore, it can be suggested that significantly close relationships of *nif*H gene abundance and tomato plant N content (correlation coefficient of 1.00 and 0.71 for low and high N supplied, respectively; data not shown) can indicate evidence for direct contribution of natural diazotrophic population to plant N nutrition.

Regression analysis to determine the relationships of those introduced diazotrophic bacteria and *nif*H gene abundance, and the significant correlation of their abundance to plant N content (Chapter 6, Tab. 21) could be employed to identify the possible contribution of biological N_2-fixation by introduced bacteria to plant N nutrition. Introducing the active members of diazotrophs to plant root increased the amount of this contribution, however, relatively less close relationships of *nif*H gene abundance and plant N nutrition of +B plants in comparison to -B plants was observed (Tab. 21). It may be due to the secondary mechanisms, like root growth promotion by phytohormonal effects (Tab. 19), of introduced bacteria contributed to general N nutrition of plant (Bashan et al. 1989, Hurek et al. 1994).

Regression analysis allowed to evaluate the effect of supplied N to the supposed activity of *nif*H gene existed in plant root. The correlation between *nif*H gene and plant N nutrition for low N supplied plants was stronger in comparison to high N supplied plants (Tab. 21) indicating that an increased N input does not only induce changes in the abundance of *nif*H gene, but also influences the potential activity of this gene suggesting that the proportion of biological N_2-fixation was higher at low N levels (Chapter 6).

7.5 PGP effect of inoculated bacteria

The importance of bacterial strain *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 was particularly significant regarding its beneficial effects on the plant growth and suppression the growth of plant pathogens. They stimulate the growth of wheat, and that PGPBs of wheat tested were not strictly plant-specific stimulating the different vegetable species.

Since, plants were grown in sand and supplied with half-strength Hoagland solution, the significant positive influence of inoculated bacteria on plant growth emphasizes that the

bacteria tested can influence plant growth even in the presence of a nutrient solution. These findings suggest that plants may be grown with lower amounts of applied fertilizers and implies (1) a reduction in the cost associated with growing plants and (2) a reduction in the pollution associated with agricultural practices.

7.6 Perspectives for further research

This thesis covered several aspects of the influence of PGPB on plant growth.

New questions arose during the study that should be investigated in more detail in further experiments. Some of these points are summarized in the following.

New technologies and methods to investigate microbial communities are being developed at a rapid pace and provide new opportunities to link community structure to ecosystem processes. In this study, the combination of *nif*H-gene quantification and plant N-uptake measurements was shown to be a possible tool to evaluate the contribution of the N_2–fixing plant-inhabiting diazotrophic community to plant N nutrition. The positive correlation between *nif*H gene abundance and plant N nutrition highlights the potential value of studying functional genes in the context of ecosystem processes. However, these results are only suggestive of this relationship, and future studies should focus on measuring the relationships of gene abundance to the target gene expression and activity simultaneously. The herein described DNA-based real-time PCR quantification of *nif*H gene abundance in plant tissues can be extended to RNA-based approaches as DNA is more likely to reflect the standing biomass of a particular community and mRNA should be more closely related to activity rates (Bürgmann et al. 2003). Additionally, the relationship between the diversity of special diazotrophic bacterial populations and their sensitivity to environmental changes should be examined.

8 SUMMARY

Diazotrophic bacteria can be beneficial for agricultural crops due to their nitrogen fixation ability and other plant growth promoting mechanisms. However, asymbiotic nitrogen fixation can not cover total nitrogen demand of plant, and requires additional mineral N supply. These characteristics of diazotrophic bacteria may be of relevance under specific plant nutritional conditions. Therefore, recently two topics gained in importance: 1. the understanding of abundance of diazotrophic bacteria and 2. the PGP effect of diazotrophic bacteria in different levels of N supply.

These topics with high practical relevance have until now only been sparsely investigated.

The present work investigates plant growth promoting effects of associative diazotrophic bacteria, its colonization in plant and supposed activity when plants are supplied with different level of mineral nitrogen. It focuses on the development and application of molecular methods to evaluate: (a) the colonization ability of inoculated bacteria in plant root and shoot (b) the abundance of natural diazotrophic population in plant tissue by quantifying the *nif*H gene (c) the potential nitrogen fixing ability of bacteria in different nitrogen supplied conditions.

In the frame of this thesis, a number of methods for the description of the diversity of root colonizing associative diazotrophs have been developed and improved to provide links between introduced diazotophic bacteria abundance and activities. The approach used was based on the sensitive real – time PCR detection/quantification of introduced bacteria and the nitrogenase reductase gene (*nif*H), which served as a marker gene for potential diazotrophs.

The amplified 16S-23S ISR sequences of studied bacteria were subjected to species – specific primer design and a highly specific bacteria quantification protocol were developed. These protocols were used to evaluate the colonization ability of studied bacteria, which was inoculated to plant root.

The application previously used universal *nif*H primers to the real – time PCR improved the detection of less abundant diazotrophs in dry land plant root. The protocols were tested and optimized using pure cultures of diazotroph reference strains, and subsequently applied to the analysis of two vegetable plant roots. The analyses of real-time PCR products obtained from plant root DNA extracts revealed that the new *nif*H PCR protocol differentiated between the diazotroph populations in different plants.

The developed methodology was applied to study *nif*H abundance of *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 inoculated to cucumber and tomato growing in non – sterile quartz sand. Treatments with nitrogen limiting conditions resulted in more diazotrophic bacteria

abundance, as well as, *nif*H gene pool while nitrogen excess suppressed diazotrophic bacteria abundance in both inoculated and non-inoculated plants (chapter 6). On low N supplied plants compared to high N supplied plants, inoculation with BL43 bacteria resulted in up to 12% and 17% more bacteria abundance 7 days and 42 days after inoculation, respectively. In contrast, 42 days after inoculation, inoculation with Xs148 resulted in higher bacteria abundance in high N supplied plants resulting in 11% more bacteria abundance compared to low N supplied plants suggesting that the response of diazotrophic bacteria abundance to N availability can be bacteria-species specific. In low N supplied plants, inoculation with both bacteria resulted in significant increase of plant dry weight (root, shoot, and total), length (root, shoot and total) and N concentrations (root, shoot and total) (chapter 6). In high N supplied plants, Xs148 colonisation of tomato increased shoot N concentrations, but did not significantly affect root N concentrations. In low N availability, bacterial inoculation (chapter 4) increased plant growth, N concentration in plants, but bacterial inoculation did not always completely meet plant N demand.

The *nif*H gene abundance was significantly correlated with measurements of N amount taken by the plant and inoculated bacteria density showing direct contribution of introduced bacteria to plant N nutrition. The correlation between *nif*H gene and plant N concentration for low N supplied plants was stronger (up to $r = 0.99$) in comparison to high N supplied plants (up to $r = 0.55$) indicating the effect of supplied N to the supposed activity of *nif*H gene existed in plant root (chapter 6).

The results presented in this thesis have shown that monitoring of *nif*H amount in plant root is a suitable and promising approach to link inoculated diazotrophic bacteria abundance and its potential nitrogenase activity. The study of *nif*H gene abundance in plant offers the opportunity to identify key players in asymbiotic nitrogen fixation, to study short-term community responses in changing environments, or to analyze the effect of regulation *in situ*.

The strong effect of N fertilisation on diazotrophic bacteria abundance demonstrates the importance to determine optimal N fertilizer levels for efficent inoculation experiments.

Zusammenfassung

Diazotrophe Bakterien können auf Grund ihrer Luftstickstoffbindungsaktivität und anderer das Pflanzenwachstum fördernder Eigenschaften Vorteile für die landwirtschaftliche Pflanzenproduktion haben. Allerdings kann über diese assoziative Luftstickstoffbindung der N-Bedarf der Pflanzen nicht vollständig gedeckt werden, so dass zusätzliche mineralische Stickstoffbindung notwendig bleibt. Diese Eigenschaften der diazotrophen Bakterien könnten unter speziellen Ernährungsbedingungen der Pflanzen unterschiedlich ausgeprägt sein. Deshalb gewannen in letzter Zeit zwei Fragen an Bedeutung: 1. zu verstehen unter welchen N-Ernährungsbedingungen der Pflanzen diazotrophe Bakterien vorkommen und 2. welche Eigenschaften der diazotrophen Bakterien zu wachstumsfördernden Effekten der Pflanzen führen.

Diese sehr praxisrelevanten Fragen wurden bisher nur wenig untersucht.

Die vorliegende Arbeit analysiert pflanzenwachstumsfördernde Effekte assoziativer diazotropher Bakterien, ihre Besiedlungsfähigkeit an Pflanzen und deren mögliche Aktivitäten bei unterschiedlicher Stickstoffverfügbarkeit. Sie konzentriert sich auf die Entwicklung und Anwendung molekularer Methoden zur Untersuchung: (a) der Besiedlungsfähigkeit inokulierter Bakterien an Pflanzenwurzeln und an Blättern, (b) der Verbreitung natürlich vorkommender diazotropher Populationen im pflanzlichen Gewebe über die Quantifizierung des *nif*H Gens, (c) der potentiellen Luftstickstoffbindungsaktivität der Bakterien bei unterschiedlicher N-Verfügbarkeit.

Im Rahmen dieser Arbeit wurden verschiedene Methoden zur Beschreibung der Diversität von rhizosphären assoziativen diazotrophen Bakterien erarbeitet und weiterentwickelt um die Interaktion zwischen inokulierten diazotrophen Bakterien und ihrer Aktivität zu analysieren. Die genutzten Versuchsansätze basierten auf der sensitiven real-time PCR Methode zur Detektion und Quantifizierung inokulierter Bakterien und des nitrogenase-reduktase Gens (*nif*H), welches als Markergen für potentiell diazotrophe Bakterien diente.

Art spezifische Primer wurden auf der Grundlage der 16S-23S ISR Sequenz der Bakterien entwickelt und hoch spezifische Protokolle zur Bakterienquantifizierung erarbeitet. Mit Hilfe dieser Protokolle wurde die Besiedlungsfähigkeit inokulierter Bakterien an Pflanzenwurzeln analysiert.

Die Anwendung der bekannten *nif*H Primer in der real-time PCR verbesserte die Möglichkeit der Detektion von diazotrophen Bakterien die nur in geringen Keimzahlen an Pflanzenwurzeln trockener Gebiete vorkommen. Die Protokolle wurden unter Nutzung von

Bakterienreinkulturen diazotropher Organismen getestet und optimiert und nachfolgend zur Quantifizierung der *nif*H Gene an Wurzeln zweier Gemüsekulturen eingesetzt. Die Analyse der real-time PCR Produkte, die nach Amplifikation der Pflanzenwurzel DNA Extrakte gewonnen wurden, zeigte deutliche Unterschiede in den Populationen diazotropher Bakterien in unterschiedlichen Pflanzenarten.

Die entwickelte Methode wurde zum Studium des *nif*H Gen Vorkommens in *Bacillus licheniformis* BL43 and *Xanthomonas sp.* Xs148 genutzt, welche zu Gurke und Tomate inokuliert wurden, die in nicht sterilisiertem Quarz Sand wuchsen. Pflanzen die unter Stickstoff limitierten Bedingungen wuchsen zeigten sowohl eine höhere Keimzahl diazotropher Bakterien als auch eine höhere Kopienzahl an *nif*H Genen, während an Pflanzen, die unter hohem N-Angebot wuchsen, die Anzahl diazotropher Bakterien reduziert war. Diese Reduktion war sowohl in Varianten mit Bakterieninokulation als auch in nicht inokulierten Kontrollpflanzen zu sehen (Kapitel 6). In gering mit N gedüngten Pflanzen wurden nach Inokulation von BL43 bis zu 12% und 17% mehr Bakterien gemessen (7 und 42 Tage nach Inokulation) im Vergleich zu hoch mit N versorgten Pflanzen. Im Gegensatz dazu wurden 42 Tage nach Inokulation des Bakterienstammes Xs148 bis zu 11% höhere Bakterienkeimzahlen an den hoch mit N gedüngten Pflanzen gefunden im Vergleich zu gering mit N gedüngten Pflanzen. Diese Ergebnisse deuten auf eine mögliche bakterienartspezifische Reaktion diazotropher Bakterien auf die N-Verfügbarkeit hin.

Bei geringer N-Versorgung führten beide Bakterienstämme nach Inokulation zu einer erhöhten Trockenmassebildung der Pflanzen (Spross, Wurzel und Gesamtmasse), zur Erhöhung der N-Konzentration in allen Pflanzenteilen als auch zu einer Vergrößerung der Pflanzenlänge (Wurzel, Spross und Gesamt) (Kapitel 6). In hoch mit N gedüngten Pflanzen war die Spross N-Konzentration durch die Inokulation des Stammes Xs148 erhöht, aber nicht die Wurzel N-Konzentration. Die Bakterieninokulation erhöhte zwar in den gering mit N versorgten Pflanzen sowohl das Pflanzenwachstum als auch die N-Konzentration in der Pflanze, sie konnte aber den gesamt N-Bedarf der Pflanzen nicht immer vollständig decken (Kapitel 4).

Das *nif*H Gen Vorkommen war signifikant positiv zur N-Aufnahme der Pflanzen korreliert und es konnte ein direkter Beitrag der inokulierten Bakterien zur N-Versorgung der Pflanzen nachgewiesen werden. Diese Beziehung war in gering mit N gedüngten Pflanzen stärker nachzuweisen (bis zu $r = 0.99$) als in hoch mit N gedüngten Pflanzen (bis zu $r = 0.55$), was auf einen Einfluss des mineralisch gedüngten Stickstoffs auf die *nif*H Gen Aktivität an den Pflanzenwurzeln hinweist (Kapitel 6).

Die Ergebnisse dieser Arbeit zeigten deutlich, dass die Messung des *nif*H Gen Vorkommens an Pflanzenwurzeln eine nützliche und vielversprechende Methode ist, um die Verbindung zwischen der Besiedlung inokulierter diazotropher Bakterien und deren potentieller Luftstickstoffbindungsaktivität zu untersuchen. Das Studium des *nif*H Gen Vorkommens in Pflanzen eröffnet die Möglichkeit, Schlüsselorganismen der assoziativen Luftstickstoffbindung zu identifizieren und kurzfristige Reaktionen der mikrobiellen Population auf Umwelt Veränderungen *in situ* zu messen. Der starke Effekt der N Düngung auf das Vorkommen diazotropher Bakterien hebt sehr deutlich hervor, dass für eine effiziente Inokulation diazotropher Bakterien optimale N-Düngermengen analysiert werden sollten.

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