Katholieke Universiteit Leuven
Faculteit Bio-ingenieurswetenschappen

DISSERTATIONES DE AGRICULTURA

Doctoraatsproefschrift nr. 807 aan de faculteit Bio-ingenieurswetenschappen van de K.U.Leuven

Phytostimulatory effect of *Rhizobium* and Plant Growth Promoting Rhizobacteria in common bean (*Phaseolus vulgaris* L.) interaction

Proefschrift voorgedragen tot het
behalen van de graad van
Doctor in de
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door

Roldán TORRES GUTIÉRREZ

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Acknowledgment for knowledge: the longest chapter

Normally it should be the longest chapter of this thesis. It is quite difficult to summarize the extensive list of people who have made this dream come true. It is even more difficult, because joining two continents, although this union involves two small countries (Cuba-Belgium), is anyway, extremely difficult. Much more when on both sides there are so many people that have influenced in the education, formation and development of who writes this letter.

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Thank you very much, Dankjewel, Muchas Gracias!!!

Roldán

Table of contents

List of abbreviations

AA	ascorbate
ABQS	Adaptive Quality Based Clustering
ACC	1-aminocyclopropane-1-carboxylate
AFLP	amplified fragment length polymorphism
ALDH	Aldehyde dehydrogenase
ANOVA	analysis of variances
AO	ascorbate oxidase
ARA	acetylene reduction assay
ARDRA	amplified ribosomal DNA restriction analysis
BAC	bacterial artificial chromosome
BLASTn	Basic Local Alignment Search Tool- nucleotide
BNF	biological nitrogen fixation
cDNA	complementary DNA
CFB	Cytophaga/Flexibacter/Bacteroides
CFU	colony forming units
CIAT	International Center for Tropical Agriculture
DAS	days after sowing
DHA	dehydroascorbate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EMBL	European molecular biology laboratory
ESTs	expressed sequence tags
FC	fusicoccin
GalK	galactokinase
GPP	grain per plant
IAA	indole-3-acetic-acid
IITA	International Institute of Tropical Agriculture
LPS	lipopolysacharides
MAS	marker-assisted selection

MDHA	monodehydroascorbate
N	nitrogen
N_2	dinitrogen
N_2O	nitrous oxide
NA	nutrient agar
NCBI	National Center for Biotechnology Information
NDW	nodule dry weight
NFW	nodule fresh weigh
NN	nodule number
NSO	National Statistic Office
NUE	nitrogen use efficiency
PGPR	plant growth promoting rhizobacteria
PGRs	plant growth regulators
PPP	pods per plant
PWP	pod weight per plant
QTL	quantitative trait locus
rDNA	ribosomal DNA
RDW	root dry weight
RFW	root fresh weigh
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDW	shoot dry weight
SFW	shoot fresh weight
SNF	symbiotic nitrogen fixation
SRA	systemic resistance acquired
SSRs	single sequence repeats
TDF	transcript derived fragment
TY	trypton yeast extract
UCLV	Central University of Las Villas
USEPA	United States Environmental Protection Agency
V.C	Villa Clara province
WP	wettable powder
YEM	yeast extract mannitol
YEP	yeast extract peptone
YMA	yeast mannitol agar

Summary

The symbiosis between plants of the *Leguminosae* family and prokaryotic partners is typically characterized by the formation of specialized organs, called nodules, on plant roots or stems that are invaded by the specific microsymbionts. These include the well-known alpha-proteobacterial group of *Rhizobiaceae* containing the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium, Azorhizobium*, and *Allorhizobium*, collectively referred as rhizobia. Legumes play a crucial role in sustainable agriculture. Symbiotic nitrogen fixation (SNF) through interaction between legumes and rhizobia, contributes to nitrogen (N) nutrition of most legumes and legume cropping systems. Common bean (*Phaseolus vulgaris* L.) is the most important legume for direct human consumption worldwide and particularly in many parts of Latin America and Africa. However, the application of SNF in common bean in the field is often low compared to the nitrogen fixing capacity of beans under optimal conditions and as compared to the amounts of nitrogen fixed by other legumes.

The aim of our study is to identify, quantify and enhance the phytostimulatory effect of the interplay between *Rhizobium*, bean genotypes and plant growth promoting rhizobacteria (PGPR) under different growth conditions and to contribute to the understanding of the molecular mechanisms involved in the *Rhizobium*-bean interaction.

To reach this objective, combinations of *Rhizobium*-PGPR were evaluated under different growth conditions in Cuba using two local bean genotypes. The nodulation and plant growth parameters were significantly stimulated with the combination of *Rhizobium-Azospirillum* and *Rhizobium-Azotobacter* under pot experiment condition, as well as in a field trial. Variations among genotypes were observed for growth parameters and yield in a second field trial. The combination *Rhizobium-Azospirillum* and the fertilizer treatments showed the best result in yield for ICA Pijao beans, while for BAT-304 beans the best result was obtained with the single *Rhizobium* inoculation. Secondly, the morphological and genetic characterization of bacterial isolates from Cuban bean fields, as well as the phenotypic characterization of Cuban *Rhizobium* isolates under controlled and field conditions, demonstrate the biodiversity of beneficial microbes in the common bean rhizosphere and the stimulatory effect of compatible interactions between common bean genotypes and

Rhizobium strains. The genetic characterization of isolated bacterial strains form Cuban soils using 16S rDNA sequencing revealed 8 groups of bacteria belonging to the genera: *Agrobacterium, Rhizobium, Ochrobactrum, Sphingomonas, Stenotrophomonas, Bacillus, Brevibacillus* and *Paenibacillus.* In nodule samples, 37.5% of isolates were 100% similar to *Agrobacterium tumefaciens* or *Rhizobium* species. This study allowed the identification of two species of *Rhizobium* isolates (*Rhizhobium etli* and *Rhizobium tropici*) in nodule samples. In nodulation tests *Agrobacterium* isolates were unable to nodulate the original host. The phenotypic characterization showed the stimulation of nodulation parameters and the N fixation through the native *Rhizobium* isolates at early stage of common bean plants. Under field trial conditions, the nodulation, growth parameters and yield were stimulated significantly for ICA Pijao as compared with BAT-304 upon inoculation with the isolated *Rhizobium* strains. Furthermore, genes differentially expressed during the bean root interaction with *Rhizobium etli* CNPAF512, infection with *Fusarium solani* f. sp. *phaseoli* and a control respectively, were identified using the cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technique. *In silico* analysis was used to determine the differential expression profiles of transcript derived fragments (TDFs). Several TDFs were isolated, cloned, sequenced and the obtained DNA sequences were compared with sequences in the GenBank database. The sequences retrieved revealed homology with genes encoding stress/defense and cell metabolism functions for *Rhizobium* treatments, as well as stress/defense functions for the *Fusarium* condition.

The results outlined in this study demonstrate the potential of selection for efficient associations among bean genotypes, rhizobia and plant growth promoting rhizobacteria in order to achieve the increase of SNF in common bean under local agro-ecosystems, as well as increase our insight of the molecular dialogue in common bean-rhizobia interaction. However, these studies should be expanded using more bean genotypes and bacterial combinations in different environmental conditions, in order to provide recommendations to farmers.

Samenvatting

De symbiose tussen planten van de familie *Leguminosae* en sommige bacteriën wordt gekenmerkt door de vorming van nieuwe plantorganen, de zogenoemde nodules of wortelknolletjes, op de wortels of stengel. De cellen van deze nieuwe organen worden geïnfecteerd door de specifieke microsymbionten. De best gekende symbiotische bacteriën behoren tot de groep van de *Rhizobiaceae* met de genera *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium, Azorhizobium*, en *Allorhizobium*, collectief rhizobia genoemd.

Vlinderbloemige planten zijn een belangrijke schakel in duurzame landbouw. Symbiotische stikstoffixatie (SNF), door de interactie van vlinderbloemige planten en rhizobia, draagt in belangrijke mate bij aan de stikstofvoeding van deze planten en als dusdanig tot de stikstofhuishouding in plantaardige productiesystemen.

De gewone boon (*Phaseolus vulgaris* L.) is wereldwijd het belangrijkste vlinderbloemige gewas voor humane consumptie en traditioneel van bijzonder belang in grote delen van Centraal- en Zuid-Amerika en Afrika. Symbiotische stikstoffixatie bij bonenteelt is echter weinig efficiënt in vergelijking met andere vlinderbloemige gewassen.

Het opzet van deze studie was het identificeren en kwantificeren van gunstige interacties tussen boongenotypes, rhizobia en plantengroeibevorderende bacteriën (PGPR). Hiertoe werden verschillende rhizobia-PGPR combinaties geëvalueerd via inoculatie van twee boongenotypes die courant gebruikt worden door de boeren in Cuba. Nodulatie- en plantgroeiparameters worden gunstig beïnvloed door co-inoculatie van gewone boon met *Rhizobium-Azospirillum* of *Rhizobium-Azotobacter,* en dit zowel in potexperimenten als onder veldcondites. Onder veldcondities werd echter duidelijk een effect van het plantgenotype waargenomen wat betreft groei en opbrengst. De combinatie *Rhizobium-Azospirillum* en de bemestingscontrole leverden de beste resultaten op voor de ICA Pijao variëteit, terwijl voor de BAT-304 variëteit de beste resultaten bekomen werden met enkelvoudige *Rhizobium* inoculatie.

Morfologische en genetische karakterisatie van bacteriën geïsoleerd uit boonvelden op Cuba lieten toe een, weliswaar beperkt, beeld te geven van de bacteriële diversiteit. Sommige van deze geïsoleerde bacteriën werden getest voor hun interactie met de gewone boon. Geïsoleerde *Rhizobium* stammen bleken een aantal interessante eigenschappen, zoals vroege nodulatie, te vertonen. Bovendien werden in co-inoculatietesten gunstige effecten waargenomen, wat wijst op een compatibiliteit tussen rhizobia en sommige PGPR in de interactie met de gewone boon.

De genetische karakterisatie van de bacteriële isolaten uit de Cubaanse bodems leidde via 16SrDNA sequenering tot de identificatie van 8 genera: *Agrobacterium, Rhizobium, Ochrobactrum, Sphingomonas, Stenotrophomonas, Bacillus, Brevibacillus* en *Paenibacillus.* In stalen genomen van nodules vertoonden 37,5% van de isolaten 100% gelijkenis met hetzij *Agrobacterium tumefaciens* of *Rhizobium* species. Twee *Rhizobium* species werden geïdentificeerd, met name *Rhizobium etli* en *Rhizobium tropici.*

In een laatste deel werd een bijdrage geleverd in het zoeken naar plantengenen die in de interactie van de gewone boon met respectievelijk *Rhizobium*, een pathogene schimmel (*Fusarium solani* f. sp. *Phaseoli)*, en een controlebehandeling, differentiëel tot expressie komen. Hiertoe werd de techniek van "cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP)" gebruikt. *In silico* analyse van een aantal geïdentificeerde DNA fragmenten leverde een aantal interessante "transcript derived fragments (TDFs)" op. DNA sequentie-analyse van een aantal van deze TDFs liet toe verwantschap op te sporen met reeds bekende genen. Deze verwante genen bleken betrokken te zijn in stressresponses en koolhydraatmetabolisme.

Resumen

La simbiosis entre plantas pertenecientes a la familia *Leguminosae* y organismos procariotas se caracteriza típicamente por la formación de un nuevo órgano especializado, comúnmente denominado nódulo, en las raíces o tallos de las plantas, los cuales son invadidos por microsimbiontes específicos. Estos se incluyen en el conocido grupo de alfa-proteobacterias de la familia *Rhizobiaceae*, el cual contempla los géneros *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium, Azorhizobium*, y *Allorhizobium*, colectivamente referidos como rizobia. Las leguminosas juegan un papel crucial en la sostenibilidad de agro-ecosistemas. La fijación simbiótica del nitrógeno (FSN) llevada a cabo mediante la interacción entre plantas leguminosas y rizobia, contribuye a la nutrición nitrogenada en muchas plantas leguminosas y en sistemas agrícolas donde estas se cultivan. El frijol común (*Phaseolus vulgaris* L.) es la legumbre más importante para el consumo humano en todo el mundo y en particular en muchos países de América Latina y África. Sin embargo, la aplicación de la FSN de este cultivo en condiciones de campo es a menudo considerada baja, comparada con la capacidad de FSN en condiciones óptimas de crecimiento, así como comparada con las tasas de fijación de nitrógeno por otras plantas leguminosas.

El objetivo de nuestro estudio se basa en la identificación, cuantificación y mejoramiento del efecto fito-estimulatorio de la interacción entre *Rhizobium*, genotipos de frijol común y rizobacterias promotoras del crecimiento vegetal (PGPR) bajo diferentes condiciones de crecimiento, así como contribuir al mejor entendimiento de los mecanismos moleculares involucrados en la interacción *Rhizobium*-frijol común.

Para alcanzar este objetivo, combinaciones de *Rhizobium*-PGPR se evaluaron en diferentes condiciones de crecimiento en Cuba, empleándose dos genotipos de frijol común. Los parámetros de nodulación y crecimiento fueron estimulados significativamente con la combinación de *Rhizobium-Azospirillum* y *Rhizobium-Azotobacter* bajo condiciones controladas, así como en los experimentos de campo. La variación genotípica fue observada en los parámetros componentes del rendimiento y el rendimiento en el segundo experimento de campo. La combinación *Rhizobium-Azospirillum* y el tratamiento de fertilización mineral mostraron los mejores resultados en cuanto al rendimiento para ICA Pijao, mientras que para la variedad BAT-304 la inoculación simple de *Rhizobium* produjo el incremento

significativo de este parámetro. La caracterización morfológica y genética de aislados bacterianos procedentes de la rizosfera del frijol común de la zona central de Cuba, así como la caracterización fenotípica de aislados de *Rhizobium* bajo condiciones controladas de crecimiento y en condiciones de campo, demostraron la biodiversidad de bacterias beneficiosas en estas condiciones y el efecto estimulante de interacciones compatibles entre genotipos de frijol común y cepas de *Rhizobium*. La caracterización genética de aislados bacterianos, basada en la secuenciación de 16S rDNA reveló grupos de bacterias pertenecientes a los géneros: *Agrobacterium, Rhizobium, Ochrobactrum, Sphingomonas, Stenotrophomonas, Bacillus, Brevibacillus* y *Paenibacillus*. En las muestras de nódulos de frijol común, 37.5% de los aislados fueron 100% similar a *Agrobacterium tumefaciens* o especies de *Rhizobium*, identificándose en este último género las especies *Rhizhobium etli* y *Rhizobium tropici*. En el ensayo de nodulación los aislados de *Agrobacterium* fueron incapaces de nodular el hospedero de origen.

Mediante la caracterización fenotípica se corroboró la estimulación de los parámetros de nodulación y la fijación de nitrógeno mediante las cepas asiladas del genero *Rhizobium* en estadios tempranos de las plantas de frijol común. Bajo condiciones de campo, la nodulación, parámetros componentes del rendimiento y el rendimiento fueron estimulados significativamente mediante la inoculación de las cepas aisladas en ICA Pijao, no siendo así para BAT-304.

Adicionalmente, genes diferencialmente expresados durante la interacción del frijol común con *Rhizobium etli* CNPAF512, *Fusarium solani* f. sp. *phaseoli* y un tratamiento control, fueron identificados usando la técnica de cADN-Amplificación Polimórfica de Longitud de Fragmentos (cDNA-AFLP). La determinación de los perfiles de expresión de los fragmentos derivados de la transcripción (TDFs) se realizó mediante el análisis *in silico* Varios TDFs fueron aislados, clonados y secuenciados. Las secuencias de AND obtenidas fueron comparadas con aquellas existentes en la base de datos de Genbank. Las secuencias recuperadas revelaron homología con genes que codifican funciones de estrés/defensa y metabolismo celular para la condición de inoculación con *Rhizobium*, así como homología con genes que codifican funciones de estrés/defensa para el tratamiento de infección con *Fusarium*.

Los resultados esbozados en este estudio demuestran las potencialidades para seleccionar asociaciones eficientes de genotipos de frijol común, rizobia y PGPR para lograr incrementos en la FSN en el frijol común en agro-ecosistemas locales. A la vez, permite la mejor

comprensión del diálogo molecular en la interacción frijol común-rhizobia. Sin embargo, estos estudios deben hacerse extensivos usando diferentes genotipos de frijol común y combinaciones de bacterias en diversas condiciones ambientales con el fin de proporcionar recomendaciones a los productores.

Introduction

Overall introduction, hypothesis, objectives and scope of the thesis

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*Understanding the interplay between common bean (***Phaseolus vulgaris** *L.) and microbes in the root zone: towards more sustainable bean production*

One major challenge for the twenty-first century will be the production of sufficient food. The United Nations Population Fund estimates that the global human population may well reach 10 billion by 2050 (www.unfpa.org). This means the need for increasing production and/or productivity of food crops, as plants form the basis of every food chain (Morrissey et al., 2004). It is estimated that an increase of agricultural production with 75-100% of today's production is needed. If such an increase in production should be realized with current agricultural management that would similarly require a doubling of the use of fossil fuel energy for fertilizer production, it will cause economic hardship and incalculable damage to the environment (Graham and Vance, 2003; Norse, 2003).

The availability of a useful nitrogen (N) source is, apart from water, the major limiting factor in agricultural productivity. This has commonly resulted in the heavy use of chemical N fertilizer to replenish soil N, an approach that suffers from high costs and severe environmental effects (Gustafson and Kreys, 2006).

Biological nitrogen fixation (BNF), a microbiological process that converts atmospheric N_2 into a plant-usable form, offers an economically attractive, agronomically viable and ecologically sound means of reducing external inputs and improving internal resources (Bohlool and Schmidt, 1974, Graham an Vance, 2000; Paredes et al., 2007).

A wide range of organisms have the ability to reduce molecular nitrogen to ammonia. However, only a very small proportion of the known bacterial species are able to do so (the so called diazotrophs): about 87 species in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs (Dixon and Wheeler, 1986; Sprent and Sprent, 1990; Zahran et al., 1995; Zahran, 1999). Nevertheless the list is expanding at fast speed because of the ongoing metagenomics projects. Amongst the different nitrogen

fixing endosymbiotic interactions, the most intensively studied is that established between legume plants and nitrogen-fixing endosymbiotic bacteria of the genera *Rhizobium, Sinorhizobium (Ensifer), Mesorhizobium, Bradyrhizobium* and *Azorhizobium*, collectively termed rhizobia (Weidner et al., 2003). Rhizobia are soil bacteria inducing root (or stem) nodules on leguminous plants, in which the process of symbiotic N_2 fixation (SNF) occurs. Major benefits of the legume-rhizobia symbiotic interaction are diminished nitrogen fertilizer requirements and improving plant growth and health (Giller, 2001). Symbiotic systems such as that of legumes and rhizobia can be a major source of N in most cropping systems with an average of 80% of the required nitrogen coming from biological N_2 fixation (Graham and Vance, 2000; Bohlool et al., 2004).

Common bean (*Phaseolus vulgaris* L.) is the most important legume for direct human consumption worldwide (Beebe et al., 2000; Drevon et al., 2001; Martínez-Romero, 2003; Broughton et al., 2003). The large amount of protein, minerals and antioxidant compounds (Xu and Chang, 2008) make this crop an excellent model food legume (Broughton et al. 2003). Together with maize (*Zea mays* L.) and cassava (*Manihot esculenta* L.), they have been a dominant staple in the low to mid-altitudes of the Americas for millennia (Graham and Ranalli, 1997). Nowadays common bean is cultivated in all continents and more than 90 countries (Hidalgo and Beebe, 1997) with special significance for Latin American (table 01).

Common beans form part of the basic Cuban diet and account for one-fifth of the total proteins consumed in Cuba (Miranda-Lorigados et al., 2006). According to FAO statistics (2005), consumption of dry bean in Cuba reaches 20.3 kg per capita per year which is far above the average consumption in Latin America (of 12.5 kg per capita per year). Bean yields in Cuba are rather low with a national average of 0.93 t ha^{-1} (National Statistic Office (SNO) Cuba, 2008; data 2006) versus optimal yields of 2.5-5 ton ha^{-1}. Losses are mainly due to a shortage of fertilizers and effective pesticides. This makes Cuba a major importer of beans, mainly from Canada and China. Estimates indicate that Cuba spends more than 60 million US dollar per year on bean imports (Gómez, 2006).

Table 01 Bean production in Latin America (from Broughton et al., 2003).

Country/Region	Area (ha x 10^3)	Production (MT x 10^3)
Brazil	5092	3055
Mexico	2259	1300
Central America (Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, Panama)	526	337
Southern Zone (Chile Argentina, Paraguay)	357	398
Andean Zone (Venezuela, Colombia, Ecuador, Peru, Bolivia)	299	265
Caribean (Cuba, Haiti, Dominican Republic)	157	141
TOTAL	8690	5496

Phaseolus vulgaris is considered a poor nitrogen-fixer pulse in comparison to other grain legumes (Hardarson, 1993, Bacem et al., 2007). Sparse nodulation and the lack of response to inoculation in field experiments is frequently reported worldwide, raising questions about the benefits of inoculation (Graham, 1981; Buttery et al., 1987). This fact is attributed to intrinsic characteristics of the host plant, particularly the nodulation promiscuity (Michiels et al., 1998), as well as the extreme sensitivity to nodulation-limiting factors, such as the high rate of N-fertilizer used in intensive agriculture, nutrient deficiency, high temperatures and soil dryness (Graham, 1981).

During the last decade much research has focused on the beneficial effect of simultaneous inoculation with *Rhizobium* and plant growth-promoting rhizobacteria (PGPR), so called co-inoculation, showing the potential to enhance plant growth, nodulation and nitrogen fixation of several legumes. Co-inoculation with *Azotobacter* spp. or *Azospirillum* spp. and *Rhizobium* strains showed a synergistic effect on nodulation, plant growth, yield and N uptake, plant health and suppressing diseases in soybean, clover, common bean, faba bean and peanut (Burns et al., 1981; Raverker and Konde, 1988; Burdman et al., 1997; Rodelas et al., 1999). The positive effects of PGPR have been observed in greenhouse experiments using hydroponic, vermiculite-based and soil-based systems as well as in field experiments (Burdman et al., 1997; Bai et al., 2003; Hamaoui et al., 2001). One of the main factors of the stimulation observed with *Azospirillum* co-inoculation is the bacterial production of phytohormones, mainly indole-3-acetic acid (IAA), a process known as phytostimulation (Okon and Vanderleyden, 1997; Lambrecht et al., 2000). The co-inoculation with *Rhizobium*

and *Azospirillum* has given promising results, but these are still variable. More detailed field studies are required (Graham and Vance, 2000). In addition, studies related to the influence of specific environmental factors on these *Rhizobium*-PGPR-plant interplays are still limited (Remans et al., 2007a). Studies on the natural genetic variation of the PGRP-*Rhizobium* stimulation, strain characterization, metabolic pathways and biotic and abiotic constraints in the interplay with common bean will provide good tools to facilitate the understanding and increase common bean production for sustainable agriculture.

Despite its economic importance, genomic data on *Phaseolus vulgaris* are still limited (Freyre et al. 1998, Ramírez et al., 2005). Recently, with the PHASEOMICS consortium support, around 21,346 ESTs from common bean (Hernández et al. 2005) and 20,120 ESTs from a related species, runner bean (*Phaseolus coccineus*) (Ramírez et al., 2005), have been deposited in GenBank's EST database. Over 92% of the ESTs deposited for the *Fabaceae* family are derived from the model legumes *Medicago truncatula* and *Lotus japonicus* and the crop legume *Glycine max* (Ramírez et al., 2005; Schlueter et al., 2007).

Partial sequencing of cDNA inserts or ESTs obtained from many plant tissues and organs has been used as an effective method of gene discovery. It is an efficient approach for identifying a large number of plant genes expressed during different developmental stages and in response to a variety of environmental conditions (Ramírez et al. 2005). cDNA-AFLP (amplified fragment length polymorphism) is an RNA fingerprinting technique to display differentially expressed genes (Bachem et al. 1996). This method needs no pre-existing sequence information, which makes it an excellent tool to identify novel genes (Qin et al., 2000). It can for instance be used to identify genes in signal transduction pathways when common bean is challenged with beneficial or pathogenic microorganisms.

Hypothesis:

The *Rhizobium-Phaseolus vulgaris* L. symbiotic interaction can be improved when Plant Growth Promoting Rhizobacteria (PGPR) are applied in co-inoculation with compatible *Rhizobium* strains. Optimization of this practice requires a better understanding of the genetic and environmental factors that contribute to the outcome of these interactions.

General objective:

To identify and quantify the phytostimulatory effect of *Rhizobium*-bean-PGPR interactions and to gain more knowledge on the mechanisms of the interplay.

Specific objectives:

1. To determine the effect of *Rhizobium* inoculation and *Rhizobium*-PGPR co-inoculation in two local common bean genotypes under different growth conditions.
2. To evaluate the host variation of the *Rhizobium* inoculation and *Rhizobium*-PGPR co-inoculation in two local Cuban bean genotypes under field conditions.
3. To characterize morphologically and genetically rhizosphere bacteria isolated from Cuban agricultural systems.
4. To determine the influence of *Rhizobium* isolates on phenotypic parameters of two common bean genotypes under controlled growth condition and under field conditions.
5. To detect genes differentially expressed in common bean in interaction with *Rhizobium* using cDNA-AFLP technique.

Scope of the thesis

The rhizosphere constitutes a valuable source of beneficial microorganisms, able to stimulate the growth of agronomically important crops. In relation to this study, two groups of bacteria are of special interest: rhizobia able to establish a nitrogen fixing symbiosis with common bean (*Phaseolus vulgaris* L) and Plant Growth Promoting Rhizobacteria (PGPR).

In this doctoral study we aim to identify and quantify the effect of *Rhizobium* and *Rhizobium*-PGPR co-inoculation on common bean in the context of the Cuban agricultural system. For that purpose bacterial strains isolated form Cuban agricultural soil were used in comparison with already well described bacterial strains. Moreover, the cDNA-AFLP technique was used to increase our understanding of common bean genes differentially expressed following a beneficial symbiotic interaction. Chapter 1 gives an overview of reported studies describing the importance of nitrogen in plant production and special attention is given to the beneficial effect of associative diazotrophs, *Rhizobium* inoculation and *Rhizobium*-PGPR co-inoculation in legumes and specifically in *Phaseolus vulgaris*. The stimulation of PGPR on *Rhizobium*-bean symbiosis in different growth conditions is analyzed in chapter 2. Pot experiments and field trials were conducted to determine the effect of PGPR and the host variation for the *Rhizobium*-bean-PGPR interaction (objectives 1, 2). The morphological and genetic characterization of rhizosphere bacteria and *Rhizobium* strains isolated form Cuban agricultural soil is discussed in chapter 3 (objective 3). The comparison of the phenotypic parameters of common bean genotypes following inoculation with isolated *Rhizobium* strains (objective 4) is reported in chapter 4. To increase our insight in the molecular dialogue on *Rhizobium*-bean interplay, chapter 5 aims the detection of differential gene expression using the cDNA-AFLP approach (objective 5).

Chapter 1

Nitrogen: seeking alternatives for sustainability

Abstract

International emphasis on environmentally sustainable development with the use of renewable resources is likely to focus attention on the potential role of biological nitrogen (N) fixation (BNF) in supplying nitrogen for agriculture to counteract the indiscriminate use of nitrogenous fertilizers. Here we explore the beneficial plant-associated microorganisms that can profoundly influence plant growth and plant health by contributing to the N cycle balance, suppressing disease, enhancing nutrient uptake and promoting plant growth. Host variability among plant genotypes or cultivars for response to beneficial microorganisms suggests potential to improve plant-microbe interactions by exploiting this natural genetic host variation and to contribute to breeding programs. Special attention is given to genetic variation in *Rhizobium*-legume symbiosis and the interaction with associative bacteria in common bean (*Phaseolus vulgaris* L.) as a model legume to achieve sustainability under low input agricultural systems. Tools to unravel common bean genetics and the natural genetic variation are also explored.

1.1 Impact of reactive nitrogen in the ecosystems

Nitrogen (N) is an essential component of DNA, RNA, and proteins, the building blocks of life. All organisms require N to live and grow. Although the majority of the air we breathe is N_2, most of the N in the atmosphere is unavailable for use by organisms (Harrison 2003).

On Earth, there are two pools of N: the gaseous dinitrogen (N_2) of the atmosphere, which makes up about 99% of total N, and the 1% of N that is chemically bound to other elements such as carbon (C), hydrogen (H) or oxygen (O) and has been described as "reactive nitrogen" (Galloway et al., 2004; Beever et al., 2007). The atmosphere contains about 10^{15} tonnes of N_2 gas, and the N cycle involves the transformation of some 3×10^9 tonnes of N_2 per year on a global basis (Postgate, 1998). However, transformations (e.g., N_2 fixation) are not exclusively biological. Lightning probably accounts for about 10% of the world's supply of fixed N (Sprent and Sprent, 1990). The fertilizer industry also provides very important quantities of chemically fixed N. World production of fixed N from N_2 for chemical fertilizer accounts for about 25% of the Earth's newly fixed N_2, and biological processes account for about 60% (Galloway et al., 2004; Beever et al., 2007).

The movement of N between the atmosphere, biosphere, and geosphere in different forms is described by the nitrogen cycle (Figure 1.1), one of the major biogeochemical cycles. Five main processes cycle N through the biosphere, atmosphere, and geosphere: 1- N_2 fixation, 2- N uptake (organism growth), 3- N mineralization (decay), 4- nitrification, and 5- denitrification (Harrison 2003). Microorganisms, particularly bacteria, play major roles in all of the principal N transformations. (Paredes et al., 2007).

In natural ecosystems, this cycle is more or less closed, with N inputs balancing N losses. The small amount of N moving in the cycle in most natural ecosystems limits biomass production. The availability of a useful N source is, apart from water, the major limiting factor in agricultural productivity (Gustafson and Kreys, 2006). In agricultural systems, the cycle is disturbed by the export of substantial amounts of N in harvested products.

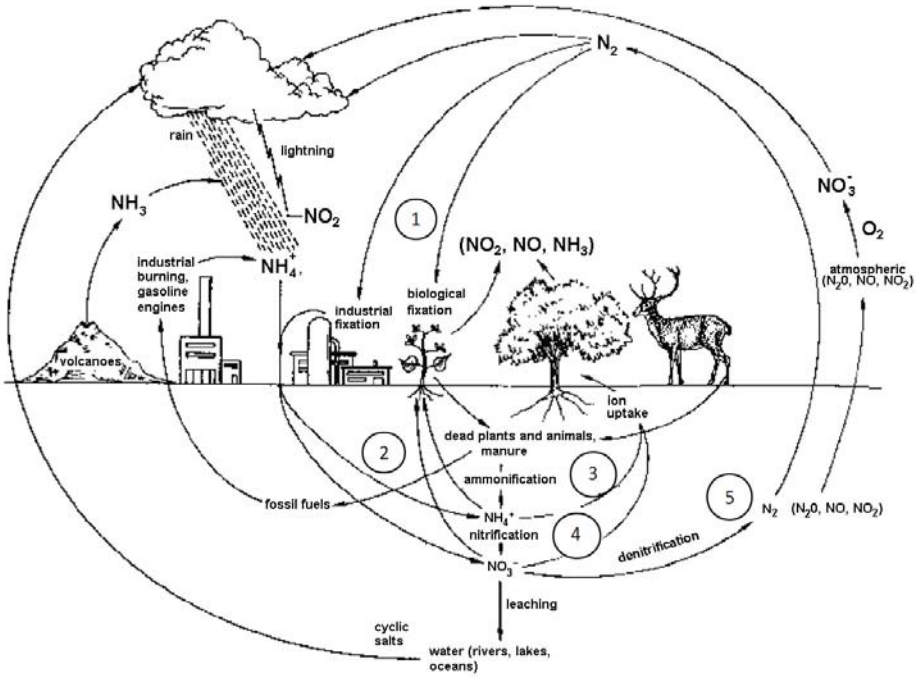

Figure 1.1 Nitrogen cycle showing the five main transformation processes: 1- N_2 fixation by biological and industrial transformation, 2- N uptake (organism growth), 3- N mineralization (decay), 4- nitrification, and 5- denitrification. Taken from Zahran, (1999)

1.1.1 Implications of irrational use of N fertilizers

Application of fertilizers containing N and other crop nutrients is essential to balance inputs and outputs and so to maintain or improve soil fertility, to increase agricultural productivity and, in turn, to preserve natural ecosystems and wild habitats from conversion to farming (Beever et al., 2007). However, it has commonly resulted in the heavy use of chemical N fertilizer to replenish soil N and to obtain desired yields, an approach that suffers from high costs and severe environmental effects (Gustafson and Kreys, 2006). The large rises in cereal grain yields in developed countries between 1959 and 1990 are directly attributable to a 10-fold increase in N fertilizer application. Concomitant with high rates of application of N fertilizers in developed countries are volatilization of N oxides into the atmosphere, depletion of non-renewable resources, an imbalance in the global N cycle, and leaching of nitrate to groundwater. By contrast, in developing countries, the high cost of N fertilizer, the energy requirements for production, and the suboptimal transportation capabilities limit its use, especially on small farms (Vance, 1997, Broughton et al., 2003).

As reported by the United Nations Population Fund (2007), the global human population may well reach 10 billion by 2050. Such growth will require an increase of agricultural production

of 75-100% to provide the needed food (Morrissey et al., 2004). If such an increase in production should be realized with current agricultural management that would similarly require a doubling of N fertilizer, the number of adverse effects on both the environment and human health, will dramatically increase (Beever et al., 2007).

Long-term projections of N use are subject to many critical assumptions about our ability to improve crop productivity as demand increases, while also improving N use efficiency (NUE). Recent projections indicate that global demand for N fertilizers in 2050 could be between 107 and 171 Mt N (Beever et al., 2007). According to the four scenarios reported by the Millennium Ecosystem Assessment (2005), global N fertilizer consumption in 2050 is anticipated to be between 110 and 140 Mt N (Bumb and Baanante, 1996; FAO, 2000; Wood et al., 2004; Galloway et al., 2004; Heffer and Prud'homme, 2006).

Lack of reactive N in the agro-ecosystem leads to soil fertility decline, low yields and crop protein content, depleted soil organic matter, soil erosion and, in extreme cases, desertification (Barbier and Bergeron, 2001). Excess amounts of nitrate may move into groundwater and drinking water supplies contaminating the fluvial sources. Although the so-called “blue baby syndrome” (methaemoglobinaemia), arises from bacteria-contamination and not from ingesting too much nitrate as originally supposed (L'hirondel and L'hirondel, 2002), the relationship between high vegetables based nitrate intake and gastric and intestinal cancer has been reported (Leifert and Golden, 2000).

Beever et al. (2007) reported that in surface water, increased loading of N-based nutrients can play a role in eutrophication, a process that contributes to ecological and resource degradation. In the atmosphere, NO_2 and particulate matter can exacerbate several human health problems, from asthma to heart disease.

Increasing the nitrous oxide (N_2O) concentration in the atmosphere contributes to global warming and undermines human health (Graham and Vance, 2003; Norse, 2003; Crutzen et al., 2007). N_2O is a “greenhouse gas” with a 100-year average global warming potential (GWP), 296 times larger than an equal mass of carbon dioxide (CO_2) (Prather et al., 2001). As a source for NOx, (i.e. NO plus NO_2, N_2O) also plays a major role in stratospheric ozone chemistry, acting as catalyst in the ozone destruction reaction (Crutzen, 1970), which derive the increase of ultraviolet (UV) radiation. There is evidence that high exposure to UV-B radiation increase the incidence of skin cancer, eye cataracts and sunburn (de Gruijl, 1999).

All ecosystems emit N_2O and more than 50% of the global emission of N_2O is considered "natural" (soils under natural vegetation, oceans, etc.). Agriculture accounts for 86% of the global anthropogenic N_2O emissions (USEPA, 2006). Of the agricultural N_2O emissions, 44% is related to the management and application of animal manure, and 14% is associated directly with the use of manufactured fertilizer (Mosier et al, 2004). Furthermore, is clear that increasing the N fertilizer application will increase the N_2O emissions by natural nitrification or denitrification processes (Merino et al., 2001, Crutzen et al., 2007).

Obviously adopting an integrated approach to nutrient management maximizing the benefits and minimizing the risks associated with the use of N sources contribute to raising crop productivity, N use efficiency and environmental and human rescue.

1.2 Biological nitrogen fixation (BNF) process.

Biological N fixation is an efficient source of N (People et al., 1995). The total annual terrestrial inputs of N from BNF as given by Burns and Hardy (1975) and Paul (1988) range from 139 million to 175 million tonnes of N, with associative plant-bacteria interactions in permanent pasture accounting for 30% (45 million tonnes of N) and with symbiotic associations (*Rhizobium*-leguminous plants) in arable land accounting for 25 to 30% (35 million to 45 million tonnes of N). While the accuracy of these figures may be open to question (Sprent and Sprent, 1995), they do help to illustrate the relative importance of BNF in cropping and pasture systems and the magnitude of the task required if BNF is to be improved in order to replace a proportion of the 80 to 90 million tonnes of N-fertilizer (Beever et al., 2007) that are applied to agricultural land. Much land has been degraded worldwide; there is need arresting the destructive uses of land and to institute a serious reversal of land degradation (Burris, 1994). BNF can play a key role in land remediation.

Organisms that can fix N, i.e., convert the stable N gas in the atmosphere into a biologically useful form, all belong to a biological group known as prokaryotes. All organisms which reduce N_2 to ammonia do so with the aid of an enzyme complex, nitrogenase **(**Zahran, 1999**).** A wide range of microorganisms have the ability to fix N. However, only a very small proportion of the known species are able to do so; about 87 species in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix nitrogen (Dixon and Wheeler, 1986; Sprent and Sprent, 1995; Zahran

and Afkar, 1995). This wide variety of diazotrophs ensures that most ecological niches will contain one or more representatives and that lost N can be replenished.

1.2.1 Associative diazotrophic interactions. Contribution of non-leguminous plants to BNF

The first associative diazotroph was reported by Beijerinck in 1925 under the name *Spirillum lipoferum*. However, it was only about half a century later, after the discovery of the highly specific *Azotobacter paspali-Paspalum notatum* association and the discovery of *Spirillum lipoferum* (now called *Azospirillum*) by the group of Döbereiner (Döbereiner et al., 1972; Döbereiner and Day, 1976), that scientists became increasingly interested in diazotrophic bacteria associated with graminaceous plants. Several genera of bacteria have now been reported to contain diazotrophs which may be loosely or more intimately (endophytes) associated with plants, including *Acetobacter, Azoarcus, Azospirillum, Azotobacter (Gluconacetobacter), Beijerinckia, Burkholderia, Enterobacter, Herbaspirillum, Klebsiella, Paenibacillus* and *Pseudomonas* (Dobbelaere, 2002). An extensive phylogenetic classification was made by Young (1994). While the capability of these organisms to fix N *in vitro* can be demonstrated easily (free living), efforts to determine rates of N fixation in natural associations with plants have produced widely varying results. In the last 30 years many crop-inoculations studies, coupled to acetylene reduction measurement (ARA), N balance and ^{15}N isotope dilution experiments, have been conducted with root-associated bacteria to determine whether the bacteria supply significant amounts of N to cultivated plants (Boddey at al., 1999; James, 2000).

The contribution of associative interactions for the global BNF evidences the economic importance of non-legume cultivation worldwide; however, the crop responses are variable due to biotic and abiotic constraints (Boddey at al., 1999; Kennedy et al., 2004; Roesch et al., 2006). One problem from which some of the studies with root colonizing diazotrophs suffer is that the amount of fixed N_2 supplied to their host plants appears to be low (Rao et al., 1998; Malik et al., 2002; Kennedy et al., 2004). This has been attributed to the fact that free-living diazotrophs do not appear to excrete reactive nitrogen, contrary to symbiotically living rhizobia. In the case of associative diazotrophs, the fixed N remains mainly in the bacterial cells and is released to the host only at later stage of the plant growth after death and decay of the bacterial biomass (Rao et al., 1998). Despite this, the probability of eventual success of N

fixation with cereals should be regarded as significant (Kennedy et al., 1997; Roesch et al., 2006).

Rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea maydis*) are the three major staple food crops for the world's population. A rice crop removes around 16–17 kg N to produce 1 t dry weight of rough rice, including straw (De Datta, 1981; Ponnamperuma and Deturck, 1993; Sahrawat, 2000). A wheat crop requires about 26–28 kg N to produce 1 t of rough grain including straw (Bhuiyan, 1995; Angus, 2001). Maize plants require 9–11 kg N to produce 1 t biomass (Anuar et al., 1995). Most of the soils are deficient in N and applications of N fertilizer are essential for good yields of such cereal crops. Generally, urea is the most convenient N source, but less than 50% of the applied urea is used by plants (Garabet et al., 1998; Choudhury and Khanif, 2001; Halvorson et al., 2002). This low efficiency of use is mainly caused by NH_3 volatilization, denitrification, and losses from leaching (De Datta and Buresh, 1989; Bijay-Singh et al., 1995). Alternative sources of N such as the use of BNF technology may supplement or replace chemical N-fertilizer. Thus, although the magnitude of BNF from bio-fertilizers may account for a fraction of total crop N requirements, the effect of reducing losses from an ecosystem may be equivalent to a much more significant contribution to the N economy of crop production (Kennedy et al., 2004).

A substantial number of studies conducted on rice suggest that on the whole 20-25% of the total N needs of this crop can be derived from associative fixation (Watenabe et al., 1987; Roger and Ladha, 1992, Wu et al., 1995; James, 2000). Sherestha et al. (1996) conducted two experiments comparing up to seventy rice varieties each. In one experiment it was estimated that the amount of N derived from the air ranged from 0 to 20.2%. In the second experiment, it was found that an equivalent of 16 to 70 kg N ha^{-1} was fixed. *Rhizobium leguminosarum* bv. *trifolii*, commonly observed in symbiosis with leguminous plant, can colonize rice roots endophytically in fields where rice is grown in rotation with Egyptian berseem clover (*Trifolium alexandrinum*), replacing 25–33% of the recommended rate of N fertilizer for rice in field conditions (Yanni et al., 1997). Field experiments demonstrated that the inoculation of this bacterium increased mean rice yield by 3.8 t ha^{-1} (Yanni et al., 2001).

Although ^{15}N isotope dilution/natural abundance studies have given much useful information on the potential for N_2 fixation of non-legumes, they have not generally provided information on the causal organisms. In rice, the systematic isolation and enumeration of the endophytic

and associative diazotroph populations in varieties showing different N_2 fixation abilities should be performed in parallel with the field work (James, 2000). Even when specific varieties have been shown to fix N_2, it will be extremely difficult to isolate the organisms responsible. According to Barraquio et al. (1997), Stoltzfus et al. (1997) and Yanni et al. (1997), the culturable diazotrophic population in rice is extremely varied and virtually uncharacterized. Other evidences have shown that the rhizosphere of rice may also contain an enormous bacterial population that has yet to be cultured (Ueda et al., 1995; Reinhold-Hurek and Hurek, 1998). Recently Sun et al. (2008) selected several groups of endophytic bacteria in rice plant roots by culture-independent molecular approaches based on 16S rDNA sequence analysis. A total of 192 positive clones in the 16S rDNA library of endophytes were identified based on the similarity of the ribosomal DNA restriction analysis (ARDRA) banding profiles. Sequence analysis revealed diverse phyla of bacteria in the 16S rDNA library, which consisted of alpha, beta, gamma, delta, and epsilon subclasses of the Proteobacteria, *Cytophaga/Flexibacter/Bacteroides* (CFB) phylum, low G+C Gram-positive bacteria, *Deinococcus-Thermus*, Acidobacteria, and archaea. However, more than 14.58% of the total clones showed high similarity to uncultured bacteria, which reinforces the above reports by Barraquio et al. (1997), Stoltzfus et al. (1997) and Yanni et al. (1997), suggesting that non-culturable bacteria are among the rice endophytic bacterial community.

Inoculation with *Azospirillum brasilense* can increase wheat grain yield by up to 30% in field conditions (Okon and Labandera-Gonzalez, 1994), but only at lower application rates of N-fertilizer (50–60 kg N ha^{-1}). At higher application rates (110–170 kg N ha^{-1}), the effects of *Azospirillum* inoculation were not statistically significant (Dobbelaere et al., 2001). This implies that there are good prospects for supplementing a substantial amount of urea-N applied to wheat while maintaining yields by inoculating *Azospirillum*. Beneficial effects of inoculation with *Azospirillum* on wheat yields in both greenhouse and field conditions have been reported by others as well (Hegazi et al., 1998; El-Mohandes, 1999; Ganguly et al., 1999). Substantial increases in N uptake by wheat plants and grain were observed in greenhouse trials with an NH_3-excreting strain of *A. brasilense*, when the soil was initially supplemented with malate (Islam et al., 2002). There were clear differences between strains of *Azospirillum* in their ability to promote growth of wheat in greenhouse trials (Han and New, 1998; Saubidet and Barneix, 1998). Although *Azospirillum* promotes growth of wheat plants and grain yield, it apparently contributes little N to wheat as a direct result of BNF. It has been

established by the ^{15}N tracer technique that *A. brasilense* and *A. lipoferum* contributed only 7 and 12% of wheat plant N by BNF, respectively (Malik et al., 2002). However, this contribution may be a critical component for obtaining a greater yield with less N application. The value of supplying even 10% of the N requirement of wheat should not be underestimated because it may increase its capacity to assimilate soil-N (Kennedy et al., 2004).

The inability of the wheat plant to release adequate C to the rhizosphere is likely to be a major constraint to realizing the BNF potential of *Azospirillum* and *Azotobacter* (Kanungo et al., 1997). Under laboratory experimental conditions, this problem can be alleviated by adding malate to the soil. While working with an NH_3-excreting mutant strain of *A. brasilense*, it was observed that the ^{15}N enrichment of wheat tissue increased by 48-fold, indicating that 20% of the wheat N had been derived from BNF after several days growth of seedlings (Wood et al., 2001). Apparently, the improved access to C compounds and a more favorable microaerobic O_2 concentration contributed to this effect. These results demonstrate the potential for BNF by *Azospirillum* to enhance the availability of N to wheat plants.

On maize, García de Salamone et al. (1996) suggested that some cultivars fix up to 60% of their N after inoculation with appropriate strains of *Azospirillum*, while other cultivars showed decreased grain yield and plant N accumulation (García de Salamone et al., 1996; Döbereiner, 1996). Biological factors like cultivar type and plant developmental stage can influence the occurrence and distribution of diazotrophic bacteria in maize plants (Roesch et al., 2006). According to Silva et al. (2003), plant genotype presents a high correlation with the diazotrophic population. Using PCR-DGGE and sequence analysis to assess the diversity of *Paenibacillus* spp. in the maize rhizosphere, these authors demonstrated that maize cultivars had an effect on the composition of the *Paenibacillus* community. Another important factor that affects the activity of diazotrophic bacteria in this crop is the availability of N. According to Tsagou et al. (2003), the presence of ammonium in the soil can inhibit the growth of diazotrophic bacteria. In this work, the authors verified restriction in the growth of *Azospirillum lipoferum* in the presence of 0.5 g NH_4Cl l^{-1} and 30 μM dissolved oxygen. This negative effect can also be seen in the diazotrophic community inhabiting plants under high levels of N-fertilization. Recently Roesch et al. (2006) studied the dynamics of associative bacteria in two genotypes of maize and the influence of N supply, reporting that the dynamics and the distribution of associative bacteria from the genera *Azospirillum*, *Burkholderia* and

Acetobacter were affected by the ontogenic stage of maize plant and the bacterial population was affected by the N-fertilization during the first stages of plant growth.

Burkholderia spp. are found in the shoots, roots, rhizosphere and rhizoplane of maize plants (Estrada-de los Santos et al., 2001; Estrada et al., 2002). Greenhouse trials using non-sterilised soils at the University of Wisconsin, USA, showed that grain yields were increased with 36–48% by inoculating seeds with *B. cepacia* AMMDR1 (Riggs et al., 2001) at planting, depending on the maize cultivar and bacterial genotype. In the field trials, this bacterium was able to increase maize yield by 5.9–6.3% (Riggs et al., 2001). Similarly as reported for rice, maize can establish associative interactions with symbiotic diazotrophs. *Rhizobium etli* bv. *phaseoli* can colonize maize roots, and increase plant dry weight (Gutiérrez-Zamora and Martínez-Romero, 2001). Riggs et al. (2001) have shown that inoculation of *R. leguminosarum* bv. *trifolii* increased maize yields by 34 and 11% in the greenhouse and field conditions, respectively. *Sinorhizobium* sp. can increase maize yields by 35–43% depending on the maize genotype (Riggs et al., 2001). These results emphasize the importance of evaluating combinations of different strains of *Rhizobium* and combinations of maize genotype.

Other non-leguminous crops like sugar cane (*Sacharum officinarum*) benefit from associative diazotrophs. Using ^{15}N isotope dilution and ^{15}N natural abundance techniques, it was reported that certain Brazilian sugar cane varieties can derive 50-80% of the plant N from BNF, equivalent to 150-170 kg N ha^{-1} $year^{-1}$ (Döbereiner et al., 1993; Döbereiner, 1995). In these studies no evidences have been given for the organism(s) responsible for BNF. It was never shown that the growth stimulation was caused by direct transfer of fixed N from the diazotroph to its plant partner. However, *Acetobacter diazotrophicus* was found to be predominantly present in sugar cane plants. Furthermore, to provide direct evidence that plants benefit from the N_2 fixed by the assumed diazotroph, plant inoculation experiments with non-N fixing (Nif $^-$) mutants as negative control were reported by Sevilla et al. (2001). The wild-type strains and *nifD* mutant of *Acetobacter diazotrophicus*, unable to fix N, were used to inoculate sterile sugar cane plantlets prepared from meristem tissue culture. Plants inoculated with the wild-type strain generally grew better and had higher N content 60 days after planting than the plants inoculated with the Nif$^-$ mutant or uninoculated plants. These results indicate that the transfer of fixed N from *A. diazotrophicus* to sugar cane might be a significant mechanism for plant growth promotion in this association.

Although the contribution of diazotrophic associations in several important crops have been demonstrated, the mechanisms involved in the stimulation still remain elusive in most of the cases (Hurek et al., 1998; Kennedy 2004; Sun et al., 2008). Recently, it has been reported that endophytic bacteria may promote plant growth and suppress plant diseases probably by means similar to plant growth-promoting rhizobacteria (PGPR) (Feng et al., 2006). Dobbelaere et al. (2003) reviewed the diazotrophic PGPR in highlighting their mechanisms of action including BNF, but also the plant growth promotion by production of auxins, cytokinins and gibberellins.

In the past decades there have been increasing evidences that besides N_2-fixation, synthesis and export of phytohormones by the diazotrophic associated bacteria may play an important role in the observed plant growth promotion. Phytohormones like auxins, cytokinin and gibberellin, also called plant growth regulators (PGRs), are well known for their regulatory role in plant growth and development. PGRs are organic substances that influence physiological processes of plants at extremely low concentrations. Because the concentration of hormonal signals is critical to the regulation of various physiological processes in plants, local changes of phytohormone levels can lead to characteristic changes in plant growth and development (Dobbelaere, 2002). Table 1.1 shows the possible mechanisms involved in plant growth promotion by several diazotrophs associated bacteria reported by Dobbelaere (2002). Some of them are briefly discussed.

Among the phytohormones, most of the attention has been given to auxin (IAA) production because this characteristic is widespread in soil and plant-associated bacteria (Vande Broek et al., 1999; Lambrecht et al., 2000; Dobbelaere, 2002). It has been estimated that 80% of the bacteria isolated from the rhizosphere can produce auxin as PGR (Patten and Glick, 1996).

The stimulating effect of IAA on plants has been extensively studied (Morgenstern and Okon, 1987; Martin et al., 1989; Lebuhn et al., 1997; Dobbelaere et al., 2003; Van Noorden et al., 2006). It has been reported that certain levels of IAA produced by bacteria, like *Azospirillum* Sp6 promote the length and the number of lateral roots in wheat (Barbieri and Galli, 1993). Recently Remans et al. (2007b) have reported the increase in nodulation parameters and the root responsiveness to auxin-PGPR in common bean, detecting QTLs for root responses to auxin.

Table 1.1 Overview of the possible mechanisms involved in plant growth promotion by diazotrophic bacteria (taken and adapted from Dobbelaere, 2002).

Bacteria	BNF	Hormones			ACC deaminase	Increase nutrient uptake	Enhanced stress resistance	P-solubilization	Vitamins	Biocontrol	Siderophores	Increased SRA	References
		auxin	cytokinin	GA									
Acetobacter diazotrophicus	x	+		+									Sevilla et al. (2001)
Azoarcus sp.	x	+											Hurek et al. (1998)
Azospirillum brasilense	-	x	+	x	0	x	x		+		+		Bashan (1991); Glick (2000)
Azospirillum lipoferum	+	+		x		+					+		Lucangeli and Bottini (1996; 1997)
Azotobacter beijerinckii	+	+	+	+									Nieto and Frankenberger (1989)
Azotobacter chroococcum	+	+	+	+			x	+	+				Stajner et al. (1997)
Azotobacter paspali	+	+	+	+									Boddey et al. (1983)
Azotobacter vinelandii	+	+	+	+					+		+		Revillas et al. (2000)
Bacillus pumilis	+			x									Gutiérrez-Moñero et al. (2001)
Bacillus licheniformis	+			x									Gutiérrez-Moñero et al. (2001)
Bacillus megaterium								-					De Freitas et al. (1997)
Bradyrhizobium elkanii	+	+				+							Biswas et al. (2000)
Bradyrhizobium japonicum	+		+								+		Guerinot (1991)
Herbaspirillum seropedicae	+	+		+									Bastián et al. (1998)

Table 1.1 continued

Bacteria	BNF	Hormones			ACC deaminase	Increase nutrient uptake	Enhanced stress resistance	P-solubilization	Vitamins	Biocontrol	Siderophores	Increased SRA	References
		auxin	cytokinin	GA									
Klebsiella pneumoniae	+	+											El-Khawas and Adachi (1999)
Paenibacillus polymyxa	+	+	+					-		x		x	Bezzate et al. (2000)
Pseudomonas putida GR12-2	-	+			x	+					+		Glick et al. (1994)
Rhizobium leguminosarum	+	x	x			+		+			+		Noel et al. (1996)
Rhizobium (Sinorhizobium) meliloti	+								+		+		Sierra et al. (1999)
Rhizobium phaseoli (*R. etli* bv. *phaseoli*)	+	+		+									Atszon et al. (1988)
Rhizobium sp.	+		+										Upadyaya et al. (1991)

0 : characteristic not presented

+ : characteristic present

x : mechanism proven/strong arguments in favour

- : characteristic present but not involved

In beans, low concentrations (up to 100 nM IAA) can enhance nodule number, while higher concentrations inhibit nodulation (Remans et al., 2007b). Interestingly, Plazinski and Rolfe (1985) described a similar dose-response curve of nodulation in response to inoculation with an increasing number of *Azospirillum* cells on bean plants.

On the other hand experiments with *Azospirillum* species have suggested that this organism specifically enhances mineral nutrient uptake (Murty and Ladha, 1988; reviewed by Dobbelaere et al., 2002). It was found that inoculation with *A. brasilense* Cd resulted in a significant increase in the proton efflux on wheat roots seedlings and a reduction in the membrane potential of the root cells in soybean seedlings, facilitating the accumulation of N, P and K at higher rates (Bashan et al., 1990; Bashan et al., 1991).

Phosphorus (P) solubilization has frequently been postulated as a possible mechanism of plant growth promotion by PGPR (Richardson, 2001). Experiments performed with P-solubilizing diazotrophs are few, and the results obtained quite diverse, varying according to plant or bacterial species (Freitas et al., 1997). However, a significant increase in plant size after *Azotobacter chroococcum* inoculation has been shown to be related with the increase in P-solubilization and P-uptake (Doneche and Marcantoni, 1992; Revillas et al., 2000). P-dissolving bacteria may have a secondary role in making extra P available from sparingly soluble sources, especially in P-deficient soils (reviewed by Dobbelaere, 2002).

Vitamins are sometimes added to the list of compounds, involved in direct plant growth promotion, that PGPR can produce. However, the possibility that plant growth can be improved by inoculation with vitaming-producing bacteria has received little attention (Oertli, 1987; Dobbelaere, 2002). Some bacteria like *Azotobacter*, *Azospirillum* and *Rhizobium* strains have been found to produce some or all of the water soluble B-group vitamins niacin, pantothenic acid, thiamine, riboflavin and biotin in defined culture media (Martinez-Toledo et al., 1996; Sierra et al., 1999; Revillas et al., 2001). Nevertheless their role in plant growth promotion has been poorly studied. There is evidence that exogenously added B vitamins can be absorbed by roots, producing favorable effects on root development, shoot length, dry matter production and nutrient uptake (Mozafar and Oertli, 1992).

In conclusion, prospects for effective microbial bio-fertilizers for cereal crops like rice, wheat and maize, and even in *Poaceae* like sugar cane and pastures, are worth pursuing. Cocking (2002) has called for concerted action to encourage bio-fertilizer production

1.2.2 Symbioses of N-fixing bacteria with plants

Symbiotic interactions of bacteria with various groups of plants are the best studied for biological N supply. A multiplicity of bacteria with different physiological backgrounds are involved in these interactions, including Gram-negative proteobacteria like *Rhizobium* sp. and *Burkholderia* sp., Gram-positive *Frankia* sp. (Benson and Silvester, 1993), and filamentous or unicellular cyanobacteria (Rai et al., 2000). The physiological and morphological characteristics of these symbioses range from extracellular communities to highly adapted interfaces within special organs or compartments. The mutualistic symbioses between various non-photosynthetic proteobacteria of the order Rhizobiales with plants of the orders Fabales, Fagales, Curcurbitales and Rosales are the most extensively studied interactions between bacteria and plants (Kneip et al., 2007). In our study we focus on the interplay among *Rhizobium* and legume plants for their substantial contribution to the N cycle and BNF.

1.2.2.1 The Rhizobium-*legume symbiosis*

An examination of the history of BNF shows that interest generally has focused on the symbiotic system of leguminous plants and rhizobia species, because these associations have the greatest quantitative impact on the N cycle. A tremendous potential for contribution of fixed N to soil ecosystems exists among the legumes (Brockwell and Thies, 1995; Peoples and Ladha, 1995; Tate, 1995). There are approximately 650 genera and about 20,000 species of legumes (Sprent, 1985), only a portion of which (about 20% Sprent and Sprent, 1990) have been examined for nodulation and shown to have the ability to fix N_2. Estimates are that the rhizobial symbioses with the somewhat greater than 100 agriculturally important legumes contribute nearly half the annual quantity of BNF entering soil ecosystems (Tate, 1995). Inputs of BNF into terrestrial ecosystems from the symbiotic relationship between legumes and their rhizobia, amount to at least 70 million tonnes of N $year^{-1}$ (Brockwell and Thies, 1995). Estimates of N_2 fixation amounts by different grain legume crops in the tropics (see Table 1.2) highlight that the amounts of N_2 fixed and the proportion of plant N derived from N_2 fixation varies enormously between grain leguminous crops, between different genotypes of the same crop and between different environments in which crops are grown (Giller, 2001).

The BNF, particularly through legume-rhizobia symbioses, plays a crucial role in increasing the sustainability of yields with minimal non-renewable external inputs (Vance, 2001). The N-fixing symbiosis between plants of the *Leguminosae* family and prokaryotic partners is

typically characterized by the formation of root or stem nodules that are induced and subsequently invaded by the specific microsymbionts (Weidner et al., 2003). Weir (2006) reports that these include the well-known alpha-proteobacterial group of *Rhizobiaceae* containing the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium, Azorhizobium*, and *Allorhizobium*, along with other taxa such as *Methylobacterium* (Sy et al., 2001) and *Devosia* (Rivas et al., 2002), *Herbaspirillum* (Valverde et al 2003), *Ochrobactrum* (Zurdo-Piñeiro et al., 2007), *Phyllobacterium* (Valverde et al., 2005) and members of the beta-proteobacteria such as *Burkholderia* (Moulin et al., 2001) and *Cupriavidus* (*Ralstonia)* (Chen et al., 2001).

Table 1.2 Estimates of N fixation by grain legumes grown as sole crops in the tropics. (taken from Giller, 2001).

Grain legume	N_2 fixed		Time period	Country	Method[a]	Ref.[b]
	kg ha^{-1}	%	(days)			
Arachis hypogaea	139-206	55-64	120	Australia	^{15}NA	1
(peanut)	85-131	47-53	144	Australia	^{15}NA	1
	32-120	22-49	140	Australia	^{15}NA	2
	43-72	45-67	90-106	Australia	^{15}NA	3
	68-116	54-78	110	Brazil	ID	4
	101	-	-	Ghana	Diff	5
	100-152	86-92	89	India	ID	6
	152-189	61-85	118-137	India	^{15}NA/Diff	7
	21-58	16-53	-	Indonesia	ID/^{15}NA	8
	101-130	59-64	90-110	Thailand	ID	9
	150-200	72-77	106-119	Thailand	ID	10
	102	68	88	Thailand	ID	11
	46	62	87-97	Thailand	ID	12
Cajanus cajan	68-88	88	-	India	ID	13
(pigeon pea)	150-166	63-86	-	India	ID	14
	0-76	0-36	95-210	India	^{15}NA	15
	30-131	59-87	-	India	^{15}NA	16
	13-163	42-85	120	Malawi	^{15}NA	17
	1-39	64-100	-	Zimbabwe	^{15}NA	18
Cicer arietinum	60-84	60-80	160	Australia	^{15}NA	1
(chickpea)	67-85	63-81	170	Australia	^{15}NA	19
	0-124	0-79	-	Australia	^{15}NA	20
	0-99	0-81	-	Australia	^{15}NA	21
	35-80	66-96	-	Nepal	^{15}NA	22
Glycine max	85-154	70-80	110	Brazil	ID	4
(soybean)	14-15	36-39	40	Congo	ID	23
	114-188	84-87	66	Nigeria	ID/Diff	24
	42-83	46-87	36-75	Nigeria	ID	25
	149-176	69-74	70-84	Philippines	ID	26
	26-57	78-87	64-73	Thailand	^{15}NA	1

Table 1.2 Continued

Grain legume	N_2 fixed		Time period	Country	Method[a]	Ref.[b]
	kg ha^{-1}	%	(days)			
	108-152	66-68	97-104	Thailand	ID	10
Phaseolus vulgaris	25-65	37-68	60-90	Brazil	ID	27
(common bean)	3-32	15-72	61	Brazil	ID	28
	4-45	12-53	60-92	Brazil	ID	29
	25-115	27-60	-	Chile	ID	29
	18-36	32-47	56	Colombia	ID	30
	9-50	24-50	63-70	Colombia	ID	31
	12-125	22-73	-	Guatemala	ID	29
	74-91	43-52	74	Kenya	ID	32
	44-50	60-73	91	Mexico	ID	33
	0-108	0-58	-	Mexico	ID	29
	34-85	30-57	-	Mexico	ID	34
	7-81	13-56	86-116	Peru	ID	35
	8-26[c]	40-51	75	Tanzania	ID	36

[a]ID: ^{15}N isotope dilution; ^{15}NA: ^{15}N natural abundance; Diff: N difference. [b]1: Peoples et al., 1991; 2: Peoples et al., 1992 ; 3: Bell et al., 1994 ; 4: Boddey et al., 1990; 5: Dakora et al., 1987 ; 6: Giller et al., 1987 ; 7: Nambiar et al. 1986 ; Yoneyama et al., 1990 ; 8 : Cadish et al., 2000 ; 9 : McDonagh et al. 1993; 10: Toomsan et al., 1995; 11: McDonagh et al., 1995; Toomsan et al., 2000; 13: Kumar Rao et al., 1987; 14: Tobita et al., 1994; Kumar Rao et al., 1996b; Kumar Rao et al., 1996a; 17: Adu-Gyamfi et al., 2007; 18: Mapfumo et al., 1999; 19: Herridge et al. 1995; 20: Herridge et al., 1998; 21: Schwenke et al., 1998; 22: Ali et al., 1997; 23: Madimba, 1996; 24: Eaglesman et al., 1982; 25: Sanginga et al., 1997; 26: George et al., 1995; 27: Rushel et al., 1982; 28: Duque et al., 1985; 29: Hardarson et al., 1993; 30: Kipe-Nolt and Giller, 1993; 31: Kipe-Nolt et al., 1993; 32: Ssali and Keya, 1986; 33: Peña-Cabriales et al., 1993; 34: Castellanos et al., 1996; 35: Manrique et al., 1993; 36: Giller et al., 1998.

The support of microscopy to examine nodule symbioses has gained new importance in light of these findings, and various studies have coupled the visual approach with the molecular characterization of symbionts (Chen et al., 2005; Elliott et al., 2007). The current rhizobial taxonomy has 6 genera and 29 species, most of which were described in the last decade using rhizobia isolated from tropical legume species (Bala and Giller, 2006). In spite of this relatively high turnover of rhizobial groups, it is likely that we are still orders of magnitude away from a true assessment of the diversity of tropical rhizobia (Giller 2001). This has led to questions being asked as to how this can be explored to enhance agricultural productivity in the tropics (Bala and Giller, 2006). This requires an ecological approach, which can help us to understand the relative environmental tolerances of the different rhizobial types and thus allow for predicting their ecology (Andrade et al. 2002). Such an approach is pertinent in view of the fact that the success of rhizobial inoculation, for instance, depends on inoculant strain competitiveness and persistence, which are both linked to the saprophytic competence of the strain. Although the description of rhizobial genera and species is now essentially based on

sequence analysis of the small subunit ribosomal DNA, phenotypic characterization still remains an essential ingredient of rhizobial classification (Graham et al. 1991; Bala and Giller, 2006).

1.2.2.2 Phenotypic characterization and genetic variation in Rhizobium-*legume symbiosis*

To enhance legume nodulation and N fixation, the introduction of bacterial inoculants to agricultural fields has been a common practice for over 100 years (Martinez-Romero, 2003). Whenever the specific rhizobia are absent, inoculation readily enhances plant growth and yield (Singleton and Tavares, 1986; Streeter, 1994; reviews of Triplett and Sadowsky, 1992, and Vlassak and Vanderleyden, 1997). On the other hand, when native bacteria exist in the fields they often out-compete the inoculant strains that only occupy a small proportion of nodules as observed in some legumes plants in Latin America (Graham, 1981; Ramos and Boddey, 1987; review in Vlassak and Vanderleyden, 1997; Burgos et al., 1999; Aguilar et al., 2001). Contrastingly, common bean (*Phaseolus vulgaris*) inoculation with *R. tropici* in Brazil has been successful (Hungria et al., 2000; Mostasso et al., 2002) and *Rhizobium* inoculated onto beans enhanced both bean and maize yields when the two were grown together in Peru (Pineda et al., 1994).

Quantitative effects of rhizobia-legume interactions can be measured in terms of how the bacteria respond to the host genotype. Several researchers have studied how the ability of strains to compete for nodule occupancy is affected by the genotype of the host. The inability of superior N-fixing strains to compete with indigenous soil strains for nodule occupancy is a major constraint in developing rhizobia inoculants (Smith et al., 1999; Snoeck et al., 2003). Cregan et al. (1989) found significant effects of soybean (*Glycine max*) genotypes on the competitiveness of closely related strains of *Bradyrhizobium japonicum*. For example, soybean PI417566 restricted the nodulation of strain USDA 129 to below 5% nodule occupancy when coinoculated with either USDA 123 or USDA 127. However, on another soybean cultivar, USDA 129 occupied over 87% of the nodules when co-inoculated with the same two strains. Josephon et al. (1991) co-inoculated two strains, KIM5 and Viking-1, on 12 cultivars of common bean, measured the percent nodule occupancy of each strain for each cultivar, and found a significant cultivar x strain interaction. Genetic analysis of host contributions to nodulation competitiveness of superior *Rhizobium* strains has been approached by Rosas et al. (1998) with a genetic tool. They made a Fix^- mutant of a wild-type

strain, KIM5, and used this mutant to screen a large collection of bean germplasm for accessions that were preferentially nodulated by the mutant when planted into soil containing indigenous rhizobia. Such preferentially nodulated accessions were yellow due to N deficiency and could therefore easily be selected.

According to early studies reported by Graham (1981) and Amarger (1986), N fixation depends on rhizobia x cultivar interaction. Consequently the process of selection of efficient rhizobia should be developed with adequate lines. Provorov and Simarov (1992) and Fesenko et al. (1994) suggested that the variability in the expression of the symbiotic functions is not only the result of simple additive contributions of both symbiotic partners, but also includes a *Rhizobium* x line interaction-composite. It reinforces the importance of examining the interaction between the diversity of native rhizobia with a newly introduced cultivar, in addition to the influence of the plant genotype on the nodulation and effectiveness in a given species. Mhamdi et al. (2002) described the variability of the rhizobia strains with the ability to nodulate common bean in different environments in Tunisia, reporting that the strains vary among regions and cultivars, with *R. leguminosarum* being found exclusively in plants in Bizerte, in contrast with *R. etli* found exclusively in Cap Bon. Recently Tajini et al. (2008) demonstrated the effective combination of native *Rhizobium etli* and *R. tropici* CIAT899 in common bean genotypes. It was evident that the native rhizobia strains were more efficient than CIAT899 strain, however, a clear effect of strain x genotype was markedly seen in the study.

All these results indicate a strong host genotype x rhizobia interaction. This interaction is supposed to result from co-evolution between *Rhizobium* strains and host genotypes (Aguilar et al., 2004). In common bean, two gene pools are recognized based on their centers of domestication in Central and South America, namely the Mesoamerican (also known as Middle American) and Andean gene pools. Consistent with the concept of co-evolution, *R. etli* strains appeared to be more common among Mesoamerican accessions as compared to Andean accessions (Kipe-Nolt et al., 1992; Montealegre et al. 1995; Montealegre and Graham, 1996; Aguilar et al., 2004). For soybean, which was domesticated in China, it was found that Asian varieties were more promiscuous for rhizobia than American varieties. Asian varieties, for example, also nodulated well in Nigerian soils whilst American varieties formed very few nodules. The difference in promiscuity can probably be explained by two processes: firstly, the American varieties have been bred from a limited genetic base and secondly, only a

limited range of inoculant strains of *B. japonicum* were introduced to North America, leading to increased cultivar-strain specificity. Based on the difference in promiscuity, breeders of the International Institute of Tropical Agriculture (IITA) in Nigeria reintroduced the ability to nodulate with indigenous strains of rhizobia into the American varieties, as they had far greater yield potential and better resistance to diseases. This resulted in the successful development and release of improved soybean varieties, that nodulate without inoculation in soils not previously cropped with soybean. The breeding program has continued and more recent materials have substantially improved ability to nodulate and fix N_2 in farmers' field without inoculation (Sanginga et al., 1999; Sanginga et al., 2000), as well as having higher yield potential (Sanginga et al., 2001).

Despite the potential to improve symbiotic N fixation (SNF) present in natural genetic resources, SNF improvement does not form part of routine cultivar improvement programs. For instance, in Cuba, common bean breeding has been focused during the last decade with strong support of participatory programs. Several genotypes obtained by conventional breeding and distributed to the farmers have shown increases in common bean yield, however, the SNF is no evaluated in this program, which diminishes the breeding potentialities through sustainable practices (Ortiz-Pérez et al., 2006; Miranda-Lorigados et al., 2006). Breeding programs for enhanced N_2 fixation have been established for some legumes (common bean, soybean, groundnut, cowpea) but apart from the case of breeding for enhanced promiscuity of nodulation in soybean, there have been few concerted efforts to enhance the potential for N_2 fixation in grain legumes through plant breeding. Incorporating selection criteria such as nodule mass, nitrogenase activity and xylem ureide content into breeding schemes while attending to other breeding objectives, remains a challenge. If marker-assisted selection (MAS) for SNF could be integrated into breeding programs that are already practicing MAS for other traits, this would avoid the necessity of additional phenotypic selection methodologies purely for N or nodule determination (Miklas et al., 2006).

1.2.2.3 Stimulation of legume–rhizobia symbioses

As demonstrated above (section 1.2.1), the diazotrophic bacterial associations contribute substantially to BNF in important non-legumes crops. Moreover in legumes the combination of PGPRs with symbiotic diazotrophs exerts a marked effect in physiological and phenotypic

parameters. Some of the mechanisms presented for the different diazotrophic bacteria in Table 1.1 are reviewed in this part.

As early as 1979, Singh and Subba Rao reported positive effects of *Azospirillum brasilense* inoculation on nodule number, nodule dry weight and shoot growth of soybean.

Rodelas et al., (1999) reported for faba bean that responses to *Azotobacter* and *Azospirillum* inoculation in combination with *Rhizobium* led to changes in total content and/or distribution of macro- and micronutrients (K, P, Ca, Mg, Fe, B, Mn, Zn and Cu) when compared with plants inoculated with *Rhizobium* alone. Some PGPR that stimulate legume–rhizobia symbioses appear to more directly influence the development of the symbioses.

There is evidence for a number of modes of action for PGPR stimulation of legume–rhizobia symbioses, but the most commonly implicated mode is phytohormone-induced (usually indole-3-acetic acid, IAA) stimulations of root growth (Molla et al., 2001; Srinivasan et al., 1996; Vessey and Buss, 2002). In this way, the stimulation of nodulation is most commonly an indirect effect; the PGPR stimulates root growth, which provides more sites for infection and nodulation. However, this is not always the case. Cattelan et al. (1999) found that a number of putative PGPR had positive effects on shoot and/or root growth in soybean and were positive for production of IAA or ACC deaminase, but these putative PGPR had no positive effects on nodulation. In fact, this study found several rhizosphere isolates which stimulated aspects of the soybean–bradyrhizobia symbiosis and which had *β*-gluconase or cyanide production. The pathways of the stimulatory effect of these substances in the soybean–bradyrhizobia symbiosis are still unclear.

Burdman et al. (1996; 2000) related *Azospirillum brasilense*-mediated stimulation in nodulation of common bean to an increased production of flavonoids by the legume host. These flavonoids are the initial chemical signals secreted by the legume host to induce *nod* genes in rhizobia and thereby initiate the legume–rhizobia symbiosis (Schultze and Kondorosi, 1998). Andrade et al. (1998) speculated that an increase in nodulation in pea mediated by inoculation with *Pseudomonas fluorescens* was due to an increase in flavonoid exudation by the host plant. Proposed alternative modes of action include toxin (i.e., tabtoxinine-*β*-lactam) release by *Pseudomonas syringae* stimulating the alfalfa–rhizobia symbiosis (Knight and Langston-Unkefer, 1988) and B vitamins secretion by *Pseudomonas* sp. enhancing the red clover-rhizobia symbiosis (Marek-Kozaczuk and Skorupska, 2001).

Other evidences are reported by Thilak et al. (2006) showing that PGPR in conjunction with efficient *Rhizobium* can also affect the growth and nitrogen fixation in pigeon pea by enhancing the occupancy of introduced *Rhizobium* in the nodules of the legume. The nodule occupancy of the introduced *Rhizobium* strain increased from 50% (with *Rhizobium* alone) to 85% in the presence of *Pseudomonas putida*. Recently Figueiredo et al. (2007) reported enhanced nodulation and N fixation in common bean with the co-inoculation of *Rhizobium* and several strains of *Paenibacillus*. In this study co-inoculation with *Rhizobium tropici* (CIAT899) and *Paenibacillus polymyxa* (DSM 36) had higher leghemoglobin concentrations, nitrogenase activity and N_2 fixation efficiency and thereby formed associations of greater symbiotic efficiency. Inoculation with *Rhizobium* and *P. polymyxa* strain Loutit (L) stimulated nodulation as well as N fixation. PGPR also stimulated nodulation (number of nodules per gram of root dry weight), increases that translated into higher levels of accumulated N. Moreover, Remans et al., (2007a) showed that the effect on nodulation of three out of four PGPR (including *Bacillus*, *Pseudomonas, Azospirillum* and *Azospirillum ipdC* minus mutant) tested strongly stimulated this parameter in common bean and the effect was dependent on P nutrition. Further, the use of specific PGPR mutant strains indicated that bacterial indole-3-acetic-acid production (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity play an important role in the host nodulation response, particularly under low P conditions.

*1.3 Common bean (*Phaseolus vulgaris *L.), a model legume to achieve sustainability under low input systems*

Previously we reviewed the phenotypic characterization and the genetic variation of *Rhizobium*-legume interactions in different plant species, genotypes and combination with associative PGPRs. In this part we describe shortly some of the aspects that make common bean a good model legume for sustainability in low input systems.

1.3.1 Common bean: a challenge legume for low-input systems

About 55 species of *Phaseolus* are known (Maréchal et al., 1978; reviewed by Martínez-Romero, 2003), but only five of them are domesticated: common beans (*Phaseolus vulgaris*), lima beans (*P. lunatus*), runner beans (*P. coccineus*), tepary beans (*P. acutifolius*) and *P.*

polyanthus. Amongst *Phaseolus* the common bean (*P. vulgaris*) is the most extensively cultivated (Martínez-Romero, 2003).

Phaseolus vulgaris L. is a basic staple food providing more than 70% of the dietary protein for poor people in Latin America and Eastern Africa. Nutritionists characterize common bean as a nearly perfect food because of its high protein content and generous amounts of fiber, complex carbohydrates and other nutritional needs including folic acid, iron, copper, potassium and zinc. Beans complement other food crops like maize and rice which are primary sources of carbohydrates. In developed countries, the nutritional benefits and contribution of beans to healthy human diets is recognized by non-profit organizations targeting human diseases like cancer, diabetes and heart disease (Hangen and Bennink, 2002; Xu and Chang, 2008). Given these characteristics, it is not surprising that common bean is the world's most important legume for direct human consumption (Broughton et al., 2003). The global bean harvest of 18 million tonnes annually has an estimated value of US$ 11 billion (CIAT, 2006). The major part (11.5 million tonnes) is produced in Latin-America and Africa, mostly by resource-poor farmers on small-scale, marginal farms ranging from 1-10 ha in size (Broughton et al., 2003). Under these conditions, beans are mainly cultivated as food crop for own consumption or for local exchange with other products or services. Cash crop beans are considered relatively profitable, although their low yields and fluctuating market prices limit the generation of a stable income based on bean production.

Beans are extremely diverse crops in terms of cultivation methods, uses, the range of environments to which they have been adapted, and morphological variability. They are found from sea level up to 3000 m above sea level, are cultivated in monoculture, in associations, or in rotations. Their growth habit ranges from bush bean varieties maturing in 3 months up to climbers that take 8 months to harvest. Their genetic resources exist as a complex array of major and minor gene pools, races and intermediate types, with occasional introgression between wild- and domesticated-types. Beans are therefore a crop that is adapted to many niches, both in agronomic and consumer preference terms (Broughton et al., 2003). Through its large biodiversity, natural genetic variation for traits of agronomic importance is extensive for beans. Both domestication and plant breeding reduced genetic diversity among cultivated varieties due to random genetic drift (bottlenecks) and selection for target genes (Singh, 2001; Rossi et al., 2007). Therefore, especially wild *Phaseolus* species are an excellent resource for traits as resistance/tolerance to biotic and abiotic stress, yield under diverse agronomical

conditions and symbiotic interactions with rhizobia and PGPR. Furthermore, wild bean germplasm is useful as a source of geographic markers in evolutionary studies allowing a more systematic search for a trait of interest in the cultivated germplasm (Snoeck et al., 2003). Interest in bean genetics is increasing with the identification of new sources of germplasm, the improvement of genetic and physical maps and mapping techniques and the identification of bean genes playing a role in SNF (Hernández et al., 2007).

1.3.2 Common bean as a promiscuous host for rhizobia

In contrast to some other legumes like soybean, common bean is highly promiscuous for both fast-growing and slow-growing rhizobia symbionts. Efficient N-fixing symbiosis, however, is only obtained with fast-growing rhizobia (Michiels et al., 1998; Bala and Giller, 2001). The promiscuity of common bean complicates management of an efficient symbiotic interaction in the field due to competition with strains that are less efficient for N fixation but more competitive in the bean rhizosphere (see Table 1.3). For that reason common bean is considered as an inefficient N-fixation crop as compared with other legumes (Hardarson, 1993, Bacem et al., 2007) (see Table 1.2). Variation among bean cultivars in favoring more efficient N-fixing rhizobia has been described and demonstrates further potential to improve SNF of common bean based on genetic variability present in nature.

The distribution of rhizobia that nodulate *P. vulgaris* varies among geographical locations (Amarger, 2001), although *R. tropici* and *R. etli* appear to be distributed worldwide. *R. tropici* are reported to be the dominant bean nodulating rhizobia in the soils of many places including tropical regions of South-Central America (Martinez-Romero et al., 1991), Brazil (Hungria et al., 2000), East and South Africa (Anyango et al., 1995); while *R. etli* is prevalent in Europe (Herrera-Cervera et al., 1999), Central and West Africa (Diouf et al., 2000), and Indonesia (Tjahjoleksono, 1993). *R. giardinii* and *R. gallicum* are less diverse and are reported to be associated with nodulation of *P. vulgaris* in France (Laguerre et al., 1993) and Spain (Herrera-Cervera et al., 1999). In many agro-ecosystems the microsymbionts have been poorly characterized. In Cuba, studies on genetic characterization of soil microorganism and specifically *Rhizobium* strains are few, therefore the knowledge about the prevalent strains are almost non existing (Hernandez et al. 1996; Loiret, et al., 2004).

Table 1.3 *Rhizobium* species isolated from *Phaseolus vulgaris* bean nodules

Rhizobium species	Site of isolation[a]
R. etli (Segovia et al., 1993)	**Mexico**, Colombia, **Ecuador-Peru**, **Argentina**, Brazil, Senegal, Gambia, Tunisia[b], Spain, Austria, USA
R. tropici (Martínez-Romero et al., 1991)	Brazil (type A, B and others), Colombia (type B), France (type A), Morocco, Kenya, Senegal and Gambia (type B)
R. leguminosarum bv. *phaseoli* (Jordan, 1984)	England, France, Spain, Colombia, Brazil, Tunisia*b*
R. gallicum (Amarger et al., 1997)	France, Austria, **Mexico** (bv. *gallicum* only), Tunisia, Spain
R. giardinii (Amarger et al., 1997)	France, Spain, Brazil

a Centers of origin are bold. *b* Mhamdi et al., (1999). Data taken from Martínez-Romero (2003)

Beans with high capacity to fix N may then be used in combination with *Rhizobium* strains with superior capacities to fix N and compete with native strains. A strategy would be to improve N fixation capacity in the native strains well adapted to different regions highlighted in the bean rhizobia diversity studies (Martinez-Romero, 2003). The improvement of bean nitrogen fixation is an important goal, biological nitrogen fixation not only lowers production costs but is also environmentally sound. The global advantages of nitrogen fixation in agriculture have often been emphasised (see Graham and Vance, 2000)

1.3.3 Natural genetic variation analysis in common bean genetics

The genome of common bean (450-650 Mbp/haploid genome) is relatively small and comparable to that of rice which is generally considered to be one of the economically most important plants with the smallest genome. Cytogenetically, common bean is a true diploid with 11 chromosomes (Broughton et al., 2003). To unravel the secrets of common bean genetics, a vast range of tools is available to date. A major tool in functional genomics is transformation of plants, which consists of introducing DNA in a plant tissue and subsequently producing transgenic plants from this transformed tissue. Through this method, direct evidence for the function of genes can be provided. Also transgenic plants can be generated providing for example resistance to diseases. Transformation of leguminous species, in particular grain legumes, is often difficult, in particular the *in vitro* regeneration

step (Svetleva et al., 2003; Broughton et al., 2003). Common bean can regenerate *in vitro* either indirectly (through callus stage) or directly (through somatic embryogenesis and organogenesis). A major bottleneck of using transformation in bean research is the low efficiency of the *in vitro* regeneration. Different techniques were found useful in advancing regeneration efficiency including cytokinine pretreatment of donor plants, thin cell layer method, utilization of embryo derived explants, and others (Veltcheva et al., 2005). Recent breakthroughs have been made with wild accessions of *P. acutifolius* (Svetleva et al., 2003) and with a root transformation technique for different species of *Phaseolus*, including *Phaseolus vulgaris* (Estrada-Navarette et al., 2006; Estrada-Navarette et al., 2007). These advancements set the foundation for functional genomics in common bean based on transformation.

The difficulties in bean transformation have stimulated the exploitation of other genetic research methods, including the analysis of natural genetic variation. Many tools to analyze bean genetic variability have been developed. Some examples are: a genome-wide anchored microsatellite map of a Mesoamerican x Andean cross (Blair et al., 2003), an integrated consensus map of the 11 linkage groups with quantitative trait locus (QTL) for traits of economic importance (Kelly et al., 2003), several bacterial artificial chromosome (BAC) and cDNA libraries for various genotypes, plant tissues and conditions. Further, the amount of available expressed sequence tags (ESTs) representing genes that function under specific conditions is increasing rapidly (Ramírez et al., 2005). This increasing amount of ESTs will allow more macroarray and microarray studies as applied recently by Hernández et al. (2007). Furthermore, these ESTs can be mined for single sequence repeats (SSRs) to refine genetic mapping (Varshney et al. 2005). Recently, a first FingerPrinted Contig (FPC) physical map of the *Phaseolus* genome has been released, containing 1183 contigs, 6384 singletons and 240 markers (http://phaseolus.genomics.purdue.edu). More markers are expected and the map will be updated regularly. This type of mapping is more accurate than genetic maps. The availability of a *Phaseolus* FPC map allows using the clones of the map as a resource to efficiently obtain stretches of the genome in large quantity and to efficiently sequence the clones to determine the DNA sequence of *Phaseolus*.

Analysis of bean genetic variation has already resulted in the detection of numerous QTL and in applications through MAS, especially for resistance against biotic stresses, but also against abiotic stresses and for yield increase (reviewed in Kelly et al., 2003; Miklas et al., 2006).

All these data demonstrate the existence of a large naturally occurring genetic variation for SNF capacity. It has even been postulated that there would be genotypic variation in the germplasm of legume species in all components of the signaling pathway. However, such a statement is based on literature in which testing of only a limited number of genotypes has been reported and therefore needs to be ascertained on a large number of genotypes (Rengel, 2002). Moreover, not every trait related to increase symbiotic N fixation may be of interest for selection. Overlap in signaling pathways between beneficial and pathogenic bacteria and the plant are not excluded (Tsai et al. 1998) and it would be unwise for example to try to enhance symbiosis by down-regulating part of the plant defense response. An increased susceptibility to one or more important diseases was observed among some selected N_2-fixing plants (Rengel et al. 2002). Advancements in high-throughout techniques (i.e cDNA-AFLP) and whole genome approaches (transciptomics, proteomics, metabolomics, interactomics) offer powerful tools to integrate different traits related to efficacy in SNF and to select appropriate groups of parameters for evaluation of germplasm accessions (Ramírez et al., 2005; Hernández et al., 2007).

Strategies for the enhancement and exploitation of BNF are assessed with attention to the likely timescales for realization of benefits in agriculture. Benefits arising from breeding of symbiotic and associative microbes with legumes and non-legumes plants for N_2-fixation have great potential to increase inputs of fixed N and alleviation of environmental stresses or changes in farming systems (Giller and Cadisch, 2004). Genetic engineering may result in substantial enhancement of N_2-fixation, particularly if the ability to fix N_2 is transferred to other crops but these are long-term goals. Immediate dramatic enhancements in inputs from N_2-fixation are possible simply by implementation of existing technical knowledge. Apart from the unfortunate political and economic barriers to the use of agricultural inputs, better communication between researchers and farmers is required to ensure proper focus of research and development of appropriate technologies (Giller, 2001). Legumes must be considered within the context of the farming systems within which they are grown and not in isolation. Proper integration of legumes requires a good understanding of the role of the legume within the system and a better understanding of the relative contributions of N sources and of the fates of fixed N.

Chapter 2

Stimulatory effect of PGPR in Rhizobium-*bean interaction under different growth conditions in Cuba*

Abstract

PGPR-*Rhizobium* combinations were studied under different growth condition in Cuba to evaluate the stimulatory effect on nodulation, plant growth and yield of common beans (*Phaseolus vulgaris* L.). One pots experiment under controlled condition and two field experiments were conducted, using two local genotypes of common beans.

The nodulation and plant growth of ICA Pijao genotype were significantly stimulated with the combination of *Rhizobium-Azospirillum* and *Rhizobium-Azotobacter* in the controlled condition, as well as in the first field trial, where the same combination of bean genotype-treatment were performed. The variations among genotypes were observed in growth parameters and yield in the second field trial. The *Rhizobium-Azospirillum* and fertilizer treatments showed the best result in yield for ICA Pijao, while for BAT-304 the best result was obtained with the *Rhizobium* inoculation.

Interestingly in all the trials, the treatments with PGPR alone or with co-inoculation showed an increase in plant growth and/or yield compared with the N fertilization. Therefore it can be stated that these treatments have potential to reduce the dependence on chemical N fertilizer.

2.1 Introduction

Common bean production in Cuba and the potential of Rhizobium *and PGPR interaction*

In Cuba, after the collapse of the Soviet Union in 1989, the nation responded to the crisis by restructuring agriculture. A transformation from conventional, high-input, mono-cropping, intensive agriculture to smaller organic and semi-organic, low-input farming system was started (Warwick, 1999). Nowadays more than 70% of the food supply is concentrated in these small producer sectors, including co-operatives, small farmers and small parcels. Beans are among the most widely distributed crops in the entire small producer sector (National Statistical Office (NSO) Cuba, 2008).

New agricultural practices associated with these low-input systems include biological control of diseases, crop diversification, intercropping, animal traction and alternatives for chemical fertilizers. In particular for common bean, which is the most important legume in Cuba, inoculation with *Rhizobium* is commonly used (Hernandez et al., 1996; Warwick, 1999; Oppenheim, 2001; Remans, 2007; NSO, 2008).

Common bean becomes a crucial crop for low input agriculture, mainly because of the beneficial symbiotic interaction with *Rhizobium* and the protein supply for human nutrition. Beans rank fifth in terms of the total proteins consumed in Cuba (Miranda-Lorigados et al., 2006).

The common bean cultivation has a long tradition and is widespread in the whole country. However, yield is rather low (see fig. 2.1 and 2.2) and the investment required for sufficient supply is above 60 million USD per year (Gómez, 2006). One of the main reasons of the low yield is the fact that this pulse is considered a poor N fixer in comparison to other grain legumes (Hardarson, 1993; Bacem et al., 2007). Sparse nodulation and the lack of response to inoculation in field experiments have been frequently reported worldwide. This is attributed to intrinsic characteristics of the host plant, particularly the nodulation promiscuity (Michiels et al., 1998), as well as the extreme sensitivity to other nodulation-limiting factors, such as nutrient deficiency, high temperatures and soil dryness (Bacem et al., 2007).

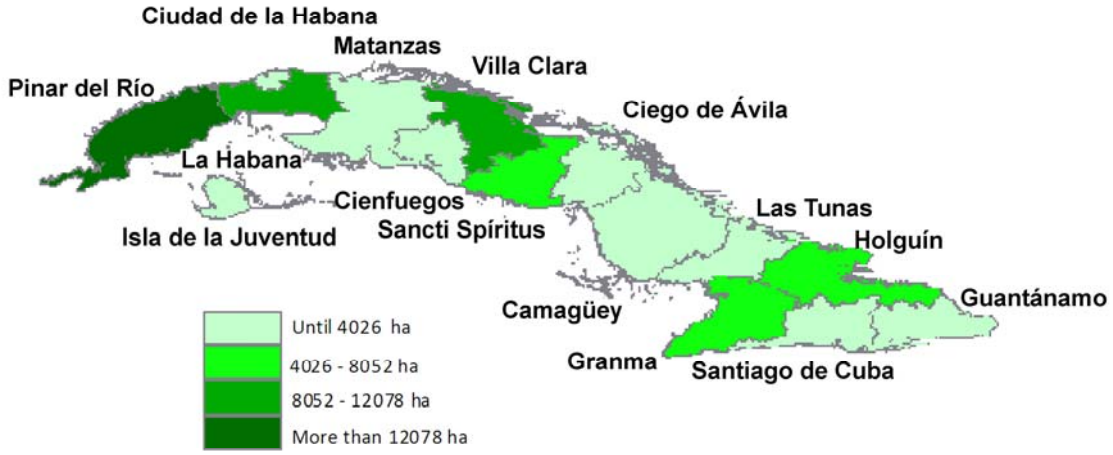

Figure 2.1 Common bean production in Cuba, 2007. Dark green represents the most productive provinces such as: Pinar del Rio, Villa Clara and La Habana. Source: National Statistical Office, Cuba (2008).

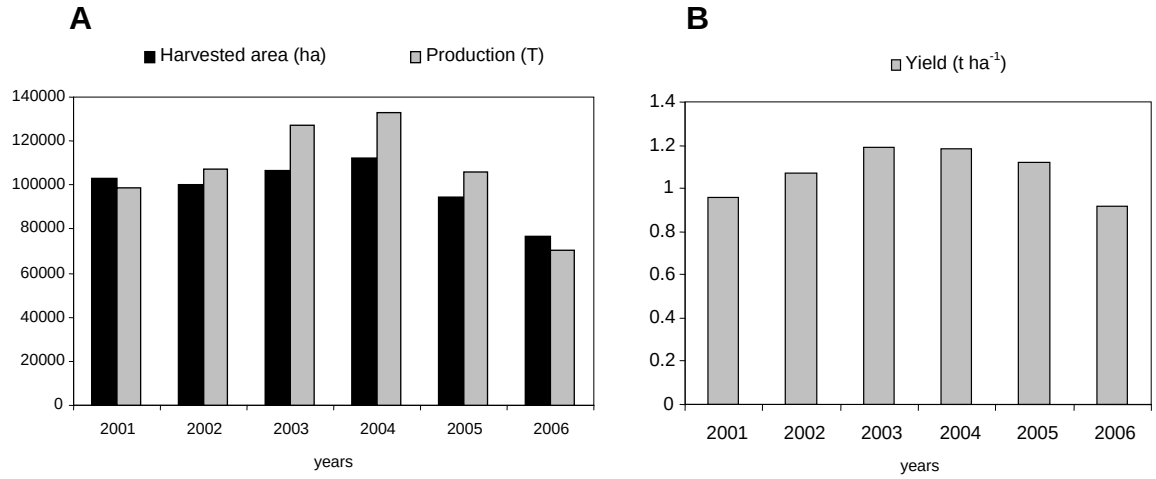

Figure 2.2 Common bean indicators in Cuba. A: Area harvested (ha) and production (tonnes) from 2001-2006. B: Nationwide yield data from 2001-2006. Source: National Statistical Office, Cuba (2008).

In the past two decades, the use of Plant Growth Promoting Rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Kloepper et al., 1980; Chen et al., 1994; Zhang, 1996; Amara and Dahdoh, 1997; Okon and Vanderleyden, 1997; Chanway, 1998; Pan et al., 1999; Bin et al., 2000; Biswas et al., 2000; Asghar et al., 2002; Vessey, 2003; Silva et al., 2006; Remans et al., 2007a; Figueiredo et al., 2007).

Generally, PGPR can function in three different ways (Glick, 1995, 2001): *i.* synthesis of particular compounds beneficial for the plants (e.g. phytohormones), *ii.* facilitating the uptake of certain nutrients from the environment (Cakmakci et al., 2006; Lucas García et al. 2004a,b; Siddiqui and Mahmood, 2001), and *iii*. biocontrol of phytopathogens (Guo et al., 2004; Jetiyanon and Kloepper, 2002; Raj et al., 2003; Zhuang et al., 2007). PGPR are capable of promoting plant growth when colonizing the plant root (Kloepper and Schroth, 1978) and this principle of plant growth promotion has become widely known as the rhizosphere effect (Khan 2005).

Positive effects of PGPR and *Rhizobium* inoculation on nodulation, N_2 fixation and plant growth of common bean, have been observed in greenhouse experiments using hydroponic, vermiculite-based and soil-based systems as well as in field experiments (Burdman et al., 1997; Hamaoui et al., 2001; Bai et al., 2003). Although multiple studies have been reported, the influence of specific environmental factors on these *Rhizobium*-PGPR-plant combinations has not been well studied yet (Remans et al., 2007a). In addition, the role of the host genotype has to be taken into account (Bashan, 1998).

Hernandez et al. (1996) reported that the benefit of rhizobial symbiotic nitrogen fixation and other bacteria (PGPR) for plant growth in Cuba is, however, limited due to environmental constraints and to suboptimal combinations of PGPR strains and bean genotypes used. Evaluation of different *Rhizobium* strains and bean genotypes under different soil and climate conditions showed potential to improve bean yields in Cuba by selecting for improved symbiosis.

In this study we describe the effect of *Rhizobium*-bean-PGPR interactions under different growth condition in the central region of Cuba. Experiments in controlled and field conditions were conducted to evaluate the stimulatory effect of several PGPR-*Rhizobium* combinations on growth parameters, nodulation and yield of common bean compared with nitrogen fertilizer and without rhizobacteria inoculation and fertilization as control. In addition, the variability of the stimulation in two local bean genotypes is evaluated in the trials.

2.2 Materials and Methods

Selected trial sites and plant material

During two consecutive periods (2005-2006; 2006-2007) experiments were conducted under different growth conditions. In the first period (2005-2006), a controlled condition experiment was performed at the Faculty of Agricultural Sciences in the Central University of Las Villas (UCLV, Santa Clara), using the local bean genotype ICA Pijao. The field experiment in this period was performed in farmer's areas of Santo Domingo's municipality (40 Km from Santa Clara, see fig. 2.3). In the second period (2006-2007), the field experiment was performed in the Experimental Station from the Faculty of Agricultural Sciences, UCLV, using the bean genotypes ICA Pijao and BAT-304.

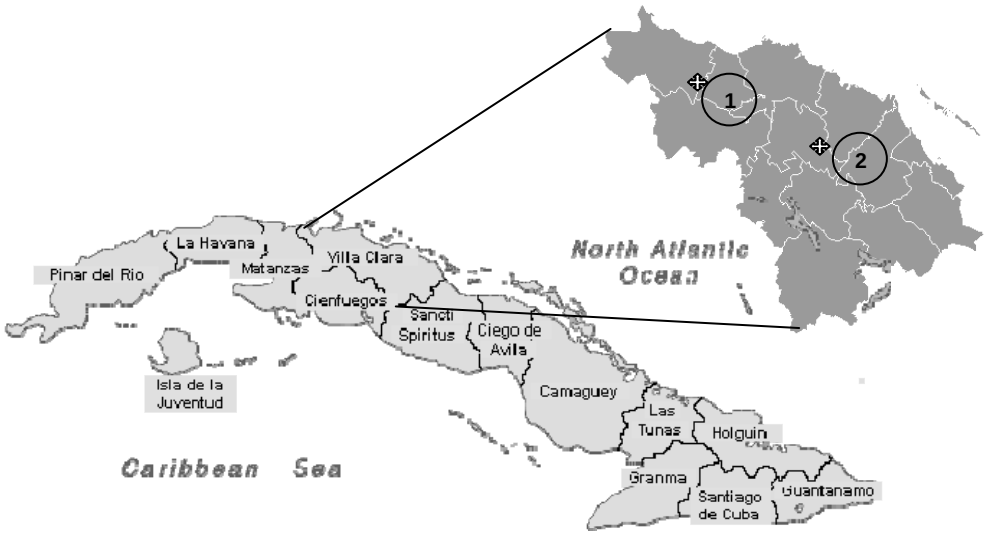

Figure 2.3 Schematic localization of the experiments conducted in two periods in Villa Clara province. 1/ Santo Domingo (22^{o} 36' 17.23''N – 80^{o} 13' 27.45''W): Controlled and field trials in the first period (2005-2006). 2/ Santa Clara, UCLV (22^{o} 25' 59.69''N – 79^{o}53' 28.23''W): Field trial second period (2006-2007).

The local genotypes used in the trials were obtained from the seeds bank of the Villa Clara (V.C) province. Both genotypes, ICA Pijao and BAT-304, are commonly used by farmers and agricultural enterprises in the country, with special use in the centre of the island. Table 2.1 shows the bean genotypes used in the study and some of the most important characteristics.

Table 2.1 Bean genotypes and relevant characteristics used in the assays

Bean genotype	Relevant characteristics					Seed provider
	Crop cycle (days)	Growth type*	Grain colour	Pod mass (g)	Yield low inputs (t ha^{-1})	
ICA Pijao	82	II	Black	7.82	0.65	Seed bank (V.C)
BAT-304	75	III	Black	9.68	0.70	Seed bank (V.C)

* undetermined growth type without climbing ability (Voysest, 2000)

One of the most important characteristics of both genotypes in Cuba is the acceptance by the farmer, especially attributed to the capacity for growth in early (September-November) and late (December- February) time. During the eighties, ICA Pijao was the most exploited genotype in Cuba, reaching more than 80% of the beans grown in the whole country (Sánchez and Scobies, 1986). The resistance against the rust (*Uromyces phaseoli*), the good culinary quality and the yield under low inputs are also important characteristics of these genotypes.

Bacterial strains, growth condition and inoculum preparation in the trials

Table 2.2 shows the strains used in the different trials. *Rhizobium tropici* strain CIAT899 and *Rhizobium etli* 6bIII were grown overnight at 30°C in adapted liquid and/or solid modified YEM medium containing per 1 liter of distilled water: 5 g Bacto Yeast Extract, 20 g sugar (from sugarcane, local production instead of mannitol); 0.5 g $K_2HPO_4.3H_20$; 0.2 g $MgSO_4.7H_20$ and 0.1 g NaCl. pH was corrected to pH 7 by adding HCl (1 M). The 6bIII strain was previously isolated from common bean nodules, further selected in a screening for nodulation and nitrogen fixation in La Renée (Experimental Station Havana). This strain is currently used as the commercial *Rhizobium* inoculum for bean across Cuba (Hernández et al., unpublished data).

Azospirillum brasilense strain Sp7 was grown overnight in liquid Yeast Extract Peptone (YEP) medium (Vanstockem et al., 1987) at 30°C. *Azotobacter chrooccocum* strain MB-9 and the *Azotobacter* sp. (isolated) were grown overnight in liquid RBA medium (Malik 1988) at 30°C. For the solid growth medium of all the strains (*Rhizobium, Azospirillum* or *Azotobacter*), 15 g of selected agar per liter growth medium was added.

Table 2.2 Bacterial strains used in different trials

Bacterial strains	Experimental condition and characteristics	Reference
Rhizobium tropici CIAT899	Controlled and field condition (2005-2006). Wild-type strain	Martínez-Romero et al. (1991)
Rhizobium sp. 6bIII	Field condition (2006-2007). Isolated strain from *Phaseolus vulgaris* nodules, Cuba	Hernández et al (unpublished)
Azospirillum brasilense Sp7	Controlled and field condition (2005-2006; 2006-2007). Wild-type strain	Tarrand et al. (1978)
Azotobacter chroococcum MB-9	Controlled and field condition (2005-2006) Wild-type strain	Malik (1988)
Azotobacter chroococcum isolated strain	Controlled and field condition (2005-2006). Isolated strain from *Sorghum bicolor* roots surface, Cuba	This work

Pre-inocula of *Rhizobium*, *Azospirillum* and *Azotobacter* for pot experiments and field condition experiments were prepared in their respective growth media. For the pot experiment, 2 ml of pre-inoculum grown overnight were transferred to 1 L of YEM, YEP or RBA respectively. The cultures were incubated at 30^{o}C and shaken during 24 h resulting in a cell density of approximately 10^8 colony forming units per ml (cfu ml^{-1}) for *Rhizobium* and 10^9 cfu ml^{-1} for *Azospirillum* and *Azotobacter*, as determined by plating of serial dilutions and based on previous experiments (La Renée and Central University). The inocula for the pot experiment were prepared 1 week before starting the experiment and stored at room temperature till the experiment was started.

To prepare the inoculum for the field experiments, 10 ml of pre-inoculum grown overnight were transferred to 5 L of the growth media (YEM, YEP and RBA). The cultures were also incubated at 30^{o}C and shaken during 24 h. 100 ml of rhizobial cell culture (with 10^8 cfu ml^{-1} YEM medium) and 100 ml of *Azospirillum* or *Azotobacter* cell culture (with 10^9 cfu ml^{-1} YEP and RBA medium) were mixed with 250 g sterile humus as inoculum carrier. This quantity of inoculated humus was used for 10 kg seeds resulting in approximately 10^6 cells per seed of *Rhizobium* and approximately 10^8 cells per seed of *Azospirillum* or *Azotobacter*. These procedures were performed in the Centre for Agricultural Development in Santo Domingo in October 2005 (1 month before starting the trial) and in the Provincial Soils Laboratory in Santa Clara in December 2006 (2 weeks before starting the trial). According to Hernandez et al. (1996), humus-inocula can be stored up to six months without loosing significant bacterial cell vitality.

Plant culture, inoculation and growth conditions in pot experiment

Seeds of ICA Pijao were surface-sterilized as described previously (Vlassak et al., 1998) and pre-germinated during two days on agar plates (10% select Agar in distilled water) in the dark at 30 °C. Seedlings were grown in 2 L pots filled with sieved non-sterile Luvisol (Ramaekers, 2007). The soil characteristics were as follows: pH (water) 6.8, 2.81% organic matter, 24.81 mg of P_2O_5 per 100 g of soil and 33.04 mg of K_2O per 100 g of soil.

For the single inoculation, 1 ml of each culture containing 10^9 cfu ml^{-1} of *Azospirillum* or *Azotobacter* (Hamaoui et al., 2001; Rodelas et al., 1999) and 1 ml of the medium were added to the bean seeds. For the co-inoculation, 1 ml of each culture (*Rhizobium-Azospirillum* or *Rhizobium-Azotobacter*) was added. For the control treatment 2 ml of distilled water were added.

The pots were placed in a plant growth chamber at room temperature. A complete randomized block experimental design with 4 replicates was performed (see annex 1). Application of urea (at sowing) as nitrogen (N) fertilizer (60 kg ha^{-1}, following national technical brochure; García, 2006) and a control without inoculation and fertilizer were the non-inoculated treatments. Irrigation twice a week with 300 ml of water was done to keep the normal moisture of the soil in all the pots.

Plant culture, inoculation, growth conditions and evaluations in field trials

A total of 36 plots in 2005-2006 and 40 plots in 2006-2007 were performed in Luvisol soil at the Centre of Agricultural Development in Santo Domingo and Santa Clara respectively. The plot dimension was 25 m^2 (5 x 5 m each plot) (see annexes 2 and 3). The soil characteristics in Santo Domingo were as follows: pH (water) 6.9, organic matter 2.65 %, 22.11 mg of P_2O_5 and 31.01 mg of K_2O 100 g $soil^{-1}$. Santa Clara: pH (water) 6.02, organic matter 2.35 %, 18.15 mg of P_2O_5 and 27.32 mg of K_2O 27.32 100 g $soil^{-1}$. The fields were prepared by traditional ploughing 2 weeks before sowing.

Both field experiments were randomized blocks designed with 4 replicates using in the first period c.v. ICA Pijao, and using both c.v. ICA Pijao and BAT-304 in the second period. The number of blocks (from 1 to 4) was taken as a random factor.

Seeds were mixed with the appropriate amount of the humus based inoculum (as described above) for single or co-inoculation treatments, approximately one hour before sowing. The treated seeds were dried in the shadow and manually planted taking into account a plant density of around 200,000-250,000 plants per hectare. The planting distance was approximately 0.7 m (between rows) x 0.025 m (between plants). The dosis of N fertilizer (urea) in each trial was 60 kg ha^{-1} (García, 2006). The fertilizer was applied to the respective plots one day before sowing.

During the course of the experiments irrigation was performed when needed. In each experiment 4 rows were left surrounding the plots to avoid the attack of pests, diseases and animals that might damage the cultivation.

Pest and disease were controlled with available local pesticides. In the second period Folpet 50 WP (Changzhou Pangu Chemical Co., Ltd; China) and Copper sulfate (Beneut, Taiwan) with doses of 3 kg^{-1} ha and 4 kg ha^{-1} respectively were applied to control bean rust (*Uromyces phaseoli* var. typica). As insecticide, 0.5 L ha^{-1} of Karate 2.5 EC (Syngenta Crop Protection Pty, Australia) was applied.

Evaluations of pot experiment and field trials

The evaluations under the different experimental conditions are displayed in table 2.3. For the pot experiment, three time point evaluations for the growth parameters were done, starting at 7 days after sowing (DAS) and repeated every 7 days until 21 DAS, measuring the height of the plants (cm) and number of leaves per plant. At 21 DAS, plants were harvested for analysis of nodulation and root/shoot parameters. Nodule number (NN) and dry weight of nodules (NDW) were measured. As for the nodules, the root and shoot dry weight (RDW and SDW) were measured after drying in a stove (Memmert, model UM/BM 100–800) for 72 h at 65 ^{o}C.

Under field trials, twenty plants per condition (5 plants per plot, Hamaoui et al., 2001) were harvested in each trial for further analysis. In the first period, nodulation and growth parameters were measured at 30 days after sowing (DAS). The same parameters as for the pot experiment were evaluated. In the second period, the growth parameters and the yield were analyzed at 81 DAS, measuring the variability in number of pods per plant, pod weight per plant, grains per plant and the yield (grain weight per plant) in the tested genotypes.

Table 2.3 Parameters, treatments and bean genotypes evaluated under different experimental condition

Experimental conditions	Trials	Bean genotypes	Parameters evaluated										Treatments tested								
			Leaves	Height	NN	NDW	RDW	SDW	PP	PWP	GP	Yield	R	RAz	RAzI	RAp	Az	AzI	Ap	Fert	Co
Pot-controlled	1	ICA Pijao	x	x	x	x	x	x					x	x	x	x	x	x	x	x	x
		BAT-304																			
	1	ICA Pijao	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Field		BAT-304							x	x	x	x									
	2	ICA Pijao							x	x	x	x	x			x			x	x	x
		BAT-304							x	x	x	x	x			x			x	x	x

Abbreviations of parameters evaluated: NN/ number of nodules, NDW/ nodule dry weight, RDW/ root dry weight, SDW/ shoot dry weight, PP/ pods per plant, PWP/ pod weight per plant, GP/ grains per plant.

Treatments: R/ inoculation with *Rhizobium*, RAz/ co-inoculation with *Rhizobium* and *Azotobacter,* RAzI/ co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain), RAp/ co-inoculation with *Rhizobium* and *Azospirillum,* Az/ inoculation with *Azotobacter*, AzI/ inoculation with *Azotobacter* (isolated strain), Ap/ inoculation with *Azospirillum,* Fert/ mineral fertilizer, Co/ control without inoculation and fertilizer application.

Statistical analysis

All the data were processed using SAS 9.1 Entreprise Guide 4. Analysis of Variance (ANOVA) mixed model was applied with specific settings: Kenward and Roger calculation as degree of freedom method and Tukey HSD as post-hoc significance test. For the pot experiment, the complete randomized block experimental design was performed. Four replications were considered as the experimental unit and the blocks as a random factor. The data of four pot replications were used to compare significant differences between the treatments. The parametric Tukey HSD post-hoc was chosen with significance level $P<0.05$.

For the field experiments similar analyses were performed. In the first period ANOVA mixed model and statistic regression with Pearson Linear Correlation was used to correlate different plant parameters with significant level $P<0.01$ and $P<0.05$. In both randomized block experimental design, four replications were considered as the experimental unit and the blocks as a random factor. In the second period the main interaction factors considered were the treatments and the genotypes, using as post-hoc Tukey HSD with significance level $P<0.05$.

2.3 Results

2.3.1 Growth and nodulation parameters in the pot experiment

The effect of PGPR inoculation and PGPR-*Rhizobium* co-inoculation for growth parameters of *P. vulgaris* c.v. ICA Pijao were evaluated at 7, 15 and 21 days after sowing. Table 2.4, shows the effects of all the conditions analyzed with respect to plant height and number of leaves. The values in bold represent the stimulation of co-inoculation with *Rhizobium* (CIAT 899) and *Azospirillum* (Sp7) for the number of leaves. This was the only treatment with statistical difference ($P<0.05$) as compared to the controls. The height of the plants was not affected significantly in the bacterial treatments either single or co-inoculated.

Table 2.4 Effect of stimulation on plant growth parameters of *P. vulgaris* c.v. ICA Pijao under pot-controlled condition evaluated at 7, 15 and 21 days after sowing

Conditions	7 DAS		15 DAS		21 DAS	
	Height (cm)	**N° Leaves**	**Height (cm)**	**N° Leaves**	**Height (cm)**	**N° Leaves**
R	3.43 [a]	2.50 [ab]	4.00 [a]	4.25 [ab]	5.48 [a]	5.75 [ab]
RAz	3.45 [a]	2.50 [ab]	4.08 [a]	4.00 [ab]	5.58 [a]	5.00 [ab]
RAzI	5.45 [a]	3.75 [ab]	6.38 [a]	5.75 [ab]	8.43 [a]	7.75 [ab]
RAp	5.73 [a]	**4.50 [a]**	6.43 [a]	**6.25 [a]**	8.50 [a]	**8.75 [a]**
Az	4.95 [a]	3.25 [ab]	5.75 [a]	5.25 [ab]	7.88 [a]	7.25 [ab]
AzI	3.70 [a]	2.50 [ab]	4.45 [a]	4.00 [ab]	5.98 [a]	5.50 [ab]
Ap	5.15 [a]	3.50 [ab]	6.08 [a]	5.50 [ab]	8.08 [a]	7.50 [ab]
Fert	3.48 [a]	2.75 [ab]	4.53 [a]	4.25 [ab]	6.20 [a]	5.75 [ab]
Co	2.33 [a]	1.50 [b]	2.83 [a]	2.75 [b]	3.88 [a]	3.75 [b]
Std. Error	0.92	0.79	1.11	1.15	1.50	1.52

Conditions evaluated: inoculation with *Rhizobium* (R); co-inoculation with *Rhizobium* and *Azotobacter* (MB-9) (RAz); co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain) (RAzI); co-inoculation with *Rhizobium* and *Azospirillum* (RAp); inoculation with *Azotobacter* (MB-9) (Az); inoculation with *Azotobacter* (isolated strain) (AzI); inoculation with *Azospirillum* (Ap); mineral fertilizer (Fert) and control (Co). Different letters in columns differ $P<0.05$ for Tukey HSD.

Interestingly, the fertilizer treatment did not differ statistically at any of the time points analyzed with the control. The positive response observed for the RAp treatment is therefore of particular importance.

Nodule number and nodule dry weight were measured at 21 DAS. Table 2.5 and figure 2.4 show the results in nodulation parameters and the root and shoot weight of the plants respectively in the different treatments.

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Table 2.5 Nodulation parameters of ICA Pijao in pot experiment

Conditions	R	RAz	RAzI	RAp	Az	AzI	Ap	Fert	Co	Std. Error
NN	3.25 ^{abc}	3.0 ^{bc}	5.0 ^{ab}	**6.25** ^{a}	3.25 ^{ab}	3.25 ^{ab}	5.25 ^{ab}	0.0 ^{d}	1.5 ^{cd}	1.35
NDW	1.6 ^{bc}	1.8 ^{bc}	**5.0** ^{a}	**6.4** ^{a}	2.5 ^{bc}	2.7 ^{b}	**6.4** ^{a}	0.0 ^{c}	1.3 bc	0.0007

Abbreviations: NN/ nodule number; NDW (mg)/ nodule dry weight. Conditions evaluated: inoculation with *Rhizobium* (R); co-inoculation with *Rhizobium* and *Azotobacter* (MB-9) (RAz); co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain) (RAzI); co-inoculation with *Rhizobium* and *Azospirillum* (RAp); inoculation with *Azotobacter* (MB-9) (Az); inoculation with *Azotobacter* (isolated strain) (AzI); inoculation with *Azospirillum* (Ap); fertilizer (Fert) and control (Co). Different letters within a row means difference at P<0.05 for Tukey HSD.

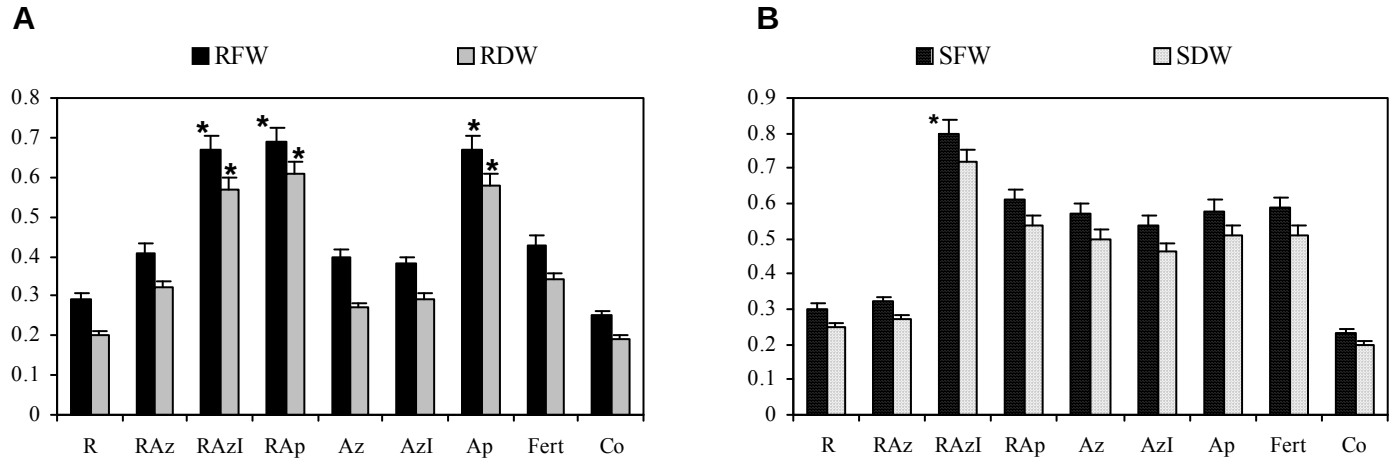

Figure 2.4 Root and shoot parameters in the pot experiment. A/ RFW: fresh weight roots (g), RDW: dry weight roots (g), B/ SFW: fresh weight shoot (g), SDW: dry weight shoot (g). Stars show the significant level P<0.05 for Tukey HSD. The conditions evaluated were R: inoculation with *Rhizobium*, RAz: co-inoculation with *Rhizobium* and *Azotobacter* (MB-9), RAzI: co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain), RAp: co-inoculation with *Rhizobium* and *Azospirillum*, Az: inoculation with *Azotobacter* (MB-9), AzI: inoculation with *Azotobacter* (isolated strain), Ap: inoculation with *Azospirillum*, Fert: fertilizer, Co: control. Stars at the top of the bars represent the best statistical result among treatments within the same parameter for Tukey HSD (P< 0.05).

The number of nodules in all the conditions evaluated is low when compared with other experiments reported (Hernandez et al, 1996, Hamaoui et al., 2001). This might be attributed to the influence of the high temperature and low organic matter of the soil (Hungria and Vargas, 2000), reducing the exchange between the legume and the microsymbiont. However, as for the parameters observed in table 2.5, the number of nodules are significantly ($P<0.05$) increased with the *Rhizobium-Azospirillum* (RAp) co-inoculation compared with *Rhizobium-Azotobacter* co-inoculation, the fertilizer and the control treatments. Dry weight of nodules shows significant stimulation in the *Rhizobium-Azotobacter* (isolated strain) and the *Rhizobium-Azospirillum* co-inoculation, and in the single inoculation with *Azospirillum*.

Surprisingly the co-inoculation of *Azotobacter* (isolated strain) with *Rhizobium* (RAzI) shows a better result than the co-inoculation with the reference *Azotobacter* strain (MB-9), although no significant differences are observed between the single inoculations. This suggests the importance of adaptation of local strains to colonize the plant roots in combination with *Rhizobium*. This result is in line with earlier reports on adaptation of local strains to the natural environment (Martínez-Romero 2003).

The nodulation parameters in the fertilizer treatment (Fert) corroborates with data reported by Caba et al. (1993) and Rodríguez et al. (2003), showing the adverse effect of nitrogen fertilization on nodule ontogeny, due to the root hair deformation, limiting the anchoring of *Rhizobium* and the consequent inhibition of the infection thread development.

Figure 2.4 (A, B) also shows the remarkable effect of co-inoculation on the root system (RFW, RDW) compared with the shoot (SFW, SDW). Both, co-inoculation of *Azospirillum* and *Azotobacter* with *Rhizobium*, as well as single inoculation of *Azospirillum* affect positively the root dry weight, while shoot fresh weight was only affected by the co-inoculation of *Rhizobium* and *Azotobacter* (isolated strain, RAzI)

2.3.2 Growth and nodulation parameters under field condition (first period, 2005-2006)

The results obtained under field conditions are similar with those reported for the pot experiment (see table 2.6 and figure 2.5). The same treatments in both trials were performed to compare the effect of PGPR single or co-inoculated with fertilizer treatment and with the control.

As for the pot experiment, the co-inoculation of *Rhizobium*-PGPR (*Azospirillum* or *Azotobacter* isolated strain) had a significant effect on nodulation and growth parameters in ICA Pijao. Table 2.6 shows the nodulation parameters under field condition, where the main positive effect is observed with the co-inoculation of *Rhizobium-Azospirillum* (RAp). The stimulation in nodule number was more pronounced in the field as compared to the pot experiment with RAp.

Dry weight of nodules is statistically increased with the *Rhizobium-Azospirillum* treatment, although without significant difference with the *Rhizobium-Azotobacter* (isolated strain) co-inoculation.

The presence of native *Rhizobium* strains is can be deduced from the nodule number and nodule dry weight in the fertilizer (Fert) and control (Co) treatments. Only the RAp treatment was able to increase the nodulation parameters as compared with those conditions without inoculation (Fert and Co). Plant responses after the other inoculated treatments (single or co-inoculated) did not differ significantly with fertilizer or control treatment. This result reinforces the clear effect of indigenous bacterial strains on root colonization and stimulation of nodulation parameters. Even in the fertilizer treatment, the native strains were able to nodulate and affect the dry weight of nodules.

Contrasting to the pot experiment, the values for root dry weight in the field trial presented in figure 2.5 A show only significant statistical difference with *Rhizobium-Azospirillum* (RAp) co-inoculation. Regarding the shoot parameter in the the pot experiment, only the SFW is affected with *Rhizobium-Azotobacter* (RAzI) combination, while for the field condition experiment, the stimulation in shoot fresh and dry weight (figure 2.5 B) is observed in *Rhizobium-Azospirillum* co-inoculation.

Table 2.6 Nodulation parameters under field conditions (first period 2005-2006)

Conditions	R	RAz	RAzI	RAp	Az	AzI	Ap	Fert	Co	Std. Error
NN	6.80 ^{b}	7.30 ^{b}	9.90 ^{ab}	**11.2** ^{a}	6.45 ^{b}	7.95 ^{ab}	8.15 ^{ab}	6.40 ^{b}	7.0 ^{b}	1.22
NDW	1.1 ^{b}	0.9 ^{b}	1.5 ^{ab}	**2.1** ^{a}	1.3 ^{b}	1.0 ^{b}	1.3 ^{b}	0.9 ^{b}	1.0 ^{b}	0.0002

Abbreviations: NN: nodules number; NDW (mg): nodule dry weight. Condition evaluated: inoculation with *Rhizobium* (R); co-inoculation with *Rhizobium* and *Azotobacter* (MB-9) (RAz); co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain) (RAzI); co-inoculation with *Rhizobium* and *Azospirillum* (RAp); inoculation with *Azotobacter* (MB-9) (Az); inoculation with *Azotobacter* (isolated strain) (AzI); inoculation with *Azospirillum* (Ap); mineral fertilization (Fert) and control (Co). Different letters within a row means difference at P<0.05 for Tukey HSD.

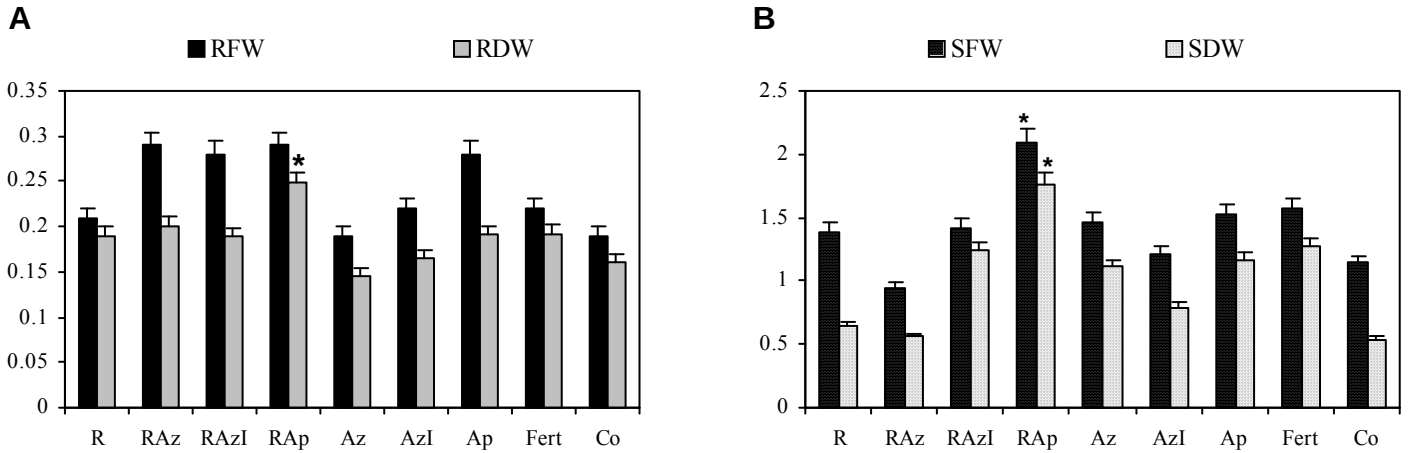

Figure 2.5 Root and shoot weight under field condition (first period 2005-2006). A/ RFW: fresh weight roots (g), RDW: dry weight roots (g), B/ SFW: fresh weight shoots (g), SDW: dry weight shoots (g). Stars show the significant level P<0.05 for Tukey HSD. The conditions evaluated were R: inoculation with *Rhizobium*, RAz: co-inoculation with *Rhizobium* and *Azotobacter* (MB-9), RAzI: co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain), RAp: co-inoculation with *Rhizobium* and *Azospirillum*, Az: inoculation with *Azotobacter* (MB-9), AzI: inoculation with *Azotobacter* (isolated strain), Ap: inoculation with *Azospirillum*, Fert: Mineral fertilization, Co: control. Stars at the top of the bars represent the best statistical result among treatments within the same parameter for Tukey HSD (P< 0.05).

To correlate the nodulation and root/shoot weight parameters, Pearson correlation was performed. Table 2.7 displays the correlation between nodulation and growth parameters. The nodule dry weight and shoot dry weight were the most significant parameters. The nodule number correlated positively with the dry weight of nodules, as well as with the shoot dry weight. Root and shoot parameters correlated positively among each other.

Table 2.7 Pearson linear correlation coefficient between nodulation and growth parameters under field condition (first period, 2005-2006).

Correlation		NN	NDW	RFW	RDW	SFW
NN	Pearson Corr.					
	Sig.					
NDW	Pearson Corr.	**0.82****				
	Sig.	0.000				
RFW	Pearson Corr.	0.07	0.12			
	Sig.	0.371	0.119			
RDW	Pearson Corr.	-0.03	0.06	0.04		
	Sig.	0.658	0.400	0.634		
SFW	Pearson Corr.	0.03	0.14	-0.12	**0.23****	
	Sig.	0.645	0.059	0.122	0.002	
SDW	Pearson Corr.	0.04	**0.16***	-0.07	**0.18***	**0.81****
	Sig.	0.581	0.038	0.364	0.019	0.000

Abbreviations NN: nodule number; NDW dry weight of nodules; RFW: fresh weight of roots; RDW: dry weight of roots; SFW: fresh weight of shoots and SDW: dry weight of shoots. Stars show the Pearson significant level: ** 0.01> P <0.05*.

2.3.3 Growth parameters, yield and variation of PGPR-Rhizobium *stimulation under field condition (second period, 2006-2007)*

The field experiment in the second period evaluated the influence of single and combined *Rhizobium* and PGPR inoculation on growth parameters and yield of two local common bean genotypes. Figure 2.6 displays the variation of the genotypes in growth parameters and the stimulation of the treatments analyzed.

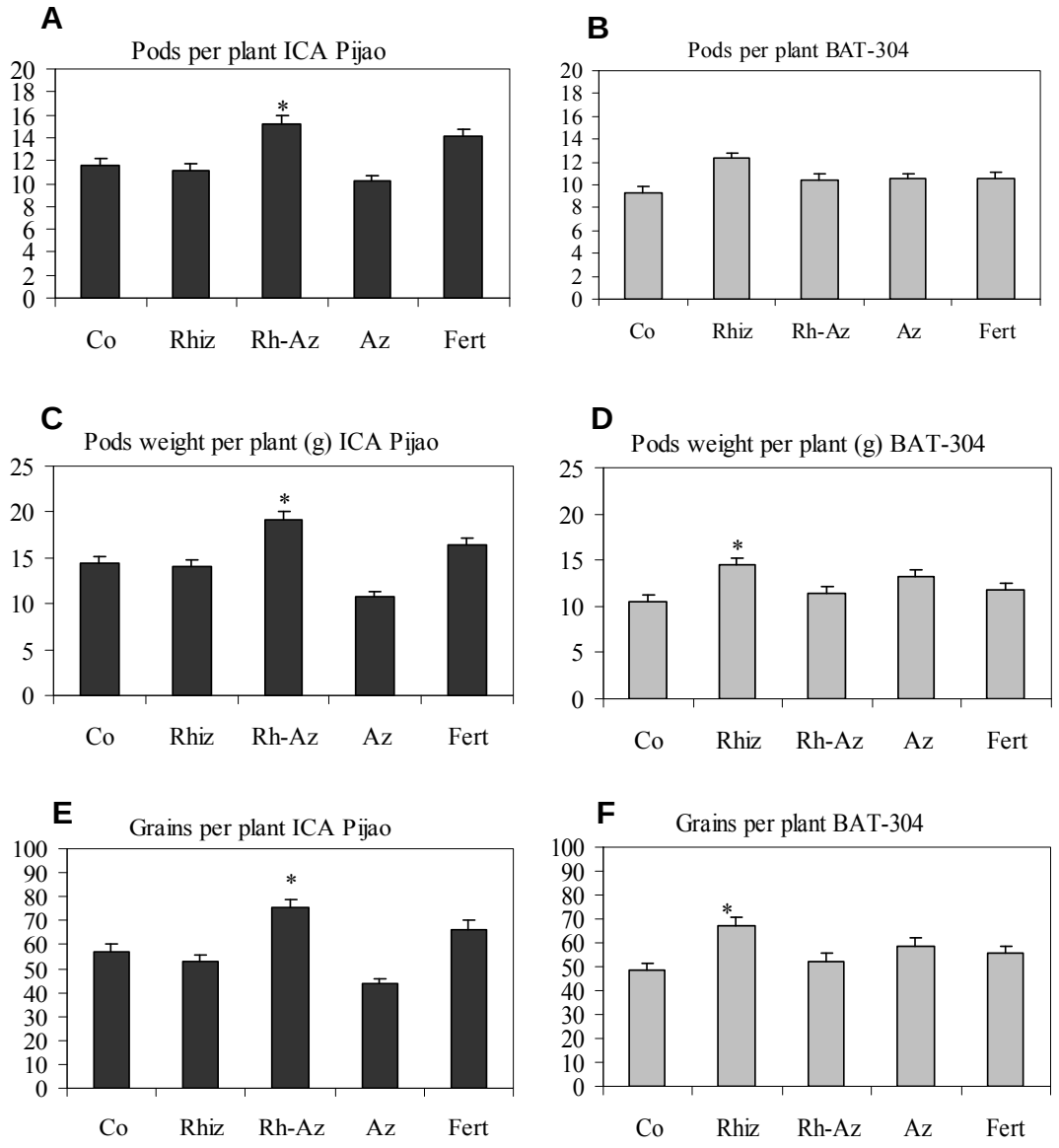

Figure 2.6 Influence of PGPR-*Rhizobium* combination compared with the control and fertilizer treatment on growth parameters under field condition (second period 2006-2007). A/ pods per plant ICA Pijao; B/ pods per plant BAT-304; C/ pod weight per plant ICA Pijao; D/ pod weight per plant BAT-304; E/ grains per plant ICA Pijao; F/ grains per plant BAT-304.. The conditions evaluated were Co: control; R: inoculation with *Rhizobium*; RAp: co-inoculation with *Rhizobium* and *Azospirillum*; Ap: inoculation with *Azospirillum*; Fert: fertilizer. Stars on top of the bars represent the best statistical result among treatments within the same parameter for Tukey HSD ($P< 0.05$).

All the parameters evaluated show the positive effect of the inoculation with *Rhizobium* alone or *Rhizobium-Azospirillum* depending on the genotype. The effect of *Rhizobium-Azospirillum* co-inoculation is striking for the ICA Pijao growth parameters with significant difference ($P<0.05$) in the number of pods per plant, pod weight per plant and grains per plant. For BAT-304, the *Rhizobium* inoculation alone has the best results, although for the number of pods per plant no significant difference was observed among the treatments for this genotype.

Results for the yield evaluation are presented in figure 2.7. For this parameter, the fertilizer treatment did not differ statistically with the co-inoculation of *Rhizobium-Azospirillum* in the case of ICA Pijao. However, taking into account previous results in the pot experiment and the first period field experiment, it can be stated that the combination of *Rhizobium-Azospirillum* affects positively the nodulation, growth parameters and yield of the ICA Pijao genotype. Such a treatment can offer an effective alternative to reduce the dependence on chemical N fertilizer. *Rhizobium* inoculation in ICA Pijao did not differ statistically with the control treatment. This might be due to the synergistic interaction between the native *Rhizobium* strains and the genotype. The inoculation with *Azospirillum* alone reduces considerably the yield in this genotype, although without significant statistical difference with *Rhizobium* and control.

Contrary to ICA Pijao, the co-inoculation of *Rhizobium-Azospirillum* has a negative influence on growth parameters and yield in BAT-304 ($P<0.05$), however, no significant difference was observed compared with the fertilizer treatment.

The yield increase for ICA Pijao with the *Rhizobium-Azospirillum* co-inoculation was rather significant having values of 13.6% increase compared to fertilizer treatment, 22.3% increase compared to control, 24.6% increase compared to *Rhizobium* treatment and 44% increase compared to *Azospirillum* treatment. For BAT-304, the yield increase observed with *Rhizobium* inoculation had values of 27.9% increase compared to control, 21.6% increase compared to *Rhizobium-Azospirillum* treatment, 10.5% increase compared to the *Azospirillum* treatment and 19.50% increase compared to the fertilizer treatment.

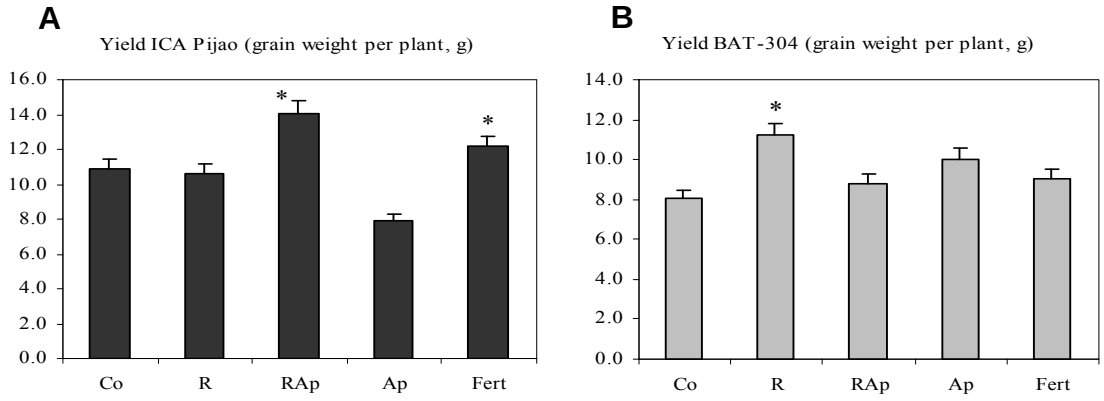

Figure 2.7 Yield analysis under field condition (second period 2006-2007). A/ ICA Pijao; B/ BAT-304. The conditions evaluated were Co: control; R: inoculation with *Rhizobium*; RAp: co-inoculation with *Rhizobium* and *Azospirillum*; Ap: inoculation with *Azospirillum*, Fert: mineral fertilizer. Stars at the top of the bars represent the best statistical result among treatments within the same parameter for Tukey HSD ($P< 0.05$).

These results evidence the variation among genotypes and treatment. As presented in figure 2.8, the control, *Rhizobium-Azospirillum* and fertilizer treatments in ICA Pijao gave positive responses in comparison with BAT-304, while *Azospirillum* inoculation alone affects significantly responses in BAT-304.

Rhizobium inoculation did not reveal statistical difference among the genotypes. This fact supports strongly the hypothesis of the PGPR stimulation on yield with the co-inoculation of *Rhizobium-Azospirillum* and the dependence of bean genotype for such treatment.

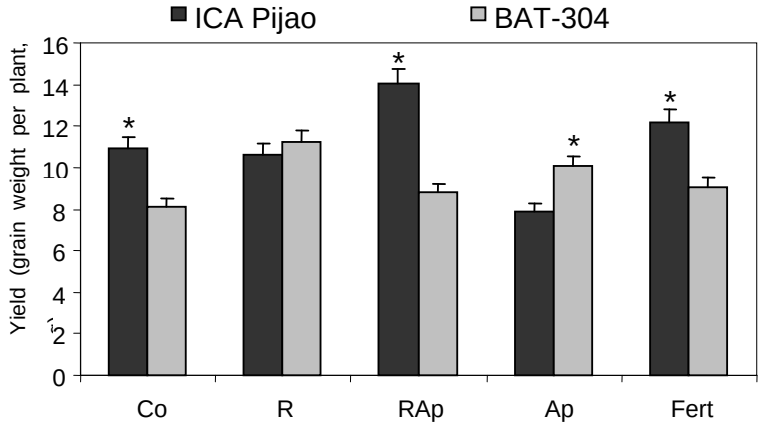

Figure 2.8 Analysis of genotypic variation among treatments under field condition (second period 2006-2007). The conditions evaluated were Co: control; R: inoculation with *Rhizobium*; RAp: co-inoculation with *Rhizobium* and *Azospirillum*; Ap: inoculation with *Azospirillum*, Fert: mineral fertilizer. Stars at the top of the bars represent the best statistical result among genotypes for Tukey HSD ($P < 0.05$).

2.4 Discussion

In this chapter the influence of PGPR-*Rhizobium* co-inoculation under different growth conditions of common bean, using local genotypes commonly used by farmers in the central region of Cuba, was studied

Results of a pot experiment using the local ICA Pijao genotype show an increase in nodulation and plant growth parameters by means of *Rhizobium-Azospirillum, Rhizobium-Azotobacter* co-inoculation and the single inoculation with *Azospirillum*. Over the past 10 years several studies on the beneficial effect of co-inoculation and single inoculation of PGPR have been reported (Okon and Vanderleyden, 1997 Pan et al., 1999; Rodelas et al. 1999; Bin et al., 2000; Biswas et al., 2000; Asghar et al., 2002; Vessey, 2003; Silva et al., 2006; Remans

et al., 2007a; Figueiredo et al., 2007). In this respect common bean has received much attention. It is often reported as a poor pulse for N fixation as compared with other grain legumes.

The increase in dry matter production, as observed in the pot experiment and field condition (figures 2.4 and 2.5) and nitrogen content of co-inoculated plants (as reported by others) might be attributed to early nodulation (Iruthayathas et al., 1983; Plazinski and Rolfe, 1985; Burdman, et al. 1997), increased number of nodules (Yahalom et al., 1987; Itzigsohn et al., 1993; Okon and Vanderleyden 1997), higher N_2-fixation rates and a general improvement of root development (Sarig et al., 1986; Huang et al. 2004; Safronova et al., 2006).

Rodelas et al. (1999) reported for faba bean that responses to *Azotobacter* and *Azospirillum* inoculation in combination with *Rhizobium* led to changes in total content and/or distribution of macro- and micronutrients (K, P, Ca, Mg, Fe, B, Mn, Zn and Cu) when compared with plants inoculated with *Rhizobium* alone. Mineral nutrient deficiencies are a major constraint limiting N fixation and yield.

In the first field trial, the co-incoulation treatments were the only ones with statistical difference in root dry weight, shoot fresh and dry weight. The dry matter responses observed with the *Rhizobium-Azospirillum* and the *Rhizobium-Azotobacter* (isolated strain) co-inoculation might be related to the overall observation that a high proportion (90%) of N compounds are transferred through the root nodules to the shoot and thus the rest of the plant (Groppa et al., 1998). The observed correlation of the nodulation and growth parameters reported here support this further, showing a significant linear correlation ($0.01>P<0.05$) between the nodule dry weight, nodule number and shoot dry weight.

The single *Rhizobium* inoculation to improve the nodulation and growth parameters was not effective. In controlled and field condition no significant difference with the control, fertilizer, *Azospirillum* and *Azotobacter* treatment was observed for nodulation and growth parameters. Poor nodulation by inoculated *Rhizobium* strains has been described in literature, particularly for *Phaseolus vulgaris* (reviewed by Giller, 2001; Broughton et al., 2003). Soils used for bean cultivation often contain large numbers ($> 10^3$ cfu g^{-1} soil) of compatible rhizobia (Giller, 2001). Absence of compatible rhizobia is particularly unlikely in the case of *P. vulgaris* due to its promiscuity of nodulation (Michiels et al., 1998). The wide range of rhizobia able to infect

P. vulgaris increases the likelihood that nodulation may occur with indigenous strains that are ineffective or poorly effective in N_2 fixation and limits the effect of inoculation.

Nodulation in in the post experiment and field conditions (tables 2.5 and 2.6) shows the ability of native *Rhizobium* strains (control and fertilizer treatments) to colonize the bean roots and to affect the nodule dry weight, as no significant differences with *Rhizobium* inoculated alone or even with the co-inoculation of *Rhizobium-Azotobacter* (MB-9 and isolated strain) were observed. Analysis of indigenous *Rhizobium* populations and research on the competitivity of rhizobial strains in the bean rhizosphere would contribute to clarify the particular reasons why the effect of inoculation was poor in some of the settings (for more details see chapters 3 and 4).

The positive effect of *Azotobacter* and *Azospirillum* inoculation alone was similarly observed in the pot experiment and the field conditions. In co-inoculation, the response is most obvious for *Azospirillum* application. Over the years, *Azospirillum* inoculation showed to have potential to increase plant growth and yield significantly in legumes, ranging from 5% to 30% increase (Bashan and Holguin, 1997). Their plant growth-promoting capacity is mainly linked to the production of phytohormones, including indole-3-acetic acid (Steenhoudt and Vanderleyden, 2000; Spaepen et al., 2007), the increase in flavonoids exudation, which are crucial plant signal molecules in the *Rhizobium*-legume symbiosis (Volping et al., 1996; Burdman, et al., 1996), the stimulation of epidermal cell formation and the formation of additional infection sites in the root hairs that are later occupied by rhizobia (Tchebotar et al. 1998) and thereby increasing the occupancy of introduced *Rhizobium* strains in the nodules (Thilak et al., 2006).

The comparison of PGPR-*Rhizobium* inoculation versus the fertilizer application revealed the positive effect of the inoculant strategy as an alternative for N fertilizer. In the first field trial, co-inoculation treatments gave the best results, with statistical significant difference compared to the other treatments. The second field trial showed the same result for all the growth parameters, although for the ICA Pijao genotype no statistical differences were observed for yield between *Rhizobium-Azospirillum* co-inoculation and the fertilizer application. For BAT-304, inoculation with *Rhizobium* alone gave the best result, statistically significant with all other treatments evaluated, including the fertilizer application.

The variability of responses among genotypes shown in figure 2.8 is an interesting issue extensively addressed during the last decade, especially for the symbiotic interaction (Riely et al., 2004; Oldroyd et al., 2005; Stacey et al., 2006). Variation among cultivars for efficacy in interactions between plants and beneficial bacteria has been described and suggests natural genetic host variation for these interactions within germplasm.

The results outlined in this study demonstrate that the use of alternatives (including PGPR inoculation) for chemical fertilizers plays a particular role in Cuban agriculture (Gersper et al., 1993). However, more research including more genotypes and PGPR-*Rhizobium* combinations should be conducted to elucidate the variation among genotypes and conditions and to evaluate other physiological, morphological and genetic parameters.

Chapter 3

Morphological and genetic characterization of bacteria in Cuban agricultural soils

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Abstract

A collection of 32 rhizobacteria isolated from Cuban agricultural soils with bean planting history in intercropping with sorghum, were morphologically and genetically characterized in this study. Samples from common bean (*Phaseolus vulgaris* L.) nodules, soil and sorghum roots (*Sorghum bicolor* (L.) Moench) were analyzed to determine the biodiversity of diazotrophic and rhizosphere bacteria in an agricultural Cuban system. The morphological analysis demonstrated several groups of isolates with differences in growth type, color, polysaccharide production and border of the colonies. Genetic characterization using 16S rDNA revealed 8 groups of bacteria belonging to the genera: *Agrobacterium, Rhizobium, Ochrobactrum, Sphingomonas, Stenotrophomonas, Bacillus, Brevibacillus* and *Paenibacillus.* 47% of the sequences matched for 100% sequences in the EMBL database, while 53% of the sequences scored above 99% of identity. In nodule samples 37.5% of the isolates were 100% similar to *Agrobacterium tumefaciens* or *Rhizobium* species. Two species of *Rhizobium* isolated (*R. etli* and *R. tropici*) were detected in nodule samples. In nodulation tests, *Agrobacterium* isolates were unable to nodulate the original host. No statistical difference was observed for nodulation between the *Rhizobium* isolates and the *R. etli* reference strain. The results presented in this study are of importance to determine the interspecies microbial relationships in the rhizosphere, possibly increasing our understanding on biotic factors interfering with the *Rhizobium*-legume symbiosis and as a source of inoculant strains for local environmental conditions.

3.1 Introduction

Microbial biodiversity, the key to unravel synergistic processes for low input systems.

In subsistence and low input agricultural systems, crop yields are directly dependent on the inherent soil fertility and on microbial processes that govern the mineralization and mobilization of nutrients required for plant growth. Furthermore, the impact of different crop species that are used in various combinations is likely to be an important factor in determining the structure of plant beneficial microbial communities that function in nutrient cycling, the production of plant growth hormones, and suppression of diseases (Giller, 2001).

Because different plant species release different types and quantities of exudates, plants exert species-specific effects on the soil microbial community that result in broad shifts in the microflora (Lynch, 1990). Although not well investigated, it can be hypothesized that, as a sequence of plant species are grown in a given soil, the predominant bacteria associated with the previous crop species will exert at least some temporary influence on the rhizosphere bacterial communities of the subsequent crop species, particularly during early growth (Alvey et al., 2003). In practice, crop rotations have been explicitly used to disrupt disease cycles (Curl, 1963), or in the case of legumes to fix atmospheric N_2 for the subsequent non-leguminous crop (Baldock et al., 1981; Pierce and Rice, 1988).

To date, only limited information exists on microbial diversity and population dynamics in agricultural soils (Dunbar et al., 2000; Smit et al., 2001). The study of the microbial diversity in agricultural soils, besides providing valuable ecological information by defining host preferences and predominance of strains, knowledge on the genetic relationships and structure of bacteria, insight in the dynamics of exchange of genetic material, is also a possible source for the selection of efficient strains to be used in inoculation trials in agricultural fields.

To enhance legume nodulation and N_2 fixation, the introduction of bacterial inoculants to agricultural fields has been a common practice for over 100 years. Whenever the specific rhizobia are absent, inoculation readily enhances plant growth and yield (Singleton and Tavares, 1986; Streeter, 1994; Vlassak and Vanderleyden, 1997). On the other hand, when native bacteria are present in the field, as observed in chapter 2, they often out-compete the inoculant strains that only occupy a small proportion of nodules as observed in some legumes plants in Latin America (Graham, 1981; Ramos and Boddey, 1987; review of Vlassak and Vanderleyden, 1997; Burgos et al., 1999; Aguilar et al., 2001).

In recent years, microbial taxonomy and specifically the classification of rhizobia that nodulate common bean (*Phaseolus vulgaris* L.*)* has been progressively revised as more rhizobial diversity is gradually discovered in different parts of the world. *P. vulgaris* is reported to originate from America (Gepts, 1990); however, this plant is known to be a relatively permissive host whose symbiotic rhizobial partners are diverse and widely spread. A broad range of *Rhizobium* species are reported to effectively nodulate *P. vulgaris* (see table 3.1). *P vulgaris* has been recognized as a promiscuous host (Bromfield and Barran, 1990; Michiels et al., 1998) like other species in the *Phaseoleae* such as *Macroptilum* (Bromfield and Barran, 1990) and *Vigna* species (Pueppke and Broughton, 1999).

Although a great deal of knowledge has been amassed concerning the diversity and genetics of bean symbionts, the basis of a successful inoculation and efficient nitrogen fixation remains elusive, as well as the influence of other rhizobacteria on nodulation and nitrogen fixation (see chapter 1). Some of the problems of bean nodulation and symbiotic nitrogen fixation detected over 20 years ago (Graham, 1981) still exist today (Martínez-Romero, 2003). Programmes to enhance bean BNF may benefit from studies on *Rhizobium* diversity, bean symbiosis genetics, environmental factors and microbial relationships in the rhizosphere (Martínez-Romero, 2003; Cooper, 2007; Muresu et al., 2008).

This chapter aims the characterization of bacteria isolated form Cuban soils. Several bacteria isolated from soil, roots of plants grown in intercroping with common bean, and bean nodules, have been morphologically and genetically characterized using 16S rDNA in order to gain a better understanding of the common bean microflora biodiversity. There are no previous reports on the genetic characterization of microorganisms and especially *Rhizobium* strains isolated from the common bean rhizosphere in Cuba. This study was undertaken to possibly identify new competitive native strains to be used as inoculant strains and thereby to increase the nitrogen fixation in selected bean ecosystems.

3.2 Materials and methods

Soil and plant collection

During the first field trial analyzed in chapter 2, samples of soil, plants cultivated in intercropping with common bean, and bean plants were taken for further analysis of the microbial population.

A total of 20 samples of Luvisol soil (Ramaekers, 2007) from the central region of Santa Clara, Cuba (data shown in chapter 2) were taken from the 0-15 cm layer and combined to represent one sample. Two grams of mixed sample were added to sterile distilled water to perform the successive dilutions from 10^{-2} to 10^{-6}. Ten samples from *Sorghum bicolor* and *Phaseolus vulgaris* L. were collected from the field 30 days after sowing. In both cases the roots system were adequate and for bean the presence of nodules was verified.

Sample preparation, culture conditions and isolation of bacterial colonies

Soil dilutions were performed as described by Jensen (1962). One ml aliquot of 10^{-6} dilution was used to transfer to plates containing nutrient agar (NA, 15 g agar L^{-1}). Plates were incubated at room temperature for 7 days.

Root segments from sorghum and bean bearing nodules were washed under running water, then surface-sterilized by immersion in 90% ethanol for 1 minute, followed by 3% sodium hypochlorite for 3 min, and finally washed ten times with sterile distilled water. Bean nodules were also surface-sterilized by immersion in 0.1% $HgCl_2$ for 2 minutes.

Sterile sorghum roots were crushed in 1 ml sterile distilled water. Bean nodules were carefully excised from the roots with a flamed-sterile scalpel. A total of 20 nodules were randomly collected and crushed in 1 ml of sterile distilled water. Both, bacterial suspension from sorghum roots and bean nodules were streaked on NA medium plates and incubated for 7 days at room temperature. All the colonies obtained from soil, sorghum or nodule samples were purified by repeated streaking (Vincent, 1970). To confirm the purity of *Rhizobium* isolates, the colonies were streaked on yeast-mannitol agar (YMA) plates supplemented with 0.025 g L^{-1} of Congo red and YMA supplemented with 0.1 g L^{-1} of bromothymol blue (Somasegaran and Hoben, 1994).

Morphological characterization

A total of 32 different colonies isolated were randomly taken from the cultures (7 from root nodules, 6 from soil samples and 19 from sorghum roots), analyzed by Gram staining and morphological visual observation, including color, growth, mucous appearance, transparency, border type and elevation of the colonies. Pure cultures were stored at -20^{o}C in 50% glycerol-YMA or NA medium.

Genetic characterization

The genetic characterization of the isolated strains was performed in the Laboratory of Microbiology at Ghent University under supervision of Prof. Ann Willems.

DNA extraction

DNA from the colonies isolated was extracted by alkaline lysis (Vanparys et al., 2007). One or two colonies per isolate were suspended in 20 µl of lysis buffer (2.5 µl 10% SDS; 5 µl 1 M NaOH; 92.5 µl MilliQ water), centrifuged for 5 min at 13000 rpm. The supernatant were transferred to glass tubes and placed at 95 °C for 15 min. Subsequently, 180 µl MilliQ water was added, the tubes were centrifuged for 5 min at 13000 rpm and the supernatant were transferred to new glass tubes. DNA extracts were stored at -20 °C until use.

PCR amplification of the 16S rRNA gene

The 16S rRNA genes were amplified with the conserved primers: 5'CTGGCTCAGGAC/TGAACGCTG3' (ARI C/T) and 5'AAGGAGGTGATCCAGCCGCA3' (pH), which amplify almost the full length of the gene (1500 bp) corresponding to the 16S rDNA (Logan et al., 2000). Each 50 µl amplification reaction contained: 5 µl dNTPs (2 mM of each), 5 µl GeneAmp 10X-PCR buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15mM $MgCl_2$, 0.01% (w/v) gelatin), 1 µl of each primer (50 ng μl^{-1}), 1 µl AmpliTaq DNA polymerase (1 U μl^{-1}), 34.5 µl MilliQ water and 2.5 µl of template (DNA extract).

The following temperature cycle sequence was used: 5 min at 95 °C to denature the DNA, 3 amplification cycles (45 sec at 94 °C, 2 min at 55 °C, 1 min at 72 °C), 30 amplification cycles (20 sec at 94 °C, 1 min at 55 °C, 1 min at 72 °C) and 5 min at 72 °C for final primer extension. All PCR-products were analyzed by electrophoresis in 1% agarose gel (1 g agarose in 100 ml TAE buffer1X) at 80V during 45 minutes.

The PCR-amplified 16S rDNA gene products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and analyzed afterwards by electrophoresis in a 1% agarose gel.

For each sequence reaction a mixture was made of 1 µl purified PCR product, 0.5 µl of the Big Dye™ Termination Ready Reaction Mix (Applied Biosystems), 3.75 µl sterile MilliQ water and 3 µl (20 ng/µl) of one of the 8 sequencing primers used (forward primer, position

339-358, 5'CTCCTACGGGAGGCAGCAGT3'; 519–536, 5'CAGCAGCCGCGGTAATAC3'; 908-926, 5'AACTCAAAGGAATTGACGG3'; 1093-1112, 5'AGTCCCGCAACGAGCGCAAC3'; reverse primers, position 358-339, 5'ACTGCTGCCTCCCGTAGGAG3'; 536-519, 5'GTATTACCGCGGCTGCTG3'; 1112-1093, 5'GTTGCGCTCGTTGCGGGACT3' and 1241-1222, 5'GCTACACACGTGCTACAATG3').

The thermal program consisted of 30 cycles (15 sec at 96 °C, 1 sec at 35 °C and 4 min at 60 °C). Sequence analysis was performed using an Applied Biosystems 3100 DNA Sequencer following the protocols of the manufacturer (Perkin-Elmer). Sequence assembly was performed with BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). The closest related sequences were found using the FASTA program (Pearson, 1994). The sequences of strains with strong resemblance to the consensus sequences of the different isolates were retrieved from the EMBL database and aligned. Sequences also were compared with EMBL Database.

Nodulation analysis

Bacterial strains, growth conditions and inoculum preparation

The strains used in the study are listed in table 3.1. *Rhizobium etli* CNPAF512 (reference wild-type) and the characterized isolated strains (see below) were grown overnight in liquid tryptone-yeast extract (TY) medium supplemented with 0.7 M $CaCl_2$ at 30^{o}C or maintained on yeast extract-mannitol (YEM) (Vincent 1970) agar plates (15 g agar l^{-1}). Cells were washed twice with 10 mM $MgSO_4$ and resuspended in 10 mM $MgSO_4$ at a density of 10^7 cfu ml^{-1}. From the five *Agrobacterium tumefaciens* strains isolated from bean nodules only two were analyzed (R35030 and R35031). R35031 and R35032 have the same accession number and both matched 100% of similarity with EMBL database. R35027 and R35044 have the same accession number than R35030.

Table 3.1 Bacterial strains used in the nodulation analysis

Bacterial strains	Relevant characteristics	Reference
Rhizobium etli CNPAF512	Wild-type reference strain, isolated from *Phaseolus vul*garis nodules, Brazil	Michiels et al., (1998)
Rhizobium etli RL-1 (CP000133)	Strain isolated from *Phaseolus vulgaris* nodules, Cuba	*This work (Segovia et al., 1993)
Rhizobium tropici RL-2	Strain isolated from *Phaseolus vulgaris* nodules, Cuba	*This work (Martínez-Romero 1991).
Rhizobium etli RL-5 (EF054889)	Strain isolated from *Phaseolus vulgaris* nodules, Cuba	*This work (Segovia et al., 1993)
Agrobacterium tumefaciens R35030 (EF620435)	Strain isolated from *Phaseolus vulgaris* nodules, Cuba	*This work (Castaldini et al., 2007)
Agrobacterium tumefaciens R35031 (AY568505)	Strain isolated from *Phaseolus vulgaris* nodules, Cuba	*This work (La Duc and Venkateswaran 2007)

* reference of the closet FASTA hit obtained in the genetic characterization using 16S rDNA.

Plant material, inoculation, growth conditions and evaluation

Seeds of bean cv. ICA Pijao were surface-sterilized as described previously (Vlassak et al. 1998) and pre-germinated during two days on water agar (15 g agar L^{-1}) in the dark at 30^{o}C. One pre-germinated seedling was planted per square Petri dish (12x12 cm) containing 50 ml of Snoeck medium (Snoeck et al., 2001). The seedlings were inoculated with 100 µl inoculum (prepared as described above) containing 10^7 *Rhizobium* or *Agrobacterium* cells. The number of cells used for inoculation is based on previous research described by Hamaoui et al. (2001) and Bai et al. (2002).

Bean plants were grown in a Sanyo Gallenkamp Fytotron plant growth chamber with a 12-h photoperiod (day/night temperature, 22°C/18°C; day/night relative humidity, 65%/75%) (Michiels et al., 1998). Complete randomized block experimental design was performed with 10 plant replicates. After 2 weeks of inoculation the number of nodules were measured for every condition.

Statistical analysis

The number of nodules was processed using SAS 9.1 Entreprise Guide 4. Analysis of Variance (ANOVA) and mixed model was applied with specific settings: Kenward and Roger calculation as degree of freedom method. The parametric Tukey HSD post-hoc was chosen

with significance level P<0.05. The nodulation analysis was performed twice with similar results.

3.3 Results and discussion

3.3.1 Morphological characterization of isolated strains

In total 32 isolated colonies were randomly selected and analyzed in this study. Table 3.2 shows the morphological characteristics of the selected isolates from soil, sorghum roots and bean nodules. The principal characteristics taken into account for the random selection were: growth, color, slime production in NA or YMA medium, borders and elevation of the colonies.

The Gram reaction was determined by the classical staining procedure as described by Süssmuth et al., (1987). About 53% of strains are Gram negative, while 47% are Gram positive bacteria. The Gram negative or positive staining appears correlated with sample origin. All the isolates from nodules were Gram negative, although not all of them belong to *Rhizobium*. From the sorghum roots 6 isolates were Gram negative and 13 were Gram positive, while for soil samples only 2 isolates were Gram positive and 4 were Gram negative.

Table 3.2 Morphological characteristics of isolated bacteria

Reference number	Sample isolation			Morphological parameters					
	S	R	N	Gram	Growth[a]	Color[b]	Slimy[c]	Borders[d]	Elevation[e]
R35027		x		-	++	2	++	+	++
R35028		x		-	+	3*	+	+	+
R35030			x	-	+++	3	+++	+	+
R35031			x	-	+++	3	++	+	++
R35032			x	-	++	3	+++	+	++
R35033			x	-	+	3**	++	+	+
R35034	x			-	+	3**	-	+	+
R35037	x			-	++	3**	+	+	+
R35038	x			-	+++	3**	++	+	+
R35039		x		+	++	2	-	+	+
R35040		x		+	++	3**	-	+	++

Table 3.2. Continued

Reference number	Sample isolation			Morphological parameters					
	S	R	N	Gram	Growth[a]	Color[b]	Slimy[c]	Borders[d]	Elevation[e]
R35041		x		+	+++	3**	-	+	+
R35042		x		+	++	3*	-	+	+
R35043		x		+	++	2	-	+	+
R35044		x		-	++	3	++	+	++
R35045		x		-	+	3*	+	+	+
R35046		x		-	++	3**	++	+	+
R35047		x		+	+++	3*	-	++	+
R35048		x		+	+++	3	-	++	+
R35049		x		+	+++	3**	+	+	+
R35052	x			+	++	3*	+	+	+
R35053		x		+	++	1	+	++	+
R35054		x		+	++	2	-	++	+
R35055		x		-	++	3	+	+	+
R35056		x		+	+++	1	+	++	+
R35057		x		+	+++	3**	+	++	+
R35137		x		+	+++	2	-	++	+
RL-1			x	-	++	3	+++	+	++
RL-2			x	-	+++	3*	++	+	++
RL-5			x	-	+++	3*	++	+	+

a/ growth: (-) none, (+) slight, (++) moderated; (+++) abundant; b/ color: (1) transparent (2) translucent, (3) opaque, (3*) white opaque, (3**) yellow opaque; c/ slimy: (-) none, (+) slight, (++) moderated, (+++) abundant; d/ borders: (+) regular, (++) irregular e/ elevation: (+) flat, (++) raised.

All of the isolated strains grew fast in NA and YMA medium. The morphology of isolates from nodules shared characteristics with *Rhizobium* strains (Martinez-Romero et al., 1991; Segovia et al. 1993) and most of the Gram positive isolates from sorghum roots and soil sample matched similarities with previous reports (Dunne et al. 1997; Donate-Correa et al., 2004; Bai et al., 2003; Cakmakci et al., 2007). Color, slime production and elevation were variable, mainly dependent on the age of the culture. Most of the Gram positive isolates became darker from 24 to 72 h growing in NA medium and the isolates from nodules increased slime production with time of growth in YMA.

3.3.2 Characterization of bacterial isolates by 16S rDNA sequence analysis

Table 3.3 gives an overview of the identification results based on the complete sequencing of 16S rDNA. Eight different taxa were recovered. As described in the table, most of the sequences showed from 99 to 100% of similarity with sequences in the EMBL database and from the entire lineages, which indicates the confidence of the characterization at genus level and often at level species. According to Stackebrandt and Goebel (1994), two strains that show 16S rDNA sequence homologies of 97.5% or lower, will have less than 60 to 70% DNA similarity and therefore do not belong to the same species. However, not all strains that have more than 97.5% sequence similarity belong to the same species

Strains from sorghum root samples represent the most microbial diversity and most of them showed 100% similarity with entries in the EMBL database. Genera observed include *Agrobacterium*, *Sphingomonas, Bacillus, Brevibacillus* and *Paenibacillus* (see Table 3.3).

3.3.2.1 Agrobacterium *in bean nodules*

The presence of *Agrobacterium* strains is a quite interesting result. This genus is present in nodules and sorghum roots, but surprisingly, was not recovered in the soil sample. Around 80% of the characterized *Agrobacterium* strains have 100% similarity with retrieved *Agrobacterium* sequences, one of them isolated from sorghum root and three from bean nodules.

Nodules can be colonized internally by several bacterial genera unrelated to rhizobial strains. *Agrobacterium* spp. have been reported in nodules of tropical legumes (De Lajudie et al., 1999). In bean nodules, Mhamdi et al. (2005) identified along with *Rhizobium*,

Table 3.3 Characterization of the bacterial isolates by 16S rDNA sequence analysis

Reference number	Groups of Characterization	Closest FASTA hit	Sample isolated form[a]			% sequence identity	Accession n°	Reference
			S	R	N			
R35027	*1. Agrobacterium*	*Agrobacterium tumefaciens*		x		100	EF620435	Castaldini et al. (2007)
R35030		*Agrobacterium tumefaciens*			x	100	EF620435	Castaldini et al. (2007)
R35031		*Agrobacterium tumefaciens*			x	100	AY568505	La Duc et al. (2007)
R35032		*Agrobacterium tumefaciens*			x	100	AY568505	La Duc et al. (2007)
R35044		*Agrobacterium tumefaciens*		x		99.7	EF620435	Castaldini et al. (2007)
RL-1	*2. Rhizobium*	*Rhizobium etli*			x	99	CP000133	Segovia et al. (1993)
RL-5		*Rhizobium etli*			x	100	CP000133	Segovia et al. (1993)
RL-2		*Rhizobium tropici*			x	100	EF054889	Martínez-Romero (1991)
R35038	*3. Ochrobactrum*	*Ochrobactrum cytisi*	x			100	AM411072	Zurdo-Piñeiro et al., (2007)
R35028	*4. Sphingomonas*	*Sphingomonas yanoikuyae*		x		100	AY574367	Dalton et al. (2004)
R35033		*Sphingomonas yanoikuyae*			x	100	AF509480	Farias et al., (2002)
R35036		*Sphingomonas yanoikuyae*		x		100	EF061133	Hong et al. (2006)
R35045		*Sphingomonas yanoikuyae*	x			100	AY574367	Dalton et al. (2004)
R35046		*Sphingomonas yanoikuyae*		x		100	AY574367	Dalton et al. (2004)
R35055		*Sphingomonas yanoikuyae*		x		100	AF509480	Farias et al. (2002)
R35034	*5. Stenotrophomonas*	*Stenotrophomonas maltophilia*	x			100	EF695449	Selvam and Raja (2007)
R35037		*Stenotrophomonas maltophilia*	x			98.7	AB294557	Tanaka et al. (2007)
R35039	*6. Bacillus*	*Bacillus* sp.		x		99.6	EF471917	Sadfi-Zouaoui et al. (2008)
R35040		*Bacillus* sp.		x		99.8	EF471917	Sadfi-Zouaoui et al. (2008)
R35041		*Bacillus* sp.		x		99.8	EF471917	Sadfi-Zouaoui et al. (2008)

Table 3.3 Continued

Reference number	Groups of Characterization	Closest FASTA hit	Sample isolated form[a]			% sequence identity	Accession n°	Reference
			S	R	N			
R35042		*Bacillus* sp.		x		99.6	EF471917	Sadfi-Zouaoui et al. (2008)
R35035		*Bacillus subtilis*	x			100	AF260750	Peppiatt and Burgess (2000)
R35137		*Bacillus licheniformis*		x		99	AB354236	Miyashita (2007a)
R35047		*Bacillus licheniformis*		x		99.5	AB354236	Miyashita (2007a)
R35049		*Bacillus licheniformis*		x		98.6	EF059752	Nayaka and Vidyasagar (2006)
R35048		*Bacillus flexus*		x		99.7	AM778192	Kuhad (2007)
R35043		*Bacillus pumilis*		x		99.8	AB354235	Miyashita (2007b)
R35052	*7. Brevibacillus*	*Brevibacillus formosus*	x			99.1	EF690427	Chang et al. (2007)
R35054	*8. Paenibacillus*	*Paenibacillus lautus*		x		99.6	AB073188	Goto et al. (2001)
R35053		*Paenibacillus lautus*		x		99.8	AB073188	Goto et al. (2001)
R35057		*Paenibacillus lautus*		x		99.6	AB073188	Goto et al. (2001)
R35056		*Paenibacillus ginsengisoli*		x		99.6	AB245383	Im and Lee (2005)

a: represent the different sites of isolation. S/ isolated from soil, R/ isolated from sorghum roots and N/ isolated from bean nodules

Agrobacterium-like bacteria, and proved that these could invade new nodules upon co-inoculation with rhizobia and affect their nodulation performance (Mrabet et al., 2006). This might possibly be related to the lack of response of legumes to *Rhizobium* inoculation in tropical conditions, as observed in previous studies and in our study described in chapter 2.

It is well known that *Agrobacterium* spp. share several characteristics and are genetically closely related to some rhizobial species (*R. tropici*, *Rhizobium* genomic species Q, *R. galegae*, *R. huautlense*, and *Allorhizobium undicola*) (Martínez-Romero, et al., 1991; Zakhia and De Lajudie, 2001). Consequently, based on 16S rDNA gene sequences, agrobacteria were recently reclassified into the genus *Rhizobium* (Young et al., 2001). N_2-fixing rhizobia resembling agrobacteria were isolated from root nodules of *Acacia* spp. and common bean (Mhamdi et al., 1999) in Africa, but the isolates were not able to maintain the symbiotic effectiveness. Their presence in nodules, according to Mhamdi et al. (2002), could be explained either by a mixed infection with rhizobia or by the acquisition of a symbiotic plasmid by the *Agrobacterium* which might be highly unstable and lost during the isolation and preservation processes.

3.3.2.2 Diversity of Rhizobium *species in nodule samples*

Two groups of isolates (2 and 3) are related with symbiotic bacteria from the genus *Rhizobium* and *Ochrobactum*, however, *Ochrobactrum cytisi* (AM411072) is only observed in soil and not in bean nodules samples. *Ochrobactrum cytisi* is a new rhizobial genus isolated from *Cytisus scoparius* in Spanish soil **(**Zurdo-Piñeiro et al., 2007). It contains *nodD* and *nifH* genes on megaplasmids that are related phylogenetically to those of rhizobial strains nodulating *Phaseolus*, *Leucaena*, *Trifolium* and *Lupinus*.

The *Rhizobium* strains isolated from the bean nodules revealed a close match with the accessions CP000133 and EF054889 representing *Rhizobium etli* and *Rhizobium tropici* respectively, giving 100% of similarity with EMBL database sequences.

In chapter 2 we observed the presence of effective native rhizobia strains in the soil used in the pot experiment and the field trials. The result obtained in this chapter with the genetic characterization of isolates in fact could explain the low response to *Rhizobium* inoculation, due to the competition for infection sites by several indigenous *Rhizobium* species, like *R. etli* and *R. tropici*.

The distribution of rhizobia that nodulate *P. vulgaris* varies among geographical locations (Laguerre et al., 2001), although *R. etli* and *R. tropici* appear to be distributed worldwide. In the three centers of bean domestication (Mexico, Ecuador-Peru and Argentina), *R. etli* bv. *phaseoli* has been found as the predominant nodule occupant and no *R. tropici* strains have been isolated from bean nodules (Martinez-Romero, 2003). Outside of their sites of origin, where *P. vulgaris* has been introduced, it seems that in some of the introduced sites bean is nodulated by other species in addition to *R. etli* bv. *phaseoli* and the co-occurrence of several species is common. Like in this study, in Brazil (Hungria et al., 2000; Martínez-Romero et al., 1991), Senegal and Gambia (Diouf et al., 2000), *R. tropici* and *R. etli* bv. *phaseoli* have been found as bean nodule occupants.

Even though common bean is a promiscuous legume, it seems to have some degree of preference for certain rhizobia (Pacovsky et al., 1984). It has been considered that the low effectivity in bean-*Rhizobium* symbiosis frequently observed may be due to the miss-pairing of host plant and bacteria (Bernal and Graham, 2001). Ecuatorian and Mexican beans, when used as traps, selected different *R. etli* strains both from Ecuatorian and Mexican soils. The efficiency of nodulation and nitrogen fixation was higher when both partners were from the same region (Bernal and Graham, 2001). While Andean cultivars form large number of nodules with *R. tropici* strains (Nodari et al., 1993), Mesoamerican beans with high capacities to fix nitrogen nodulated poorly with *R. tropici* strains and in these beans, *R. tropici* blocked *R. etli* nodulation when both strains were inoculated together (Martínez-Romero et al., 1998).

3.3.2.3 Diversity of rhizosphere bacteria

In group 4, all *Sphingomonas* isolates were identified as *Sphingomonas yanoikuyae* (100% identity with EMBL database) and for group 5 the *Stenotrophomonas* (EF695449, AB294557) were characterized by the specie *maltophilia* (100% and 98.7% sequence similarity, respectively). From the Gram positive isolates, only one sequence matched 100% (AF260750) with a *Bacillus subtilis* strain in the EMBL database; however; all the other identifications were below the limit (97.5%) of species level characterization and represent the presence of *Bacillus*, *Brevibacillus* and *Paenibacillus* strains.

Representatives of the genera *Sphingomonas, Stenotrophomonas, Bacillus* and *Paenibacillus*, have been reported to establish beneficial interactions with plants. In some cases like *Bacillus* sp., *Bacillus subtilis*, *Bacillus licheniformis* and *Paenibacillus* (Hervás et al., 1998; Bai et al.,

2003; Cakmakci et al., 2007) have been reported as plant growth promoting rhizobacteria (PGPR). Furthermore, *Sphingomonas* isolates, though reported previously in legumes (Dunne et al., 1997; Donate-Correa et al., 2004), have also been shown to be effective for bioremediation soils contaminated with heavy metals (Baranieki et al., 2002).

In our study most of the Gram positive isolates (66.7%) are related to *Bacillus* species and the remainders are belonging to *Brevibacillus* and *Paenibacillus*. Although their presence is most obvious in sorghum roots and soil sample, some studies showed co-habitation of symbiotic and Gram positive bacteria in legume nodules. Sturz et al. (1997) showed that rhizobia recovery from red clover nodule tissue could yield up to $4.3x10^9$ cfu g^{-1} fresh weight, but that at the same time, $3.0x10^5$ cfu g^{-1} of non-rhizobial endophytes, belonging to 12 different species, could be cultured from the same nodules. Bai et al. (2003) showed that *Bacillus subtilis* and *Bacillus thuringiensis* can naturally co-inhabit soybean nodules along with *Bradyrhizobium japonicum*, and that these Gram-positive bacteria can enhance plant productivity in co-inoculation experiments. A more recent report (Zakhia et al., 2006) described the association of 14 bacterial genera with wild legume nodules in Tunisia.

3.3.3 Analysis of nodulation tests

The nodulation of the *Rhizobium* and *Agrobacterium* isolates was compared with *Rhizobium etli* wild-type reference strain CNPAF512. Figure 3.1 shows the abundant nodule formation by *R. etli* (reference and isolated strains) and *R. tropici* (RL-2, EF054889), while none of *Agrobacterium* isolates did elicit nodules on ICA Pijao roots.

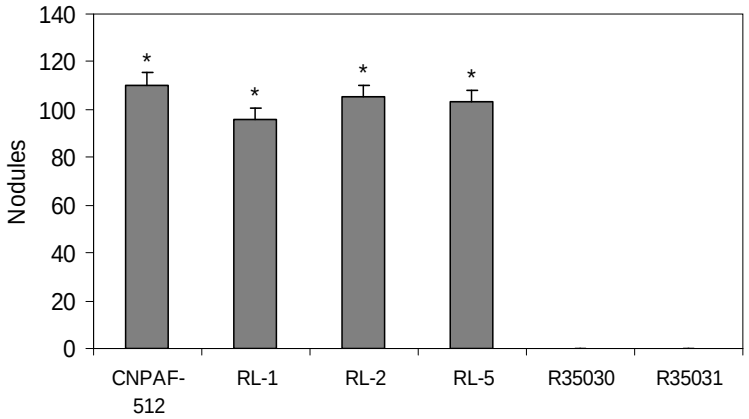

Figure 3.1 Nodulation test on *P. vulgaris* c.v. ICA Pijao. Strains analyzed: *Rhizobium etli* (CNPAF512, reference wild-type), *R. etli* (RL-1), *R. tropici* (RL-2), *R. etli* (RL-5), *A. tumefaciens* (R35030), *A. tumefaciens* (R35031). Stars on top of the bars represent the best statistical result among treatments for Tukey HSD ($P<0.05$).

Several studies have reported the presence of *Agrobacterium* in several tropical legumes. Chen et al. (2000), Hungria et al. (2001), Hungria et al. (2006) and Muresu et al. (2008) have detected *Agrobacterium* in nodules of *Hedysarum spinosissimum, Hippocrepis unisiliquosa, Scorpiurus muricatus, Glycine max, Phaseolus vulgaris,* but the plant tests showed that the isolates were unable to nodulate their original host.

The bean plants showed more roots with the *Agrobacterium* inoculation than for the *Rhizobium* inoculation, which is in line with has been reported by Plazinski and Rolfe (1985): non-nodulated bean plants form more root hairs and lateral roots than nodulated plants (see schematic representation in figure 3.2). No significant differences ($P<0.05$) in nodulation among *Rhizobium* isolates and the reference strain (CNPAF512) were observed, which evidences the ability of both, *Rhizobium etli* and *Rhizobium tropici*, to colonize bean roots. However, in soil condition, the possible competence between both species could decrease the nodule formation and N fixation due to the competition for nodule occupancy in legume roots.

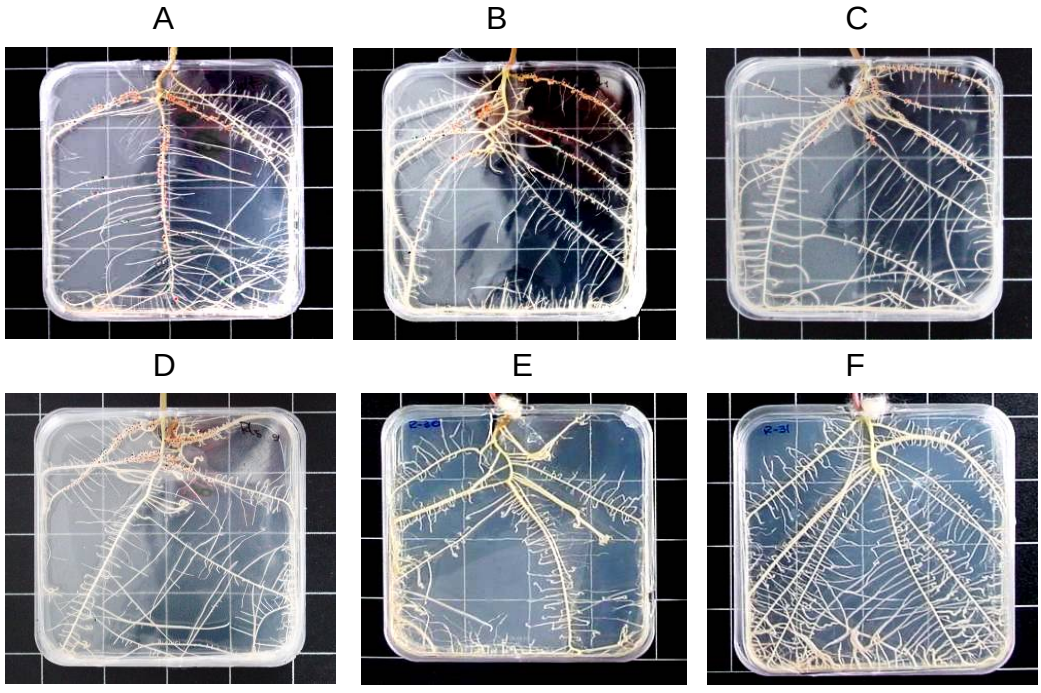

Figure 3.2 Schematic representation of ICA Pijao roots showing the root system with: A/ *Rhizobium etli* (CNPAF512, reference wild-type), B/ *Rhizobium etli* (RL-1), C/ *Rhizobium tropici* (RL-2), D/ *Rhizobium etli* (RL-5), E/ *Agrobacterium tumefaciens* (R35030), F/ *Agrobaterium tumefaciens* (R35031).

R. tropici type (type A) isolates from France were moderately effective or ineffective, while *R. leguminosarum* bv. *phaseoli* isolates were effective on bean plants. While the

Mesoamerican bean cultivar RAB39 nodulates preferently with *R. tropici* (Montealegre et al., 1996), wild *P. vulgaris* accessions do not nodulate with some *R. tropici* strains (Kipe-Nolt et al., 1992).

In this chapter only the capacity of nodule formation by the isolated strains was analyzed, however, additional phenotypic characterization in common bean genotypes and N fixation are studied in detail in chapter 4.

The results presented in this chapter illustrate the importance of characterizing the rhizobacteria locally in order to unravel the microbial biodiversity in intercropping systems and to discover new sources of microbes that are able to enhance or that interfere in processes like SNF.

In conclusion, the identification of several groups of bacteria in common bean-sorghum rhizosphere, some of them belonging to PGPR, could contribute positively to the plant growth and the yield of the intercropped plants. However, in some cases, like *Agrobacterium,* the indigenous bacterial population can also negatively affect the *Rhizobium*-bean symbiosys.

Chapter 4

Phenotypic characterization of Rhizobium *isolates*

Abstract

Rhizobium isolates, previously characterized in chapter 3, were phenotypically studied under optimal growth condition to determine the ability of nodule formation at early stage and N_2 fixation with *Phaseolus vulgaris* L. on c.v ICA Pijao. In addition a field trial was performed in Cuba to unravel the effect of the isolated strains on the variability of nodulation, growth parameters and yield of the ICA Pijao and BAT-304 bean genotypes.

The nodulation kinetics showed a significant increase in nodule number for *Rhizobium etli* (RL-1), *Rhizobium tropici* (RL-2) and *R. etli* (RL-5) at early stage as compared with the *R. etli* reference strain CNPAF512. All the strains tested were able to reduce acetylene in symbiosis with common bean cv. ICA Pijao, although significant differences were observed among CNPAF512, RL-2 and RL-5 as compared with Rl-1. Under field condition, nodulation was stimulated with the inoculation of *R. etli* (RL-1) for *P. vulgaris* c.v. ICA Pijao but no responses were observed for BAT-304. The growth parameters and the yield were significantly stimulated for *P. vulgaris* c.v. ICA Pijao with the control and the inoculation of *R. tropici* (RL-2) treatments respectively, while for BAT-304 no statistical differences were observed for yield among the treatments.

4.1 Introduction

The native rhizobial soil population is dependent on the edapho-climatic conditions but the presence of leguminous plants also is a strong determinant. Evaluation of rhizobia diversity, as described in chapter 3, will enable a better strain selection for inoculation of common bean genotypes, resulting in efficient nodulation and N fixation under field conditions. Furthermore it will also improve our knowledge of microorganism population dynamics and contribute to our understanding of phenotypic and genotypic characteristics, distribution patterns found in an ecosystem and changes due to the effects of management in an ecosystem (Martins et al., 1996).

It is well known that common bean is a promiscuous legume (Bromfield and Barran, 1990; Martínez et al., 1985; Michiels et al., 1998). It has long been recognized that native isolates recovered from the nodules of *Phaseolus vulgaris* show considerable genetic diversity, suggesting that several different *Rhizobium* species can associate with beans (Michiels et al., 1998; Martinez-Romero 2003). However, a systematic analysis of nodulation phenotypes on bean plants in support of this hypothesis has not yet been made.

Nitrogen fixing capacity of some commercial beans is amongst the lowest of the widely cultivated legumes. Crop management and plant selection can possibly improve symbiotic N fixation in common bean (Hungria and Vargas, 2000), especially considering cultivars that have been identified with high capacities to fix N and by breeding for bean lines that are less dependent on chemical N fertilization (Elizondo-Barrón et al., 1999). Beans with high capacity to fix N may then be used in combination with *Rhizobium* strains with superior capacities to fix nitrogen and compete with native strains in the soil. A strategy would be to improve N fixation capacity of the native strains well adapted to different regions.

In this chapter the phenotypic characterization of *Rhizobium* isolates identified in chapter 3 is described. The isolated rhizobia strains were shown to effectively nodulate the original host without significant differences among the tested strains. However, this chapter focused on a more profound analysis of the strains in interaction with common bean genotypes.

Analysis of the nodulation kinetics was performed to detect possible differences among strains for early nodulation. The nitrogen fixation capacities were compared using the acetylene reduction assay (ARA). Both analyses were performed with bean plants grown in a

plant growth chamber. Thirdly a field trial was conducted with two beans genotypes (ICA Pijao and BAT 304) to evaluate the variation in nodulation parameters, growth and yield of local genotypes.

4.2 Materials and methods

Nodulation test and nitrogenase activity assays

Bacterial strains, growth conditions and inoculum preparation

The strains used in this study and under field condition are listed in table 4.1. For nodulation parameters and nitrogenase activity (acetylene reduction assay, ARA), *Rhizobium etli* CNPAF512 (wild-type reference strain) and the characterized isolated strains (see chapter 3) were grown overnight in liquid tryptone-yeast extract (TY) media supplemented with 0.7 M $CaCl_2$ at 30°C or maintained on yeast extract-mannitol (YEM) (Vincent 1970) agar plates (15 g agar l^{-1}). Cells were washed twice with 10 mM $MgSO_4$ and resuspended in 10 mM $MgSO_4$ at a density of 10^7 colony forming units (cfu) ml^{-1}.

Plant material, growth conditions and evaluation

Seeds of bean cv. ICA Pijao were surface-sterilized as described previously (Vlassak et al. 1998) and pre-germinated during two days on water agar (15 g agar L^{-1}) in the dark at 30°C. For nodulation kinetics, one pre-germinated seedling was planted on agar based medium (8 grams Agro gum agar L^{-1}) per square Petri dish (12x12 cm) containing 50 ml of sterile Snoeck medium (Snoeck et al., 2003). For ARA, pre-germinated seeds were grown in 250 ml cylindrical flasks (one seedling per flask). For both the nodulation kinetics and N fixation experiments, the seedlings were inoculated with 100 µl of inoculum containing 10^7 *Rhizobium* cells. The number of cells used for inoculation is based on previous research described by Burdman et al. (1996), Burdman et al. (1997), Hamaoui et al. (2001) and Bai et al. (2002).

Bean plants were grown in a Sanyo Gallenkamp Fytotron plant growth room with a 12-h photoperiod (day/night temperature, 22°C/18°C; day/night relative humidity, 65%/75%) (Michiels et al., 1998). Complete randomized block experimental design was performed for both trials, using 10 plant replicates for nodulation parameters and 12 plant replicates for the acetylene reduction assay.

Table 4.1 Bacterial strains used in the nodulation kinetics, nitrogenase activity and field experiment

Bacterial strains	Relevant characteristics	Reference
Rhizobium etli CNPAF512	Wild-type strain, isolated from *Phaseolus vulgaris* nodules, Brazil. Used for nodulation kinetics and nitrogenase activity experiments	Michiels et al. (1998)
Rhizobium tropici CIAT899	Wild-type strain, isolated from *Phaseolus vulgaris* nodules, Colombia. Used for field experiment	Martinez-Romero et al. (1991)
Rhizobium etli RL-1	Strain isolated from *Phaseolus vulgaris* nodules, Cuba. Used for all the experiments	*This work (Gonzalez et al., 2005).
Rhizobium tropici RL-2	Strain isolated from *Phaseolus vulgaris* nodules, Cuba. Used for all the experiments	*This work (Martínez-Romero 1991).
Rhizobium etli RL-5	Strain isolated from *Phaseolus vulgaris* nodules, Cuba. Used for all the experiments	*This work (Gonzalez et al., 2005).

* gives the reference of the closest FASTA hit of 16S rDNA sequence obtained in the genetic characterization (chapter 3).

Evaluation of the nodulation kinetics started from the third day until 2 weeks after inoculation. Nodules were counted daily. Acetylene reduction activity was determined with a Hewlett-Packard 5890A gas chromatograph equipped with a "PLOT fused silica" column 4 weeks after inoculation. Ethylene production was quantified with propane as an internal standard.

Comparison of the Rhizobium *strains on nodulation, growth parameters and yield of common bean genotypes under Cuban field condition*

To compare the effect of the characterized isolated strains under natural conditions, a field trial was performed in a farmer's area of Quemado de Güines, Villa Clara province (22^o 47' 18.05" N – 80^o 15' 05.86" W).

Both the ICA Pijao and BAT-304 genotypes used in the second field trial discussed in chapter 2 were analyzed in these experimental conditions to determine the effect of the isolated strains compared with a wild-type reference strain (CIAT 899), N fertilizer and a control.

Bacterial strains, growth condition and inoculum preparation

Table 4.1 shows the bacterial strains used in the field trial. Bacterial cultures and inoculum were prepared similarly as described in chapter 2. *R. tropici* strain CIAT899 and the isolated

strains RL-1, RL-2 and RL-5 were grown overnight at 30°C in adapted liquid YEM medium, containing per 1 liter of distilled water: 5 g Bacto Yeast Extract, 20 g sugar (of sugarcane, local production instead of mannitol), 0.5 g $K_2HPO_4.3H_2O$; 0.2 g $MgSO_4.7H_20$ and 0.1 g NaCl. The pH was adjusted to pH 7 by adding HCl (1 M).

To prepare the inoculum, 10 ml of pre-inoculum grown overnight (as described above) was transferred to 5 L of the YEM growth media. The cultures were incubated at 30°C and shaken during 24 h. 100 ml of rhizobial cell culture (with 10^8 cfu ml^{-1} YEM medium) were mixed with 250 g sterile humus as inoculum carrier. This quantity of inoculated humus was used for 10 kg seeds resulting in approximately 10^6 cells per seed. These procedures were performed in the Provincial Soils Laboratory of Villa Clara 1 month before the trial set up and the inoculum was stored at room temperature until use. According to Hernandez et al. (1996), humus-inoculum can be stored up to six months without loosing significant bacterial cell viability.

For inoculation, seeds were mixed with the appropriate amount of the humus based inoculum (as described above), approximately one hour before sowing. The inoculated seeds were dried in the shadow and manually planted taking into account a plant density of about 200,000-250,000 plants per hectare.

Plant culture, growth conditions and evaluations

The different treatments were distributed in a randomized blocks design with 4 replicates (see annex 4). A total of 48 plots (5 x 5 m for each plot) were performed in Luvisol soil with the following characteristics: pH-water 6.5, organic matter 2.26 %, 6.68 mg of P_2O_5 and 15.00 mg of K_2O per 100 g of soil. The soil samples were analyzed in the Soil Laboratory from the Faculty of Agricultural Sciences, Central University of Las Villas, Cuba. The fields were prepared by traditional ploughing 2 weeks before sowing.

The N fertilizer (urea 60 kg ha^{-1}) was applied to the respective plots before sowing (García, 2006). Irrigation and weeding were controlled and performed when needed during the experiment. No application of chemical pesticides was needed.

Five plants per plot (Hamaoui et al., 2001) were carefully removed from the soil for nodulation parameters at 21 days after sowing (DAS). Nodule number (NN), nodule fresh weight (NFW) and nodule dry weight (NDW) were measured. The growth parameters and

yield were analyzed at 92 DAS, measuring the variability in number of pods per plant (PPP), pod weight per plant (PWP), grains per plant (GPP) and yield (grain weight per plant).

Statistical analysis

Data were processed using SAS 9.1 Entreprise Guide 4. Analysis of Variance (ANOVA) mixed model was applied with specific settings: Kenward and Roger calculation as degree of freedom method and Tukey HSD as posthoc significance test. For the nodulation test and the nitrogen fixation assays, complete ramdomized experiments were designed. Ten replicates for the nodulation kinetics and 12 replicates for ARA were considered as the experimental unit and the blocks as a random factor. The data of plates and flasks replicates were used to compare significant difference between the treatments with the mixed model. The parametric Tukey HSD post-hoc with significance level $P<0.05$ was used.

For the randomized block experimental design under field condition, four replicates were considered as the experimental unit and the blocks as a random factor. The main interaction factors considered were the treatments and the genotypes, using as post-hoc Tukey HSD with significance level $P<0.05$.

4.3 Results

4.3.1 Influence of Rhizobium *isolates at early stage and N fixation in the interaction with bean c.v. ICA Pijao*

To phenotypically determine the effect of the isolated strains in the *Rhizobium*-bean (c.v ICA Pijao) interaction, nodulation kinetics were monitored. As described above, common bean seedlings were inoculated with isolated strains: *R. etli* RL-1, *R. tropici* RL-2 and *R. etli* RL-5, and compared with the wild-type reference strain *R. etli* CNPAF512.

Figure 4.1 shows the results of the nodulation kinetics for each treatment separately. The emergence of the nodules was recorded daily, counting (marking from the outside of the dishes) the nodule number per day. The evaluations started from 3 days until 14 days after inoculation. The data in figure 4.1-A (inoculation with CNPAF512) and 4.1-B (inoculation with RL-1) show statistical difference ($P<0.05$, Tukey HSD) in nodule emergence at 6^{th}, 7^{th} and 8^{th} days after inoculation as compared with all the other days for those treatments. The

data in figure 4.1-C (inoculation with RL-2) and 4.1-D (inoculation with RL-5) show statistical differences at 6th and 7th, and 7th and 8th days post-inoculation respectively. This result indicates that the maximum nodule formation in cv. ICA Pijao under optimal growth condition is between 6 and 8 days after inoculation.

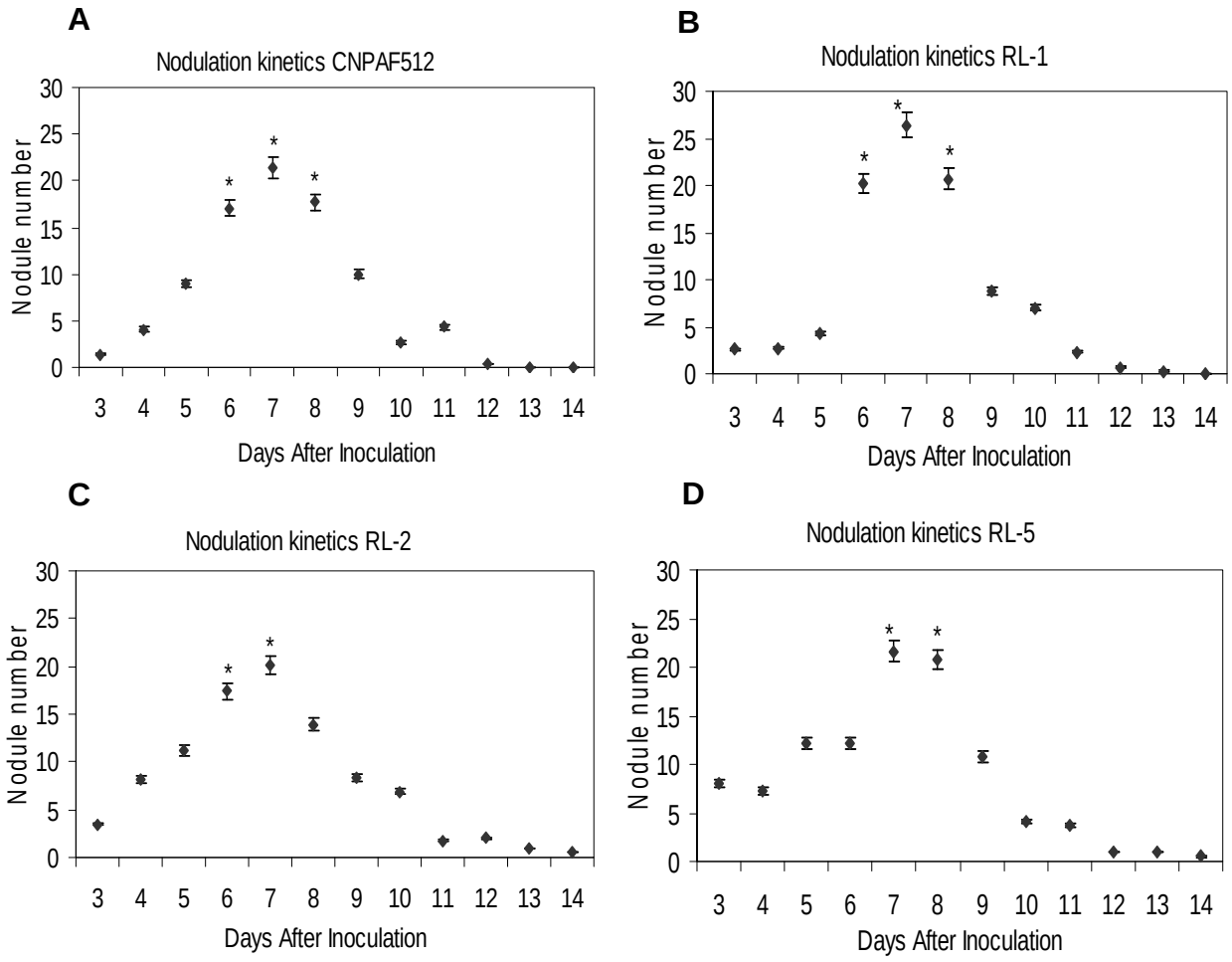

Figure 4.1 Differences among the strains at the early stage of the nodule formation. The treatments analyzed were: CNPAF512/ inoculation with *Rhizobium etli* wild-type reference strain CNPAF512, RL-1/ inoculation with *Rhizobium etli* RL-1, RL-2/ inoculation with *Rhizobium tropici* RL-2, RL-5/ inoculation with *Rhizobium etli* RL-5. Stars in the different days evaluated represent the best statistical result among the time point evaluated for Tukey HSD ($P<0.05$).

For CNPAF512, RL-1 and RL-5 strains, the number of nodules formed starts to decline after 8th days post-inoculation. For RL-2 the reduction starts from the 7 day after inoculation. When comparing the differences among the strains (see figure 4.2), it can be seen that strain RL-5 and RL-2 performed the best for early nodulation (3rd, 4th and 5th day after inoculation).

For the sixth and seventh day after inoculation, RL-1 showed the best results. For the 10th day post-inoculation, RL-1 and RL-2 differed significantly with CNPAF512 and RL-5. At the 11th day after inoculation, the reference strain CNPAF512 gave significant difference with all the treatments.

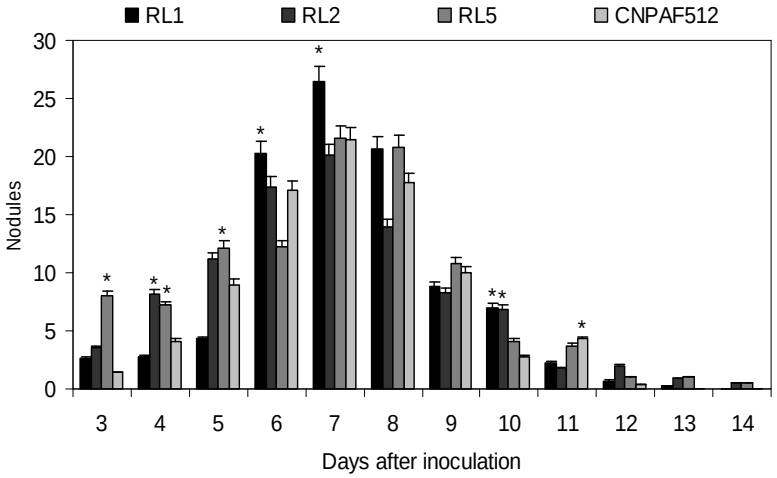

Figure 4.2 Differences among the strains at the early stage of the nodule formation. The treatments analyzed were: CNPAF512/ inoculation of *Rhizobium etli* wild-type strain CNPAF512, RL-1/ inoculation with *Rhizobium etli* RL-1, RL-2/ inoculation of *Rhizobium tropici* RL-2, RL-5/ inoculation of *Rhizobium etli* RL-5. Stars on top of the bars represent the best statistical result among the strains within the same time point evaluated for Tukey HSD ($P< 0.05$).

The precocious nodulation with RL-5 at the third day after inoculation increased the number of nodules significantly: 82.5% compared to CNPAF512, 67.5% compared to RL-1 and 56.25% compared to RL-2. At the maximum nodulation level (7th day post-inoculation) the increase of nodule number with RL-1 revealed an increase of 23.86% compared to RL-2, 18.94% compared to CNPAF512 and 18.18% compared to RL-5.

The RL-1 and RL-5 isolates belong to the same species as the wild-type reference strain CNPAF512 (*R. etli*). However, in both cases the number of nodules between the 3rd to 7th day after inoculation is significantly higher as compared with the reference strain. The effect of *R. tropici* (RL-2) on nodule emergence is most pronounced between the 4th and 10th day post-inoculation when compared with the reference *R. etli* strain.

The nitrogenase activity was determined by the acetylene reduction test to estimate the N fixation capacity in common bean c.v ICA Pijao. Figure 4.3 shows the quantities of µmol of ethylene produced per plant per hour with the different strains inoculated.

All the strains evaluated were able to reduce acetylene 4 weeks after inoculation. As observed in figure 4.3, there were no significant differences among CNPAF512, RL-2 and RL-5 and

those three strains differed statistically with the RL-1 inoculation. The significant stimulation when comparing RL-5, RL-2 and CNPAF 512 with RL-1 in ethylene production revealed a 37.5%, 32,3% and 23.3% difference respectively.

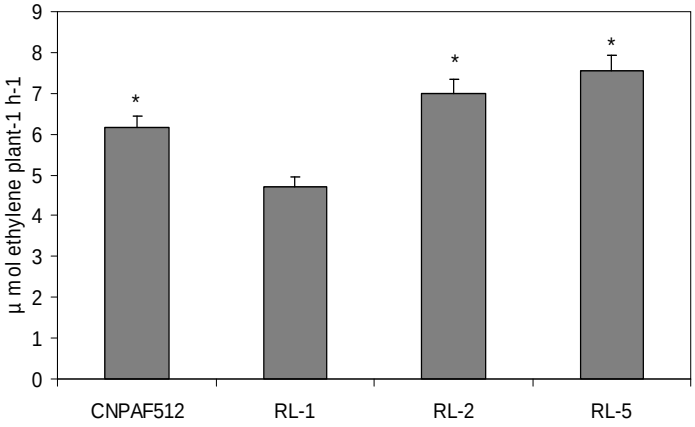

Figure 4.3 Nitrogenase activity of *P. vulgaris* c.v ICA Pijao inoculated with *R. etli* CNPAF512, *R. etili* RL-1, *R. tropici* RL-2, *R. etli* RL-5. Stars on the top of the bars represent the best statistical result among the strains for Tukey HSD ($P< 0.05$).

The increased nodule number at an early stage and the nitrogenase activity of *R. etli* RL-5 reinforces the positive effect of this strain at the early stage of rhizobia-ICA Pijao interaction. Surprisingly, the inoculation with RL-1, although able to form rather high number of nodules at early stage, resulted in lower N fixation as compared with all the other treatments under optimal growth condition.

4.3.2 Phenotypic characterization of isolated strains under Cuban field conditions

Two genotypes of common bean, ICA Pijao and BAT-304, were analyzed in field trial upon inoculation with RL-1, RL-2 and RL-5 strains, a control and fertilizer treatment. The characteristics of the *Phaseolus* genotypes were described in chapter 2. The nodulation parameters of ICA Pijao and BAT-304 are displayed in table 4.2.

Both ICA Pijao and BAT-304 genotypes did not differ significantly for the nodule number in any of the treatments evaluated. For nodule fresh and dry weight, the inoculation of ICA Pijao with *R. etli* RL-1 isolate gave the highest result and was the only treatment with statistical difference as compared with the fertilizer treatment. However, no differences were observed among RL-1 and the treatments Co, CIAT899, RL-2 and RL-5. As for the field experiment in the first period showed in chapter 2, the nodule parameters for both genotypes are low when

compared with previous reports (Hernandez et al, 1996, Hamaoui et al., 2001). These results are in line with those presented in chapter 3, where no statistical diferences were observed among the strains in the nodulation test (see chpater 3, Fig 3.1).

Table 4.2 Nodulation parameters of ICA Pijao and BAT-304

Treatments	NN		NFW (mg)		NDW (mg)	
	ICA Pijao	BAT 304	ICA Pijao	BAT 304	ICA Pijao	BAT 304
Co	9.70 [a]	6.40 [a]	5.83 [ab]	3.60 [a]	1.93 [ab]	1.23 [a]
CIAT 899	7.62 [a]	7.10 [a]	5.73 [ab]	4.51 [a]	1.90 [ab]	1.62 [a]
RL-1	13.05 [a]	7.95 [a]	**9.78 [a]**	5.04 [a]	**3.87 [a]**	1.54 [a]
RL-2	12.00 [a]	8.75 [a]	5.08 [ab]	5.16 [a]	1.89 [ab]	1.71 [a]
RL-5	8.25 [a]	7.95 [a]	5.37 [ab]	4.46 [a]	1.77 [ab]	1.53 [a]
Fert	7.50 [a]	7.70 [a]	2.96 [b]	3.13 [a]	1.23 [b]	1.20 [a]
Std. Error	0.59	0.35	0.0584	0.0031	0.0023	0.0009

Abbreviations: NN/ number of nodules, NFW/ nodule fresh weight, NDW/ nodule dry weight. Treatments evaluated: Co/ control without inoculation or fertilization, CIAT899/ inoculation with *Rhizobium tropici* (wild-type reference strain CIAT 899), RL-1/ inoculation with *Rhizobium etli* (isolated strain RL-1), RL-2/ inoculation with *Rhizobium tropici* (isolated strain RL-2), RL-5/ inoculation with *Rhizobium etli* (isolated strain RL-5), Fert/ application of fertilizer (urea 60 kg ha^{-1}). Different letters in columns differ $P<0.05$ for Tukey HSD.

The variation in the inoculation responses is more pronounced for ICA Pijao than for BAT-304. Figure 4.4 shows the comparison among the common bean genotype in nodulation parameters and the responses with the different treatments evaluated. In panel A it can be seen that the influence in nodule number is more pronounced for ICA Pijao as compared with BAT-304. The control (Co), the inoculation with *R. etli* RL-1 and *R. tropici* RL-2 were the best treatments for ICA Pijao. The inoculation with *R. tropici* CIAT899, *R. etli* RL-5 and the fertilizer treatment showed the same responses for ICA Pijao and BAT-304. In panel B (Fig. 4.4) it can be seen that only the RL-1 inoculation gave significant difference for nodule dry weight for ICA Pijao as compared with the other treatments.

The results for nodule number in the control treatment indicate the potential of indigenous *Rhizobium* strains to nodulate common bean roots. For both, ICA Pijao and BAT-304, the control treatment did no show statistical difference with the other treatments evaluated (see table 4.2), but comparing the genotypes, ICA Pijao scored 34% better than BAT-304.

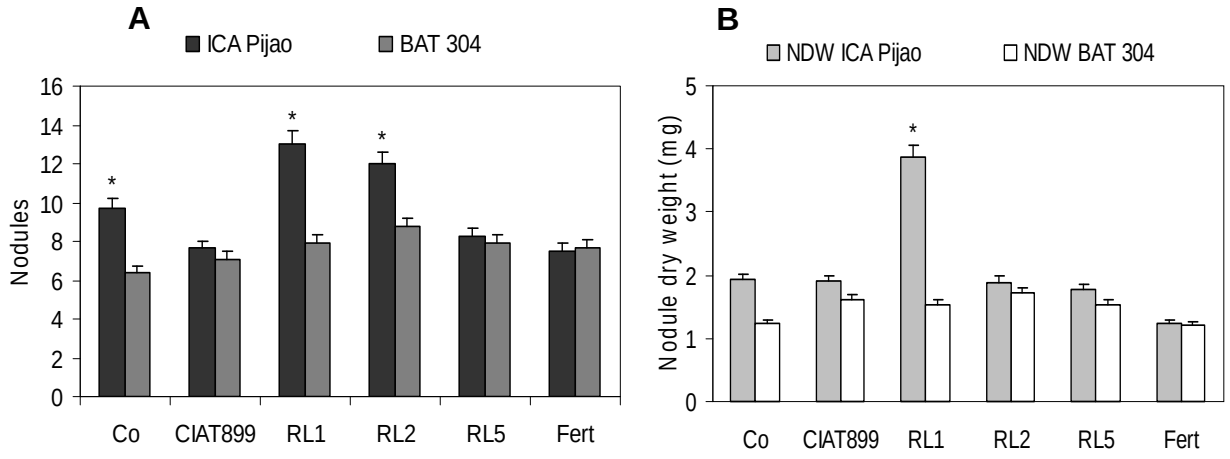

Figure 4.4 Comparison of variability on nodulation parameters in ICA Pijao and BAT-304. A: nodule number, B: nodule dry weight (NDW). Treatments analyzed: Co/ control without inoculation and fertilization, CIAT899/ inoculation with *R. tropici* (wild-type reference strain CIAT 899), RL-1/ inoculation with *R. etli* (isolated strain RL-1), RL-2/ inoculation with *R. tropici* (isolated strain RL-2), RL-5/ inoculation with *R. etli* (isolated strain RL-5), Fert/ application of fertilizer (urea 60 kg ha^{-1}). Stars on top of the bars represent the best statistical result among the genotypes within the same treatment evaluated for Tukey HSD ($P< 0.05$).

The nodule number for ICA Pijao with *R. etli* RL-1 increased with 39% as compared with BAT-304 and for ICA Pijao with *R. tropici* increased with 27% as compared with BAT-304. For nodule dry weight (Fig 4.4-B), statistical difference was only observed with RL-1 in ICA Pijao, increasing the dry weight of nodules with 60.45% as compared with BAT-304.

The fertilizer (Fert) treatment did not significantly affect the nodulation parameters. It could be due to the fact that the doses of urea used in the study are low and therefore do not inhibit the nodulation under field condition.

The significant increase in nodule number in ICA Pijao for RL-1 and RL-2 is in line with the nodulation parameters at early stage analyzed as described in 4.3.1. No significant differences were observed among *R. etli* (RL-1) and *R. tropici* (RL-2), emphasizing the promiscuity of common bean to be colonized by both species.

The genotypic variability is, as for the nodulation parameters, also observed for growth parameters. Table 4.4 shows the values of PPP, PWP, GPP and the yield of ICA Pijao and BAT-304 genotypes evaluated at 92 days after sowing. The differences among the treatments (see Table 4.4) are small for all the parameters evaluated, also among the bean genotypes studied (see Fig. 4.6). The best results with significant differences are flagged in bold.

For PPP, no significant differences were observed in ICA Pijao, while for BAT-304 the fertilizer treatment had the best result, although without significant difference as compared

with the control and the *Rhizobium* treatments. The PWP was not statistically affected in BAT-304 with any of the treatments analyzed, but for ICA Pijao, the control treatment showed the best results, although no statistical differences were observed with the *Rhizobium* treatments (RL-1, Rl-2 and Rl-5). For GPP, ICA Pijao showed stimulation in combination with *R. tropici* RL-2, being the only treatment with statistical difference as compared with the inoculation of the reference strain CIAT899. For BAT-304 no differences were observed among the treatments. For the yield, the results were higher with the inoculation of *R. tropici* RL-2, but without significant difference with *R. etli* RL-1 and *R. etli* RL-5 in ICA Pijao. The plant response with the inoculation of the reference strain CIAT899 did not affect the PWP, GPP and the yield for ICA Pijao having the lower statistical value. This illustrate the potentialities of native *Rhizobium* isolates to increase the bean parameters as compared with the reference strain. These results can be explained by the adaptation of the native strains to specific environments.

The yield for *R. tropici* RL-2 inoculation in ICA Pijao is significantly increased as compared with the other treatments. An increase of 26% is observed as compared with the fertilizer application, 25.8% compared with the reference strain CIAT899 inoculation, 24.4% compared with the control, 16.4% compared with *R. etli* RL-1 and 12.7% compared with *R. etli* RL-5 inoculation.

Figure 4.5 shows the comparison among both plant genotypes for the growth parameters and the yield. Panel A shows no significant differences in pods per plant for both genotypes in all the treatments evaluated. Panel B shows the weight per plant, where the control and *R. tropici* RL-2 inoculation had the best statistical results for ICA Pijao as compared with BAT-304. Panel C shows the stimulation of *R. etli* RL-1 for ICA Pijao, being the only treatment with statistical differences among genotypes. The yield, observed in panel D, is statistically increased with the inoculation of *R. tropici* RL-2 for ICA Pijao.

Table 4.4 Growth parameters and yield for ICA Pijao and BAT-304 under field conditions

Treatments	PPP		PWP (g)		GPP		Yield (g)	
	ICA Pijao	BAT 304	ICA Pijao	BAT 304	ICA Pijao	BAT 304	ICA Pijao	BAT 304
Co	8.15 [a]	7.30 [ab]	**17.13 [a]**	10.52 [a]	42.90 [ab]	34.25 [a]	8.98 [b]	7.92 [a]
CIAT 899	6.90 [a]	6.40 [b]	11.01 [c]	10.26 [a]	34.45 [b]	30.80 [a]	8.81 [b]	7.62 [a]
RL-1	7.35 [a]	6.65 [ab]	13.76 [ab]	11.08 [a]	42.25 [ab]	30.85 [a]	9.91 [ab]	8.44 [a]
RL-2	8.30 [a]	7.80 [ab]	16.05 [ab]	11.39 [a]	**46.95 [a]**	37.45 [a]	**11.87 [a]**	8.07 [a]
RL-5	8.15 [a]	8.40 [ab]	13.97 [ab]	13.15 [a]	41.55 [ab]	40.60 [a]	10.35 [ab]	9.38 [a]
Fert	7.65 [a]	**8.60 [a]**	12.44 [bc]	12.28 [a]	39.25 [ab]	41.20 [a]	8.77 [b]	10.17 [a]
Std. Error	1.14	1.15	2.23	2.03	5.83	5.69	1.45	1.64

Abbreviations: PPP/ pods per plant, PWP/ pod weight per plant, GPP/ grains per plant. Treatments evaluated: Co/ control without inoculation or fertilization, CIAT899/ inoculation with *R. tropici* (wild-type reference strain CIAT 899), RL-1/ inoculation with *R. etli* (isolated strain RL-1), RL-2/ inoculation with *R. tropici* (isolated strain RL-2), RL-5/ inoculation with *R. etli* (isolated strain RL-5), Fert/ application of fertilizer (urea 60 kg ha^{-1}). Different letters in columns differ $P<0.05$ for Tukey HSD.

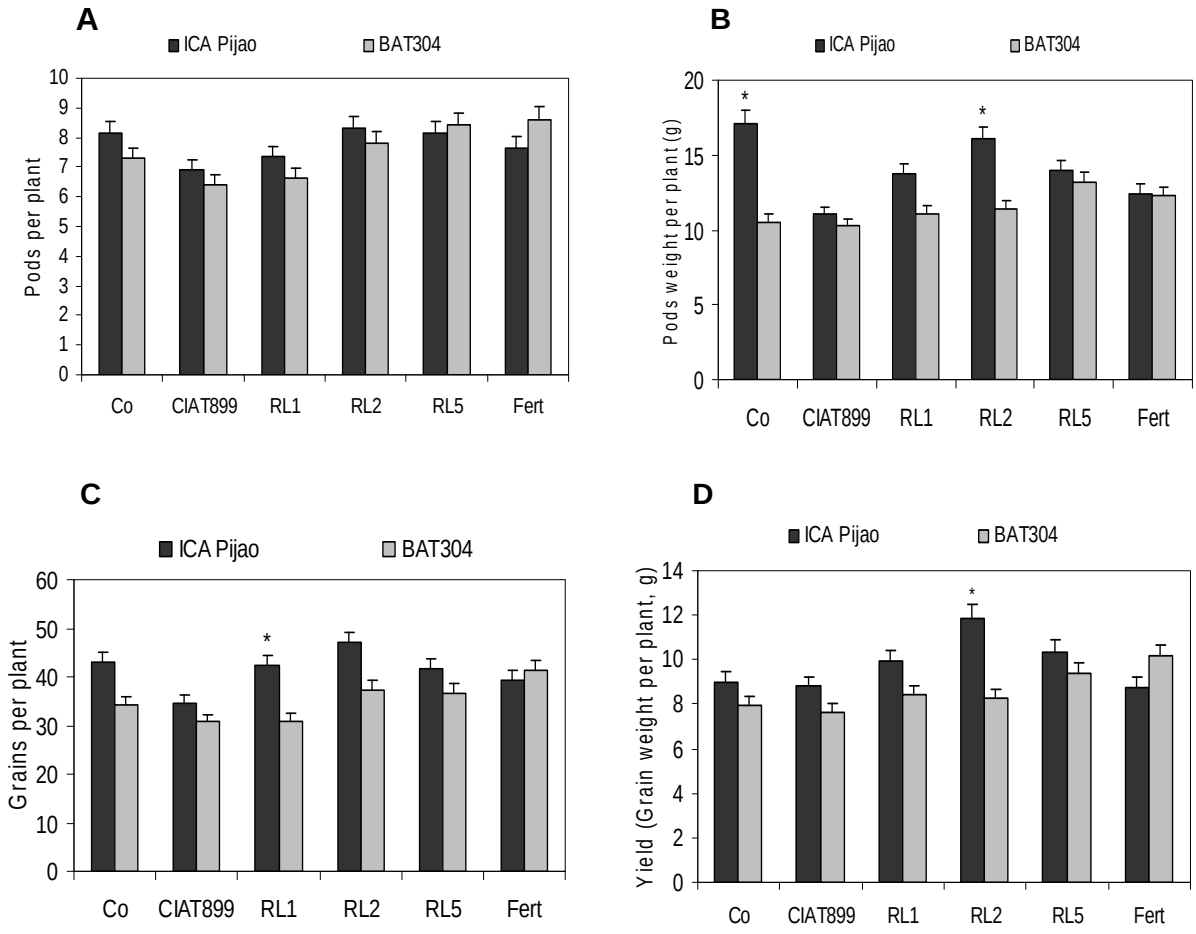

Figure 4.5 Comparison of the genotypic variability on growth parameters and yield for ICA Pijao and BAT-304. A: pods per plant, B: pod weight per plant, C: grains per plant, D: yield (grain weight per plant). Treatments analyzed: Co/ control without inoculation or fertilization, CIAT899/ inoculation with *R. tropici* (wild-type reference strain CIAT 899), RL-1/ inoculation with *R. etli* (isolated strain RL-1), RL-2/ inoculation with *R. tropici* (isolated strain RL-2), RL-5/ inoculation with *R. etli* (isolated strain RL-5), Fert/ application of fertilizer (urea 60 kg ha^{-1}). Stars on top of the bars represent the best statistical result among the genotypes within the same treatment evaluated for Tukey HSD ($P< 0.05$).

The increase in pod weight per plant for the control and *R. tropici* RL-2 treatment in ICA Pijao as compared with BAT-304 was 38.5% and 29% respectively. *R. etli* RL-1 increases the grains per plant in ICA Pijao with 27% compared with BAT-304. *R. tropici* RL-2 increases the yield in ICA Pijao with 27% as compared with BAT-304.

Similarly as with the comparison of genotypic variability on nodulation parameters (see Fig. 4.4), the control, *R. etli* RL-1 and *R. tropici* RL-2 treatments stimulated some of the growth parameters or the yield for ICA Pijao as compared with BAT-304. The inoculation with the reference strain CIAT899, *R. etli* RL-5 and the fertilizer treatment yielded the same results for ICA Pijao and for BAT-304.

Obviously the inoculation with *R. tropici* RL-2 gave the most stable response as compared with all the other treatments for ICA Pijao.

4.4 Discussion

This study demonstrates the influence of the *Rhizobium* strains isolated from Cuban field sites on *Rhizobium*-bean symbiosis under controlled growth conditions and in Cuba field conditions.

All the strains isolated were able to nodulate the roots of ICA Pijao, indicating the compatibility in the interaction. The early stage analysis was focused on the phenotypic characterization of the *Rhizobium*-bean interaction in terms of nodulation kinetics under controlled conditions. The isolated strains (Rl-1, RL-2 and RL-5) indeed showed differences among each other and in comparison with the wild-type reference strain CNPAF512. The nodule formation was stimulated through inoculation with the *R. etli* isolates (RL-1 and RL-5), although the inoculation of *R. tropici* (RL-2) had some positive results at the 4^{th} and 10^{th} day after inoculation

The onset of nodulation is most likely linked to the early steps in the symbiosis, mediated by mutual signaling between the plant roots (flavonoids) and the bacteria (synthesis of lipochitooligosacharides (Nod factors). Legume signals activate the production of the rhizobial Nod factors, which in turn signal back to the plant (Mulder et al., 2005).

The variation in the amount and structures of Nod factors produced by a rhizobial species is a key factor determining its host range (Perret et al., 2000). However, other bacterial factors play a role as well. It is well known that rhizobial cell surface polysaccharides are involved in attachment, penetration, and invasion of the emerging nodules by the microsymbiont (Laeremans and Vanderleyden, 1998). For successful infection of determinate nodules (e.g. common bean and soybean), the presence of lipopolysacharides (LPS) is strictly required (Pellock et al., 2000; reviewed by Fraysse et al., 2003), although they are not the primary determinant for the host range specificity (Laeremans and Vanderleyden, 1998).

Taking into account that bean plants are able to recognize Nod factors with distinct substitutions, different chain lengths and different decorations from at least 10 different characterized *Rhizobium* species (including *R. etli, R. tropici, S. fredii, M. loti, R.*

leguminosarum bvs. trifolii and *viciae, B. japonicum, S. meliloti*, and *Rhizobium* spp. NGR234 and GRH2) (Spaink, 1996; Michiels et al.,1998); it can be inferred that differences among the isolated strains in nodulation at the early stage as compared with the wild-type reference strain CNPAF512, could be related with the Nod factors cultivar-specific receptor or a hierarchy of Nod factor chemical modifications, which are implicated in efficient nodulation (Cullimore et al., 2001)

Although the flavonoids released by the ICA Pijao cultivar are not known, it might well be that they differentially activate the *nod* genes in the different *Rhizobium* strains tested. A clear effect of the bean cultivar on the nodulation phenotype has been observed (Michiels et al., 1998). In the case of the natural bean symbionts *R. tropici* strain CIAT899 and *R. etli* strain CNPAF512, the amount and types of flavonoids released from bean seeds were shown to affect initial root nodule formation (Kato and Arima, 2007). Differences between RL-2 on the one hand and RL-1 and RL-5 on the other hand are most likely due to differences in Nod factor structures, as has been observed for *R. etli* and *R. tropici* type strains.

Other *R. etli* bv. *phaseoli* or *R. tropici* functions that play a role in the early interaction with beans have been identified (reviewed by Martinez-Romero, 2003). In *R. tropici, R. etli* and *R. gallicum* bv. *phaseoli*, ABC transporters for uptake of root exudates are required for optimal nodulation.

The effect of the early interaction and nodulation by RL-1, RL-2 and RL-5 could also possibly be related to the different flavonoids excreted for ICA Pijao. A clear effect of the bean cultivar on the nodulation phenotype of rhizobia inoculation has been observed (Michiels et al., 1998). Cardenas et al. (1995; 1996) demonstrated that the absence of nodulation on common bean was caused by a lack of appropriate *nod* gene inducers. Similarly, in the case of the natural bean symbionts *R. tropici* strain CIAT899 and *R. etli* strain CNPAF512, the amount and types of flavonoids released from bean seeds were shown to affect initial root nodule formation (Hungria et al., 1993, D'Haeze and Holsters, 2002).

The N fixation showed in figure 4.3 demonstrates the ability of all the strains to reduce acetylene, however, a marked reduction in ethylene production is observed with the inoculation of *R. etli* RL-1. This result does not correlate with the previous experiment of nodulation kinetics, in which *R. etli* RL-1 elicited a high number of nodules at early stage on ICA Pijao. As reported by Jaramillo et al. (2003) and González-Ruiz et al. (2008), the

nitrogenase activity varies within and among species of diazotrophs when used for inoculation of legumes. Ceccatto et al. (1988) reported that the nodule number does not substantially contribute to high rate of N fixation in common bean plants, but the nodulins (leghaemoglobin), which may contribute to increase nodule activity and sustain nodule longevity, could be crucial to obtain significant yield increases in common bean cultivars.

Under field conditions, the nodulation parameters (Table 4.2) were invariable for BAT-304, where no statistical difference was observed among treatments. For ICA Pijao, although the only treatment with statistical difference with the fertilizer was the *R. etli* RL-1 inoculation, no differences were observed with the other isolates (RL-2 and RL-5), the reference strain CIAT899 and the control treatments. These results highlight the ability of the isolated, wild-type and indigenous strains to effectively colonize the ICA Pijao roots. Similar results were showed previously in the chapter 2 of this thesis.

Comparing the bean genotype variability (Fig. 4.4), the control, *R. etli* RL-1 and *R. tropici* RL-2 treatments increased significantly the nodule number for ICA Pijao as compared for BAT-304. These results evidence that the competitiveness of each *Rhizobium* strain depends not only on its genetic intrinsic characteristics for nodulation ability, but it is also influenced by the genotype of the host legume (Brutti et al., 1999; Raposeiras et al., 2006).

The results of the growth parameters and the yield (Table 4.4) under field condition show more variation for ICA Pijao than for BAT-304. The best results are exhibited in ICA Pijao with the control treatment for the pod weight per plant and with the inoculation of *R. tropici* RL-2 for the grains per plant and for the yield. This could be explained by the competitivity of the native strains in the soil and the assertive adaptation of *R. tropici* RL-2 to the local environmental conditions. For BAT-304, the only difference among treatments was observed for the pods per plant with the fertilizer treatment.

For ICA Pijao, *Rhizobium etli* (isolated strains, RL-5) and *R. tropici* CIAT899, did not affect significantly any of the growth parameters nor the yield under field condition. Several reports have shown that *R. etli* bv. *phaseoli* has been found to be more competitive for bean nodule formation than *R. tropici* (Martínez-Romero and Rosenblueth 1990; Anyango et al., 1998; Martinez Romero 2003). In contrast, in conditions where *R. etli* bv. *phaseoli* is not adapted, such as in acidity or high temperature, *R. etli* bv. *phaseoli* strains are not more competitive than selected *R. tropici* strains (Tajini et al., 2008).

The evidence of the low responses by the reference strain *R. tropici* CIAT899 is supported by Thies et al. (1992), reporting that the native rhizobia are generally more competitive than the introduced ones. However, a recent report (Tajini et al., 2008) reinforces the conclusion of a better adaptation of *R. tropici* to various adverse environments. Indeed this strain was originally isolated in an acid soil of Colombia (Martinez Romero et al., 1991), and was found to nodulate efficiently in many field trials.

The analysis of the genotypic variation among ICA Pijao and BAT-304 on growth parameters and yield shown in figure 4.5 demonstrates that the control, *R. etli* RL-1 and *R. tropici* RL-2 treatments were the best combinations to increase those parameters in ICA Pijao. For yield, the combination ICA Pijao x *R. tropici* RL-2 had the best result as compared with BAT-304. For all the other treatments analyzed no statistical differences were observed among the genotypes.

The results outlined in this chapter represent an example for the utilization of native rhizobia population to increase plant parameters, focused on the natural genetic variation. The variation among cultivars for efficacy in interactions between plants and beneficial bacteria has been described and suggests natural genetic host variation for these interactions within germplasm. There is some evidence that breeding efforts in crop plants inadvertently have selected against hosting such beneficial microflora (Hetrick et al., 1995). This suggests untapped potential to exploit genetic variation in the host through breeding to enhance beneficial interactions with microorganisms (Smith and Goodman, 1999).

The phenotypic characterization showed in this study and specifically the phytostimulation of common bean through the inoculation of native *Rhizobium* strains open the door to the detection of proper combinations of bean genotypes and *Rhizobium* strains and the possible use of them -in short term- in co-inoculation with PGPR to achieve the potential yield of common bean and to use new sources of efficient strains in the inoculants for bean production under low input systems.

Chapter 5

Detection of genes differentially expressed in **Phaseolus vulgaris** *L. following interaction with symbiotic or pathogenic micro-organisms*

Abstract

Micro-organisms can establish either mutualistic beneficial or pathogenic associations with plants. Although the outcome is completely different, common molecular mechanisms that mediate communication between the interacting partners have already been reported. In contrast to model plants such as *Arabidopsis thaliana* and *Medicago truncatula*, the signal transduction pathways involved in the biotic response of crop plants such as *Phaseolus vulgaris* are not widely studied. Large-scale gene expression analyses, for instance using micro-arrays, are difficult to perform since the genome of *P. vulgaris* has not been sequenced yet and the number of available expressed sequence tags (ESTs), although growing, is still relatively limited.

In our research, we focus on the understanding of the molecular dialogue between *P. vulgaris* (BAT 477) and root symbiotic interacting microorganism, comparing with a pathogenic interaction. Genes, differentially expressed during the root interaction with *Rhizobium etli* CNPAF512 were identified using the cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technique, using as controls the infection with *Fusarium solani* f. sp. *phaseoli* and a treatment without microbial inoculation or infection. Differentially expressed transcript profiles were quantitatively analyzed. Key genes were isolated, sequenced and compared to sequences in the available databases. Our data revealed similarity with several legume genes, some of them encoding plant stress/defense for symbiosis and pathogenesis and cell metabolism genes for symbiosis. Cluster analysis revealed groups of TDFs with similar expression patterns based on the ABQC clustering algorithm.

5.1 Introduction

Microbial symbionts and pathogens are comparable in that they colonize eukaryotic hosts. Whereas pathogenic interactions result in damage or death of the respective host, symbiotic interactions are characterized by an overall benefit (Hentschel et al., 2000).

Co-evolution of plants and microbes may result in intimate and durable interactions. A well-characterized example of co-evolution of plants and microbes is the symbiosis of legumes and rhizobia. Events that lead to establishment of these interactions are triggered by microbial recognition of specific plant-associated signal molecules, which are detected by dedicated microbial sensory proteins (Brencic and Winans 2005). Symbiosis arises from an extensive exchange of molecular signals between two partners. As mentioned in chapter 1, under appropriate environmental conditions, rhizobia and host plants can initiate a symbiotic interaction, resulting in the development of root nodules, which the bacteria inhabit as N-fixing endosymbionts. Development of a *Rhizobium* plant symbiosis is a complex process (Bladergroen and Spaink 1998; Broughton et al. 2000). It involves a highly coordinated exchange of signals between the plant and the bacteria and leads to a gradual and coordinated differentiation and adjustment of physiology and metabolism in both partners (Perret and Broughton 2000). In contrast to rhizobia, pathogenic biotrophic bacteria, fungi, and nematodes often flourish at the expense of the hosting plant and release pathogenicity factors in order to reach a feeding site, counteract plant defense responses, and extract food (Qin et al., 2000).

During the plant-microbe co-evolution, plants have also developed a complex defense system against microbial pathogens (Chisholm et al., 2006). When a plant recognizes a potentially infectious pathogen, local defense responses aid to sequester the pathogen away from non-infected plant tissue (Nimbalkat et al., 2006). Many plant pathogen interactions are governed by specific interactions between pathogen avirulence (*avr*) genes and corresponding plant resistance (*R*) genes. An interaction where a corresponding pair of *R* gene and *avr* gene is present and expressed, results in incompatibility and the plant is resistant. When one of the two is inactive or absent, the interaction is compatible and the plant is susceptible.

Events of recognition and defense by a host plant to its fungal pathogen and ability of the pathogen to overcome the plant's defenses implies a complex, dynamic and interactive molecular network. Induction of these molecular responses necessitates up- and down-

regulation of numerous but specific genes. Differential large-scale gene expression analysis in plant–pathogen interactions has resulted in identification of several defense-related transcripts (Schenk et al., 2000; Badri et al., 2008). A direct or indirect role of these transcripts in controlling pathogen invasion to the plant tissue is also demonstrated in a number of cases. However, these studies are restricted to model plants (Schenk et al., 2000) and few crops such as sugarcane, tomato, coffee, cassava and rice (Fernández et al., 2004; Carmona et al., 2004; Zhang et al., 2004).

Several publications have described similarities between symbiosis and pathogenicity (Parniske, 2004; Sesma and Osbourn, 2004, Guimil et al., 2005; Paszkovski, 2006). Indeed, both for symbiosis and pathogenicity, plant defense mechanisms have to be avoided or misled. Moreover, plants have developed complex and integrated signal transduction pathways allowing to adapt their reaction to individual invaders and thereby preserving the balance between prohibiting pathogens on the one hand and allowing beneficial symbionts on the other hand (Harrison and Baldwin, 2004).

In contrast to the model plant *Arabidopsis thaliana*, the signal transduction pathways involved in the defense of legumes in general and in *Phaseolus vulgaris* in particular are still insufficiently studied (D'Ovidio et al., 2004). Recently, more and more insight in this molecular communication is becoming available from the model legumes *Medicago truncatula* and *Lotus japonicus*. However, this information is more limited in other *Leguminosae*, such as common bean (Graham et al., 2006; Hernandez et al., 2007).

One of the major constraints in common bean genomics is the fact that the analyses of signaling processes have focused traditionally, in contrast with the model legumes, on only one or a few genes at a time (including Salzer et al., 2000; Guenoune et al., 2001). From such studies it has not been possible to assess the extent of overlap or difference of gene activation by different micro-organisms in the plant response.

In this chapter we aim to identify differentially expressed genes in *Phaseolus vulgaris* L. following inoculation with *Rhizobium* as compared to infection with a pathogen and a control treatment by using the cDNA-AFLP approach. It has been shown to be an effective RNA fingerprinting technique to display differentially expressed genes (Bachem et al. 1996). In contrast to hybridization-based techniques, such as cDNA microarrays, cDNA-AFLP can distinguish between highly homologous genes from individual gene families. In addition,

cDNA-AFLP does not need any pre-existing sequence information, which makes it an excellent tool to identify novel genes.

5.2 Materials and methods

The study of differentially expressed genes in common bean using the cDNA-AFLP protocol has been started by members of the Plant Fungi Interaction (PFI) group from the Centre of Microbial and Plant Genetics (CMPG) within the framework of a GOA project. The work previously carried out in this project by the Dr. Miguel F.C. De Bolle, Dr. Janick Mathys and Inge Goderis under the supervision of Prof. Bruno P.A. Cammue, was crucial for the results obtained in this chapter. My own contribution starts with the isolation and reamplification of transcripts derived fragments (TDFs). However, for the sake of clarity, all the steps preceding this are described and discussed as well.

The implementation of cDNA-AFLP approach was done by the efforts of Dr. Miguel F.C. De Bolle and Inge Goderis, while the bioinformatics and data processing (software implementation) were done by Dr. Janick Mathys. The results shown in this chapter are the result of a joint effort.

Plant growth conditions and inoculations

Seeds of *Phaseolus vulgaris* L. (BAT477) were sterilized and germinated as described by Vlassak et al. (1998) and grown *in vitro* according to Snoeck et al. (2003). *Rhizobium etli* CNPAF512 was used as described by Michiels et al. (1998). The test fungus *Fusarium solani* f.sp. *phaseoli* was used according to Mohr *et al.* (1998). Control-grown beans were submerged in a 20mM Mg_2SO_4 solution. Per treatment and for each time point, ten beans were germinated. After the various treatments, the germinating plants were randomly distributed in the plant growth chamber (Sanyo Gallenkamp Fytotron) with a 12-h photoperiod (day/night temperature, 22°C/18°C; day/night relative humidity, 65%/75%) (Michiels et al., 1998). Roots of inoculated or mock-treated beans were collected from 8 h, 16 h, 32 h, 2 up to 6 days post-inoculation. At each time point for each treatment, 3 to 5 roots from randomly chosen, different plants were sampled, shock-frozen in liquid nitrogen and disintegrated using mortar and pestle. The obtained powders were stored at -80° prior to total RNA preparation.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen roots using Concert Plant RNA Reagent (Invitrogen), according to the manufacturer's protocol. Both quantity and quality of the isolated total RNA were tested prior to further use. All manipulations were performed using RNAse-free consumables. The cDNA synthesis was performed as described by Bachem *et al.* (1998) with slight modifications. The first strand of the cDNA was synthesized from 2 µg total RNA, mixing 700 ng biotinylated d(T)25 oligonucleotide (Eurogentec), 20 nmol dNTPs (Roche), 200 U reverse transcriptase (Superscript II; Invitrogen), 4 µl DDT (0.1 M; Invitrogen) and buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$) in a total volume of 40 µl and incubating for 2 h at 42°C. The second strand of the cDNA was synthesized adding 15 U *E. coli* DNA ligase (Invitrogen), 50 U *E. coli* DNA polymerase I (Invitrogen), 1.6 U RNAse H (Invitrogen), 30 nmol dNTPs, 3.7 mM DTT and *E. coli* ligase buffer (18.8 mM Tris-HCl pH 7.0, 4.6 mM $MgCl_2$, 90.6 mM KCl 150 µM NAD^+ and 10 mM $(NH_4)_2SO_4$) in a total volume of 160 µl. The reaction mixture was incubated 1 h at 12°C and 1 h at 22°C. Double stranded cDNA was purified using QIAquick Spin Purification according to the manufacturer's protocol (Qiagen), loaded on a 1% (w/v) agarose gel for quality control and finally the concentration was determined using the NanoDrop device prior to forthcoming manipulations (ND-1000 Spectrophotometer, Nanodrop Technologies).

Implementation of cDNA-AFLP protocol

The cDNA-AFLP protocol was performed as described by Breyne et al. (2003). In short, 500 ng biotinylated cDNA was first digested with 10 U *BstY*I (New England Biolabs) using the appropriate buffer (New England Biolabs) in a total volume of 40µl at 60 °C for 2 h. Next, the 3' ends of the cDNA fragments were immobilized using streptavidine beads (Dynabeads M-280 Streptavidin, Dynal) as recommended by the manufacturer. The bead-linked cDNA fragments were digested using 10 U *Mse*I (New England Biolabs) for 2 h at 37 °C in the proper buffer (New England Biolabs). Finally, the released cDNA fragments were separated from the beads and used for adaptor ligation. The *BstY*I and *Mse*I adaptors were made by heating a mixture of two complementary oligonucleotides at 65 °C for 10 min followed by a slow cooling at room temperature for each adaptor, respectively. The ligation of both adaptors occurred as follows: to 40 µl of the digestion mixture, 5 pmol *BstY*I and 50 pmol *Mse*I adaptors, 1 U T4 DNA ligase (Invitrogen), 1 mM ATP (Invitrogen) in the appropriate buffer

(50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$ and 1 mM DTT (New England Biolabs) were added and incubated at 37 °C for 3 h.

The pre-amplification reaction was carried out with adaptor-ligated cDNAs as template and non-selective primers, complementary to the corresponding adaptors. The reaction components were as follows: a two-fold diluted mixture containing the adaptor-ligated cDNA fragments (in $T_{10}E_{0.1}$-buffer [10mM Tris-HCl (pH 8.0), 0.1mM EDTA]), was mixed with 1 U AmpliTaq DNA polymerase (Applied Biosystems), PCR buffer (10 mM Tris-HCl (pH 8.3), 50mM KCl and 2.5 mM $MgCl_2$), 0.2 mM dNTPs (Roche), 75 ng *Mse*I+0 primer and either 75 ng *BstY*1T+0 or 75 ng *BstY*IC+0, ending up in a total volume of 50 µl. The amplification reaction was performed in 20 cycles (each cycle 94 °C, 30 s; 56 °C, 60 s; 72 °C, 60 s). Next, the selective amplifications were done using samples of pre-amplified cDNA fragments as template and seven selective primer combinations of the 512 possible combinations were used (see table 5.1), which are identical to the pre-amplification primers but extended by two oligonucleotides at the 3' end (*BstY*I+2 and *MseI*+2; see table 1).

The AFLP products were loaded onto high resolution polyacrylamide gels (called preparative gels). Two primer combinations were used per gel (2x3x8 lanes) together with a 50-500 bp ladder. Gels were silver-stained (Silver sequence kit, Promega) and dried vertically for at least 24 h.

Table 5.1 Primer combinations used in the selective amplification reaction

Preparative gels identification	Primer combinations	Primers sequence*
004	411-259	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAAAA
006	411-262	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAAAT
007	411-263	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAACA
010	411-266	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAACT
011	411-267	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAAGA
014 & 017	411-271	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAATA
019	411-274	5'GACTGCGTAGTGATCTAA – 5'GATGAGTCCTGAGTAATT

* *BstY*I+2 and *MseI*+2 used for the selective amplification. The same adaptors were used for the pre-amplification reaction but without the extension with two nucleotides at 3' (*BstY*I+0 and *MseI*+0). Selective amplification was done for 7 primer combinations in 8 preparative gels. Gels 014 and 017 shared the same primer combination.

Data analysis and quantitative measurements of the expression profiles

A fluorescence image (called analytical gel) was taken and analyzed with the ImageMaster 1D Elite v.4.20 (Amersham Biosciences) software. This software automatically matches fragments with the same length in the different lanes and calculates the length and the intensity of each fragment. As an output, an MS Excel file is generated containing the lengths and the intensities of all the fragments in each lane. In order to prevent division by zero and log transformation of zero or negative values, all zero values were set to the minimum positive value of the data set. Additionally, a Boolean flag was added to each row to indicate whether or not the corresponding data is trustworthy. The flags are set after visual inspection of the images of the gels to identify errors introduced by the 1D Elite software. In this way, a tab delimited text file was generated for each primer combination, containing the lengths, the adjusted intensities and the flag of the bands. These data were used to determine the quantitative expression profile for each transcript derived fragment (TDF).

Prior to the analysis of differentially expressed genes, the data must undergo a number of preprocessing steps to reduce the amount of experimental noise. The first step in the preprocessing consists of lane correction. To correct for differences in total lane intensities, a lane correction factor was calculated for each lane, based on the assumption that there is no global difference in total expression between different lanes (conditions or time points).

The total intensity of a lane relative to the sum of the total intensities for all the lanes of the preparative gel and the intensities in each lane are divided by their corresponding lane correction factor. The lane correction is based on the complete data of all the fragments, regardless the value of their flags because the total lane intensities are assumed to be equal. Lane correction is performed for each primer combination separately. After lane correction, the flagged data are removed from the data set. Since the objective of our assay is to compare two biological samples (R or F with W), the intensities are transformed into the logarithm base two of the ratios of the measured expression levels for the two samples.

$$\log_2 R/W = \log_2 R - \log_2 W,$$
$$\log_2 F/W = \log_2 F - \log_2 W.$$

Where R stand for *Rhizobium* treatment, F stands for fungal infection and W stands for water control.

Without the log transformation, the ratios would be squashed between 0 and 1 for down-regulated TDFs, while the ratios of up-regulated TDFs can vary between 1 and infinity. First of all, this would hamper the interpretation of the results. Moreover, in clustering, pairwise distances between expression profiles are calculated and without the log transformation, down-expressed ratios would contribute much less to the distance than over-expressed ratios. Additionally, after log transformation the data approximate a normal distribution which allows more powerful statistics.

Since we are interested in TDFs that show the most difference between R or F and W, we want to select fragments with log ratios that differs the most from 0. Therefore, we used the variance about 0 as a metric of variability of the TDFs. A number of scripts have been generated in Matlab v.6.5.1 to preprocess the data that are generated by cDNA-AFLP (using ImageMaster 1D Elite software) and to identify highly variable TDFs.

Transcript- derived fragments (TDFs) isolation and reamplification

After quantitative data analysis, the selected polymorphic fragments were cut from the gel with a sharp razor blade, with maximum care to avoid any contaminating fragment(s) and eluted in 50 µl of MilliQ water. Five microliters of the aliquot was used for reamplification in a total volume of 50 µl, using the same set of corresponding selective primers as for the selective amplification. The reamplification reaction was performed in 30 cycles (94^{o}C, 30s; 52^{o}C, 30s; 72^{o}C, 30s). The PCR product was resolved in a 2% 1x TBE-agarose gel and each single band was compared with the length (base pares) in the preparative gel.

Cloning and sequence analysis

The PCR product of the TDFs with similar length as compared with the preparative gel were purified using QIAquick PCR purification kit (Qiagen, Hilden) and cloned into *E. coli* strain DH5α (Sambrook et al., 1999) using TA Cloning kit (Invitrogen). Sequencing of the cloned TDFs was carried out on Applied Biosystems apparatus (Sequence Analyzer 3100-Avant). The sequences were compared with Genbank database using BLASTn and BLASTx Network Service (NCBI, National Center for Biotechnology Service).

Adaptive Quality Based Clustering (AQBC)

Clustering was performed to select groups of TDFs with similar expression pattern. Preprocessing and filtering of the data were performed as described above. Standardization of the data was not performed since standardization is performed in the core of the AQBC algorithm (De Smet *et al.,* 2002). Two parameters have to be defined for the AQBC algorithm: the minimal number of TDFs (genes, as for the algorithm output) in a cluster and the minimal probability of TDFs belonging to a cluster. In our analysis, the former was set to the default value of two, and for the latter a number of settings were tested (0.95, 0.9 and 0.85). The results showed here were performed with 0.85 minimal probability of TDFs belonging to the cluster. . The access to the cluster algorithm is possible at the following URL: http://homes.esat.kuleuven.be/~thijs/Work/Clustering.html

For clustering, the results of several primer combinations are analyzed simultaneously, therefore unique fragment identifiers were generated consisting of the primer combination identifier and the length of the fragments. The clustering is performed on log transformed ratios. An important step is to filter the data prior to clustering (Tavazoie *et al.,* 1999). During filtering we selected the most variable genes using the variance about zero as a metric of variation. The final data transformation step consists of standardization of the log ratios. The expression values for a TDF across all time points are standardized (linearly scaled) to have mean 0 and standard deviation 1, and these standardized values are used to calculate a distance matrix.

5.3 Results

5.3.1 Plant analysis and cDNA-AFLP protocol implementation

Common bean inoculations with *R. etli* strain CNPAF512 were conducted as described by Snoeck et al. (2003). The same system was also possible to use for bean inoculation with *F. solani* f. sp. *Phaseoli* and the control treatments. The successful interaction with *R. etli* was clearly visible starting 5 days post inoculation by the appearance of root nodule primordia. At the same time point, the first visible symptoms of *Fusarium* infection appeared as reddish discolorations. From 6 days post inoculation onwards both the number of nodules and the visible symptoms caused by *Rhizobium* and *Fusarium* proliferation, respectively, increased.

However, none of the fungus-inoculated plants were stunted or showed an altered growth profile. Clearly both root-interacting microorganisms showed infection patterns, allowing us to use this test system for reliable subsequent analyses. Control-inoculated plants did not show any root alterations at all. At 8h, 16h, 32h and next daily during 4 consecutive days, roots from 3 to 5 plants per treatment were collected. Roots from all the conditions were crushed in liquid nitrogen for the further RNA isolation and cDNA synthesis.

For the implementation of cDNA-AFLP protocol, *BstY*I and *Mse*I adaptors were made by heating a mixture of the two complementary oligonucleotides. The ligation of both adaptors was performed and incubated at 37^{o}C. Furthermore, the pre-amplification reaction was done with the adaptor-ligated cDNA as template and the selective primer combinations. The selective amplifications were performed using the pre-amplified cDNA and the selective primer combinations shown in Table 5.1. After loading the cDNA-AFLP product, electrophresis and drying of the preparative gels, the fluorescence images were taken for the *in silico* analysis.

5.3.2 Quantitative expression profiles using a novel tool for differential gene expression analysis

TDFs were visualized and analyzed using the ImageMaster 1D Elite software prior to systematic quantitative analysis (see Fig. 5.1). After visual inspection of the 1D Elite gel images, software was written in Matlab with the objective of preprocessing the data and selecting TDFs which show the largest difference in expression in plant roots treated with *R. etli* (R) or *F. solani* (F) compared to untreated plants (W). To analyze the early interaction of common bean roots with the inoculation of beneficial and pathogen microorganisms, the starting material consisted of the cDNA-AFLP data, originating from the 7 different primer combinations. The resulting data set consisted of the expression levels of 504 TDFs with varying lengths between 100 and 500 base pares (bp), measured upon the 3 conditions (R, F, W) over a time course of 8 time points (8h, 16h, 32h, 2d, 3d, 4d, 5d, 6d).

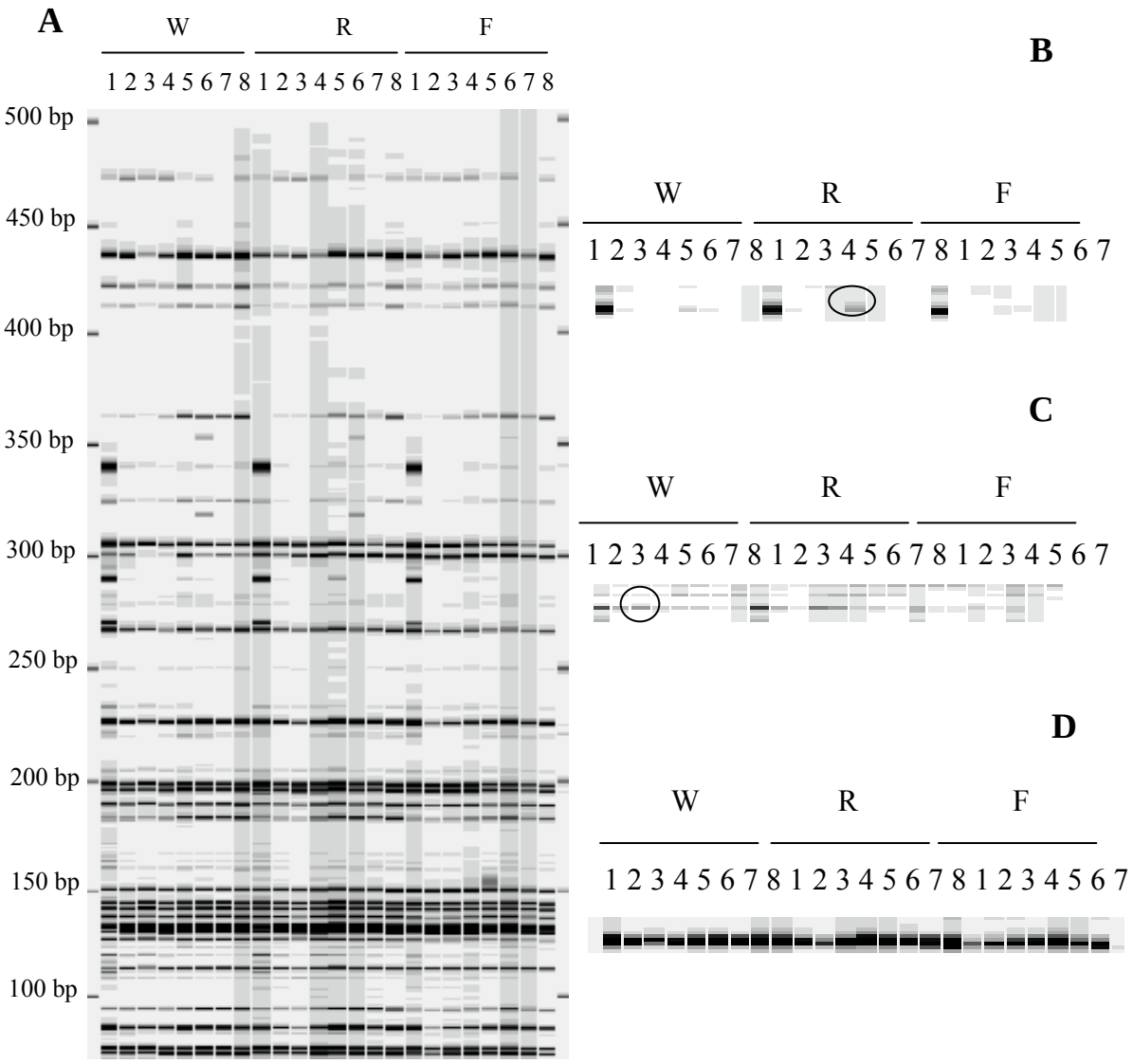

Figure 5.1 Representation of the quantitative analysis by ImageMaster 1D Elite software (Amersham Biosciences). Section A represent an example of one analytic gel with the 3 conditions (W: control; R: inoculation with *Rhizobium* CNPAF512; F: infection with *Fusarium solani* f.sp. *phaseoli*) and 8 time points (see above). Section B shows an example of TDFs differentially expressed for *Rhizobium* (R) treatment at time point 5 (3 days post-inoculation). Section C shows an example of TDFs differentially expressed for control (W) treatment at time point 3 (32 h post-inoculation). Section D shows the same expression in TDFs for all the condition and time points.

The expression profile for each TDF was separated taking into account the data preprocessing and the variance among conditions. Following this approach, the software was subsequently used to select 78 TDFs that showed no signs of errors introduced by the 1D Elite software and with high expression profile for R/W or F/W. Although very time-consuming, this step is required since the analysis of repeat experiments showed that the majority of the noise is

introduced during the 1D Elite image analysis e.g. erroneous matching, merging or division of bands.

The data preprocessing procedure can be used to preprocess cDNA-AFLP data generated on a classical gel system. Other systems, e.g. based on capillary gels, need a different type of lane correction than the one that is implemented with this software. The variance analysis can be used on all expression data in which the reference sample is not common but varies together with the test sample e.g. in time. Therefore, this technique is also applicable to microarray data when the reference sample is not common. However, it should be noted that such microarray data should be subjected to preprocessing prior to variance analysis especially to compensate for global intensity differences on different arrays since different time points or conditions are measured on different microarrays and not on the same gel as is the case for the cDNA-AFLP technique (Durrant et al. 2000; Qin et al. 2000; Sutcliffe et al. 2000; van der Biezen et al. 2000; Kornmann et al. 2001).

Figure 5.2 shows a demonstration of the $\log_{10}$ intensities generated by Matlab for the selected TDFs. Both intensities for R, F, W and the ratios for R/W, F/W were released from preprocessing and the variance analysis to select the differential expression profiles. Taking into account this variance for R/W or F/W, a total of 1008 different profiles for the complete dataset was generated.

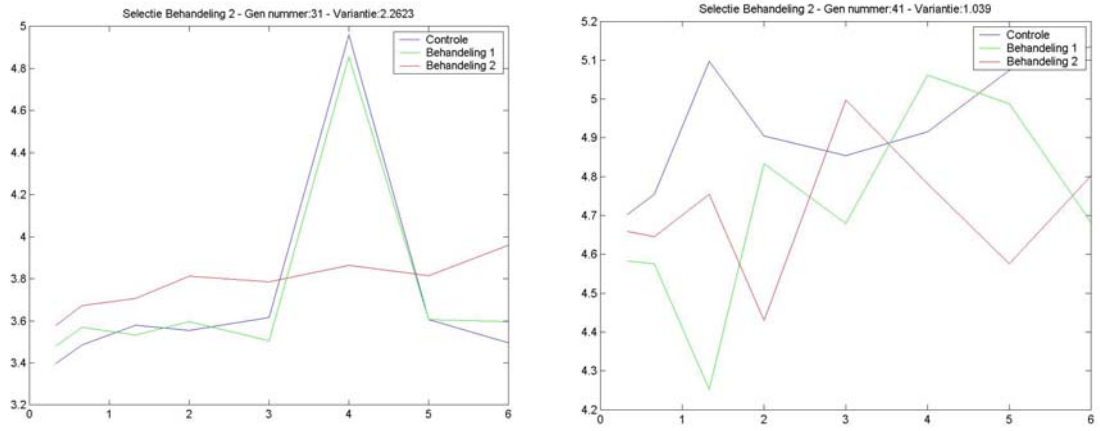

Figure 5.2 Demonstration of the $\log_{10}$ intensities generated by Matlab showing differential expression profile of TDFs in the different time point analyzed for: — intensity for *Rhizobium* (R) treatment, — intensity for *Fusarium* (F) treatment inoculation, — intensity for control (W) inoculation.

As mentioned, a total of 7 primer combinations (only 1.36% of the possible 512 combinations) were used in our study. Although it seems to be a low value as compared with all the possible primer combinations, our data set is rather large (504 transcripts) taking into

account the 8 time points and the 3 conditions analyzed. A large number (85%) of transcripts were expressed under the three treatments, thus representing non-differentially expressed genes for *Rhizobium*, *Fusarium* or non-treated condition.

Table 5.2 shows the number of TDFs differentially expressed and excised from the preparative gels, as well as the positive reamplified fragments for each condition after the quantitative analysis by the novel tool applied. Several TDFs displayed an altered expression pattern. However, after the reamplification reaction with the selective primer combination and the electrophoresis analysis, unfortunately only 33 TDFs could be reamplified, matching the same length as compared with the preparative gel. Of the 33 TDFs positively reamplified, 16 of them were cloned and sequenced.

Table 5.2 Differential expressed TDFs after quantitative analysis

Origin of TDFs	N° of differential expressed TDFs and excised	N° of positive TDFs reamplified
Fragments for *Rhizobium* treatment	52	19
Fragments for *Fusarium* treatment	7	3
Fragments for control treatment	19	11
Total of TDFs	78	33

5.3.3 Identification of differentially expressed genes

Table 5.3 shows the summary of the sequence analyses compared with NCBI-BLASTn Genbank database (Altschul et al., 1997). The TDFs denomination was represented by the number of the preparative gel analyzed, the line number and the position in the gel form which they were excised. However, to facilitate the identification, we describe them with simple numbers. For some of them, like 2.1 or 4.1, they belong to the same TDF (previous one) but to different clones, representing different sequences (i.e 2.1 represents a sequence of a clone of the TDF 2).

Table 5.3 Summary of the TDFs clones identified by cDNA-AFLP and the homology with hitherto known sequences (NCBI-BLASTn)

TDFs-sequence denomination	Length (bp)	Selection*		Accession	Homology (BLASTn)	Organism	E-value
		Cond.	Time				
1	413	R	8	AF532628	14-3-3 protein	*Glycine max*	6E-04
2	309	R	1	AB236791	putative galactose kinase	*Trifolium pratense*	2E-61
2.1	309	R	1	AJ630104	galactokinase (galK gene).	*Pisum sativum*	2E-54
4	301	W	5	AF529300	ascorbate oxidase precursor	*Glycine max*	6E-74
4.1	301	W	5	Y15295	L-ascorbate oxidase	*Medicago truncatula*	9E-17
6	202	R	7	DQ455283	cDNA-AFLP fragment BT11M24_216	*Medicago truncatula*	1E-05
7	149	R	7	AP004488	chromosome 2, clone LjT02F05	*Lotus japonicus*	2
8	436	F	3	AB020746	ALDH2C4; aldehyde dehydrogenase	*Arabidopsis thaliana*	2E-08
9	217	R	1	AM475652	contig VV79X000412.3	*Vitis vinifera*	7E-48
10	166	F	7	AJ132212	MADS domain transcription factor GGM6	*Gnetum gnemon*	2E-96
11	274	W	2	AB255435	plasmid pO86A1 DNA	*Escherichia coli*	2E-108
12	177	R	6	AB069650	16S rRNA gene, JEYF16	*Rhizobium* sp	3E-100
13	175	R	5	AM712156	16S rRNA gene, clone Geo18-Geo825R	*Geobacter* sp.	1E-72
14	175	R	5	AJ851868	Immunoglobulin heavy chain locus	*Mus musculus*	3E-100
15	196	R	8	-	No match homology	-	-
16	269	F	3	-	No match homology	-	-
17	191	W	2	-	No match homology	-	-
18	301	R	5	-	No match homology	-	-

* selection of the TDFs for the condition and time point. R: *Rhizobium* inoculation, F: infection with *Fusarium*, W: control. TDFs 7.1 and 10.1 represent different clones.

In our study we focused on genes which were differentially expressed in symbiosis and/or pathogenesis versus control condition in common bean (c.v BAT477). A total of 11 sequences corresponding to the *Rhizobium* condition, 4 sequences for the control and 3 sequences for *Fusarium* condition were selected. Interestingly 56% represent sequences with homology to plant sequences and from them, 78% show homology with legumes-related genes. 22% of the sequences reveal matches with other organisms and another 22% do not match with hitherto known sequences.

The TDFs were grouped according to putative function and presented in figure 5.3. Several TDFs differentially expressed for the *Rhizobium* condition show homology with genes that represent different functions, mainly related with stress/defense. The TDF 1 shows homology to the 14-3-3 protein in *Glycine max*. The TDF 2 and the clone 2.1 are showing homology with genes related with galactose kinase activity in *Trifolium* and *Pisum* respectively. Other TDFs for this condition like, 6, 9, 12 and 13 show homology with non-plant genes and positioned in different functional categories.

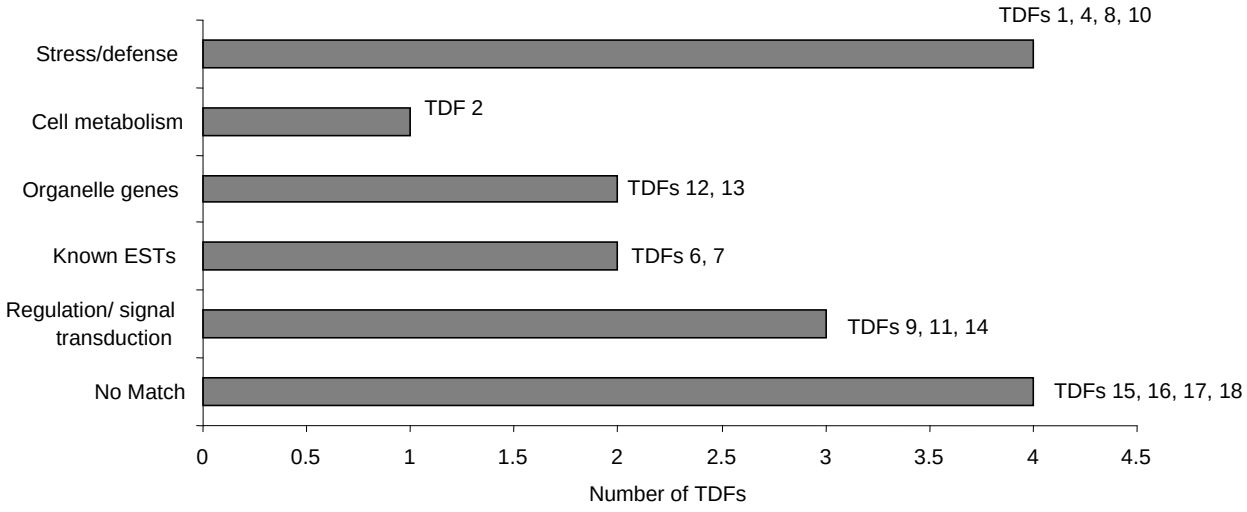

Figure 5.3 Classification of the TDFs according to the putative genes functions of the sequences retrieved.

For the *Fusarium* condition, the TDFs 8 and 10 showed homology with *Arabidopsis thaliana* genes encoding aldehyde dehydrogenase ALDH2C4 and homology with the *Gnetum gnemon* gene encoding the transcription factor GGM6 respectively. For both cases, the functions have been related with stress/defense genes. For the control condition, the TDF 4 and the clone 4.1 matched homology with genes encoding ascorbate oxidase in *Glycine max* and *Medicago*

truncatula respectively. A total of 4 TDF-sequences did not match homology with any known sequence in GenBank database.

5.3.4 Identifying groups of TDFs with similar expression pattern

The cluster analysis was performed using AQBC algorithm in collaboration with Dr. Janick Mathys. The entire dataset generated from the quantitative analysis using ImageMaster 1 D Elite software was analyzed simultaneously to identify groups of TDFs with similar expression patterns.

We generated identifier characters for each TDF differentially expressed taking into account the primer combination and the length of the fragments. In our study we have clustered the log ratios for R/W (1) or F/W (2). This means that the results of the clustering are groups of fragments that show the same changes in expression, relative to the expression of these fragments in control plants.

In the filtering step, TDFs expression profiles that show no clear difference between R/W or F/W were discarded. The filtering was adjusted to remove half of the TDFs from the dataset and retaining only the most variable data. The 252 remaining TDFs (504 expression profiles for log ratios R/W and F/W) were standardized and subjected to clustering. The minimal number of TDFs in a cluster was set to 2 and the minimal probability of TDFs belonging to a cluster was set at 0.85.

A total of 9 clusters with different expression pattern were obtained. As observed in figure 5.4, each cluster shows the variability in expression pattern for the 8 time points evaluated. The identifiers from the differentially expressed TDFs selected were compared with the clustering output to detect the number of TDFs included in each cluster. Unfortunately only 3 selected TDFs of which the sequence was determined were found to be included in clusters. These results were due to the high number of transcripts discarded during the data filtering. Obviously, reducing the level of TDFs discarded increases the number of TDFs belonging to each cluster. However, discriminating the less variable data provides a strict expression pattern of the similar TDFs.

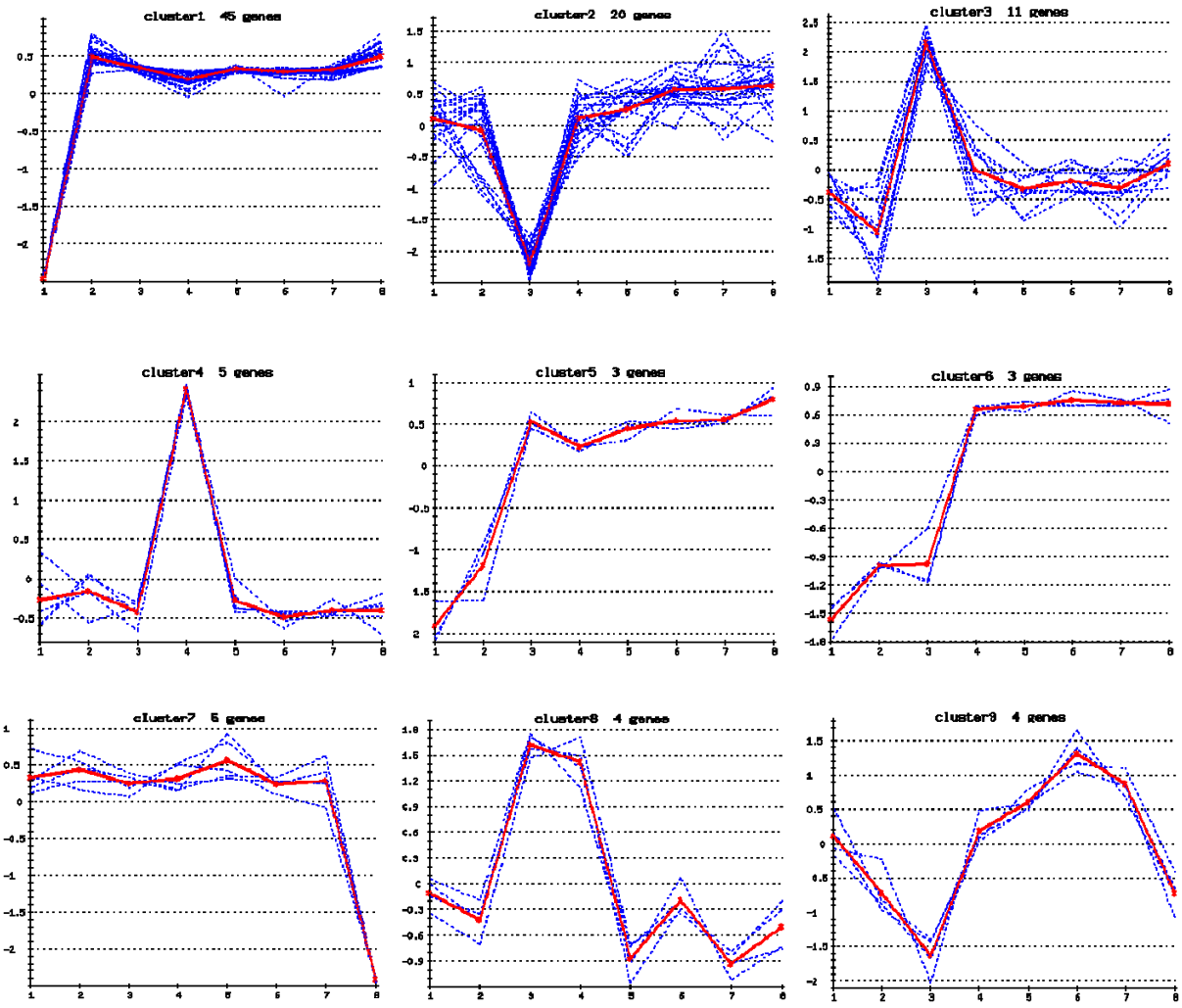

Figure 5.4 Clustering result for 50% of the most variable TDFs generated form the quantitative analysis. The 9 clusters represent the expression profiles of the log ratios for 1 (R/W), 2 (F/W) or both (1 and 2) for the entire 8 time point analyzed. ---- log ratios plots, —— cluster mean.

The TDF 8 was included in cluster 3, showing the same expression pattern for log ratio 1 (R/W, symbiosis) and 2 (F/W, pathogenesis), having similarity with 10 TDFs. The TDF 15 was included also in cluster 3, showing the same expression pattern for log ratio 2 (F/W, pathogenesis), having similarity with 10 TDFs. The TDF 4 and the clone 4.1 were included in cluster 8, showing the same expression pattern for log ratio 2 (F/W, pathogenesis), having similarity with 3 TDFs. The variability of the gene expression pattern belonging to the clusters 3 and 8 at different time points analyzed are discussed below.

5.4 Discussion

In our study we analyzed the quantitative profile of differentially expressed transcript for symbiosis and pathogenesis in common bean cv. BAT 477.

Following the cDNA-AFLP protocol, images from the preparative gels were analyzed by using ImagenMaster 1D Elite and Matlab software. Although differential expression can be discriminated by visual scoring, automated analysis with the appropriate software is more sensitive and reliable, generating stronger quantitative expression data profiles. In total 504 transcripts were retrieved with the 1D Elite software corresponding to the 7 primer combinations, 3 conditions and 8 time points analyzed. The expression profiles of the data were analyzed in Matlab to select the most variable profiles for R or F with W. The lane intensities obtained for all the conditions were transformed into the logarithm base two of the ratios of the measured expression levels for R/W and F/W.

Thorough quantitative analysis is indispensable to understand large-scale gene expression studies. So far, only a minority of cDNA-AFLP mediated gene expression studies are supported by quantitative data analysis (e.g. using Quantar Pro software (Breyne et al., 2002 and 2003)). The results of the majority of the studies, however, are based on just visual or arbitrary scoring of differentially expressed genes (Durrant et al., 2000).

Despite the recent development of high-throughput full-genome expression systems like microarrays, which rely on comparison of two samples and prior knowledge of gene sequences, cDNA-AFLP remains a useful technique since several transcript pools can be compared in the same experiment. In this study we compared three different conditions in common bean plants: a control, symbiosis challenge (*Rhizobium*) and pathogenicity challenge (*Fusarium*). This allowed us to choose transcripts expressed only in one or several conditions, offering an advantage to effectively obtain transcripts for symbiosis, pathogenesis or both. Another important feature of this study is comparison with transcripts from cDNA libraries. Libraries of cDNA are in use since quite some time, mainly for cloning specific genes, and recently for generating ESTs (Hernández et al., 2007). We have described here the cDNA-AFLP technique to demonstrate successful isolation of differentially expressed transcripts. This has several advantages: *i.* it requires only a simple PCR with flanking vector primers to rescue the cDNAs in the library; *ii.* based on the TDF sequence, full-length cDNA can be isolated from the library either by PCR with primers designed from the TDF or by screening

the library with the TDF. One disadvantage in the PCR amplification before AFLP may be the reduced sensitivity to differences between the transcript levels that may lead to failure in discriminating the marginally differing transcripts (Nimbalkar et al., 2006).

Form the selected TDFs, 16 of them were successfully cloned and sequenced (see table 5.3). The retrieved sequences with plant origin revealed in 78% of the cases homology with genes form legumes.. Around 25% of the TDFs did not match homology with any sequence, which could be associated to unknown function (Borras-Hidalgo et al., 2006).

The results of the sequence analysis revealed that 25% of the sequenced TDFs were related with stress/defense (e.g: ALDH2C4, 14-3-3 protein, ascorbate oxidase and transcription factor GGM6) and 6.25% with cell metabolism (galactose kinase genes). Here we will discuss only the most important characteristics of these two functional groups to link the possible roles with our study.

Induction of stress/defense resistant gene TDFs in common bean

- *The 14-3-3 protein*

In our work we detected gene encoding 14-3-3 protein in *Rhizobium* condition at time point 8, having homology with *Glycine max* and with a high score of E-value. 14-3-3 has emerged as a group of multifunctional proteins that bind to and modulate the function of a wide array of cellular proteins. More than 50 signaling proteins have been reported as 14-3-3 ligands. This broad range of partners suggests for 14-3-3 protein a role as a general biochemical regulator of diverse biological processes in mammalian, including neuronal development, cell growth and viral and bacterial pathogenesis (Fu, et al., 2000). In plants, the role in regulation of primary metabolism, ion transport, cellular trafficking, gene transcription and stress/defense has been reported (Bunney et al., 2002; Gévaudant et al. 2007).

Several studies have focused on the influence of 14-3-3 genes in plant defense responses (Collinge et al., 1997; Gregersen et al., 1997; Bunney et al., 2002; Nimbalkar et al. 2006; Gévaudant et al., 2007). One of the earliest reports of a 14-3-3 protein in plant resulted from a subtractive cDNA library screen for transcripts accumulating in barley leaves after inoculation with the non-host powdery mildew fungus, *Blumeria* (syn. *Erysiphe*) *graminis* f.sp. *tritici* (Brandt et al., 1992; Thordal-Christensen et al., 1992). The transcript for the 14-3-3 protein was seen to accumulate early, though weakly, in the defense response concomitantly with a

number of other defense-related transcripts (encoding pathogenesis-related proteins and peroxidase among others). In contrast to other interactions, no difference in the accumulation of 14-3-3 transcripts was noted between compatible and incompatible interactions with the barley powdery mildew fungus (*Blumeria graminis* f.sp *hordei*). This suggests that the transcript accumulation is associated with the fungus penetration stage.

Evidence that 14-3-3 proteins have a role in defense response is explained through regulating the proton pump (H^+-ATPase) to activate the hypersensitive response: the pH drops under epidermal tissue undergoing a hypersensitive response (HR), the HR is stimulated by the fungal toxin fusicoccin (FC). FC prevents dissociation of the H^+-ATPase/14-3-3 complex (Baunsgaard et al., 1998; Olsson et al., 1998; Piotrowsky et al., 1998; Oecking and Hagemann, 1999; Kanczewska et al., 2005). Furthermore, FC binding activity of an epidermal microsomal fraction increases upon pathogen attack, and a 100 kDa protein which co-migrates with the H^+-ATPase accumulates and binds the 14-3-3 proteins (Finnie et al., 2002).

The activity of the H^+-ATPase is deregulated by FC, resulting in membrane hyperpolarization and alteration of ionic gradients. This affects a number of plant processes, including cell expansion, seed germination, stomatal behavior, and nutrient uptake (for review, see Marré, 1979). The H^+-ATPase has also been proposed to play a direct role in the regulation of growth and development. It is regulated at both the transcriptional and posttranslational levels by auxin, a major growth hormone, and has been proposed to be a key player in cell elongation. According to the acid growth theory, upon activation by auxin, the H^+-ATPase acidifies the apoplasm and thus activates enzymes involved in cell wall loosening (for review, see Rayle and Cleland, 1992; Hager, 2003). Changes in membrane potential are also associated with the initiation of a number of other signal transduction pathways, in particular those involved in pathogen and in stress responses (Ward et al., 1995).

The detection of the 14-3-3 protein in the *Rhizobium* condition could possibly be related to a signal transduction pathway in compatible *Rhizobium*-legume interaction. Although the role of 14-3-3 protein genes in plant defense has been well documented, no available information about the influence in symbiosis has been reported. The finding of a 14-3-3 gene in common bean roots treated with *Rhizobium* at time point 8 (6 days after inoculation) and the link between the high affinity of 14-3-3 protein with H^+-ATPase, could be related to the mechanisms of colonization and nitrogen fixation efficiency in *Rhizobium*-bean symbiosis,

taking into account the high energy cost of biological nitrogen fixation are partly caused by hydrogen production during the reduction of N_2 to ammonia (Stam et al., 1987).

- *Aldehyde dehydrogenase (ALDH2C4) gene*

Aldehyde dehydrogenase (ALDH) genes belong to a large family of genes related with plant stress (Kotchoni, 2004). Aldehydes are long-lived molecules that can be generated from various endogenous sources (metabolism of amino acids, carbohydrates, vitamins and lipids) and exogenous sources such as abiotic and biotic stress (Sophos and Vasiliou 2003, Sunkar et al. 2003). The majority of the genes in the aldehyde dehydrogenase family are related with abiotic stress. Recent reports described the finding of a reduced epidermal fluorescence1 (*REF1*) gene encoding ALDH involved in ferulic and sinapic acid biosynthesis, which has been designated ALDH2C4 (Nair et al., 2004, reviewed by Krich et al., 2005). *REF1* has been reported to have a useful application in crop improvement because of its role in cross-linking cell wall-bound polysaccharides to lignin (Grabber et al 2000, Grabber et al 2002 reviewed by Kotchoni, 2004).

Ferulic and sinapic acid are phenolic compounds (secondary metabolites) synthesized in a wide range of monocotyledonous and dicotyledonous plants (Nair et al., 2004) involved in plant defense, specifically in relation to antifungal activities (Tamari and Kaji 1954; Punja 1985; Demyttenaere et al., 1997; Sarma and Singh 2003).

In our study the TDF 8 shows homology with the ALDH2C4 gene in *Arabidopsis*. This TDF was selected in the *Fusarium* condition at time point 3. Sarma and Singh (2003) have reported the antifungal activity of ferulic acid in chickpea against *Sclerotium rolfsii*. The fungal growth was decreased with the increase of ferulic acid concentrations and the mycelium was completely inhibited at a concentration of 1000 $\mu g\ ml^{-1}$.

- *Ascorbate oxidase related genes*

The concept that plants respond to environmental stress by inducing defense pathways and slowing vegetative growth is widely accepted (Pignocchi et al., 2006). Key changes in gene expression are engaged leading to a decrease in cell division and elongation growth and an increase in pathogen resistance (Knight and Knight, 2001). Although the nature of the mechanisms that control these processes is poorly understood, it is considered to involve the coordinated regulation of antioxidant defenses and plant hormones (Pignocchi et al., 2006). In

this regard, the apoplast and cell wall act as a reservoir of information on the biotic and abiotic environment surrounding the cell as well as a major conduit of information between cells. Similarly, the plasmalemma has major functions in stress perception and the subsequent appropriate control of growth and defense (Fath et al., 2002; Achard et al., 2006).

The TDF 4 and the clone 4.1 retrieved from the control condition, were found to have homology with ascorbate oxidase (AO) genes. Ascorbate oxidase is a cell wall-localized enzyme that uses oxygen to catalyse the oxidation of ascorbate (AA) to the unstable radical monodehydroascorbate (MDHA) which rapidly dissociates to yield dehydroascorbate (DHA) and AA, and thus contributes to the regulation of the AA redox state (Fotopoulos et al., 2006).

Apoplastic AA is thought to represent the first line of defense against potentially damaging external oxidants, and may play an important role in mediating response to stresses generating an enhanced oxidative burden (Barnes et al., 2002; Pignocchi and Foyer, 2003). Many environmental and metabolic triggers, including pathogen attack, ozone, and physical and chemical assaults, alter the redox state of the apoplast by triggering an oxidative burst at the plasmalemma (Foreman et al., 2003).

Another interesting function of AO in plants is the fact that its activity and its expression is modulated by complex transcriptional and translational controls (Esaka et al., 1992), and is closely correlated with cell expansion (Kato and Esaka, 2000). It has been shown that the AO transcript levels increased by growth promoters (e.g. auxin, Pignocchi et al., 2003; jasmonates, Sanmartin, 2002) and reduced by growth suppressors (e.g. salicylic acid; Sanmartin, 2002; Pignocchi et al., 2003).

Both functions of AO, controlling the redox state and cell expansion, have been reported to modify the hormone signaling expression and the orchestration of defense processes in pumpkin (*Cucurbita maxima*) and tobacco (*Nicotiana tabacum*) respectively (Pignocchi et al., 2006; Esaka et al., 1992). In legumes, Bashor and Dalton (1999) reported the positive effect of AA mediated by the AO in *Glycine max*, affecting positively the nodule maintenance (reducing senescence) and the increase in nitrogenase activity, which is critical for N_2 fixation.

- *MADS domain transcription factor GGM6*

TDF 10 shows homology with the transcription factor GGM6 in *Gnetum gnemon*. This gene has several homologues of MADS-type floral homeobox genes (Winter et al., 1999). In our

work, the homolog has been detected in the *Fusarium* condition at time point 7 (5 days after infection). Although not many reports are available about the functional analysis of this transcription factor, recently Borras-Hidalgo et al. (2006) have reported the identification of GGM6 transcription factor in *Nicotiana megalosiphon* in response to tobacco blue mold (*Peronospora hyoscyami* f. sp. *tabacina*). By northern blot analysis they have shown the expression 4 days after the infection.

- *Carbohydrate metabolism genes in common bean with* Rhizobium *inoculation*

TDFs 4 and the clone 4.1 were detected in the *Rhizobium* condition after 8 h of inoculation and show homology with genes involved in metabolism. The sequences retrieved of these TDFs were homologues to *Trifolium pretense* and *Pisum sativum* respectively. The E-values of the sequences were considered as statistical significant.

Galactokinase is a key enzyme in the Leloir pathway of D-galactose metabolism in yeast and mammals. Galactokinase phosphorylates galactose to galactose-1-phosphate as the first committed step on the galactose catabolic pathway. The gene encoding the galactokinase protein has been isolated from several species of yeast and bacteria and two galactokinase genes have been isolated from human subjects. In humans, galactokinase deficiency results in elevated galactose levels (galactosemia) which can lead to cataract formation, and in some cases mental retardation (Kaplan et al., 1997).

The finding of GalK related genes in this study suggests a role of gatalctose catabolism in common bean after the *Rhizobium* inoculation, making possible the utilization of galactose at early stage of the interaction (Boucher et al., 2003). In plants galactokinase activity has been demonstrated in and purified from extracts of legumes plant such as: *Phaseolus aureus* (Neufeld et al., 1960; Chan and Hassid 1975); *Trigonella foenum-graecum* (Foglietti 1976); and *Vicia faba* (Dey 1983). Galactokinase is a single copy gene in *Arabidopsis*, which has been designated AGK1, and is expressed in all the major organs of the plant (Kaplan et al., 1997). Sherson et al. (1999) have found that the arabinose kinase gene of *Arabidopsis* ARA1 is a novel member of the galactokinase gene family. Only basic kinetic parameters have been reported and more genes have yet to be isolated (Kaplan et al., 1997).

Cluster analysis

Although a low number of TDFs sequenced were included in the clustering results, with this approach was possible to group TDFs with the same expression pattern. By analyzing the cluster 3 and 8 presented in figure 5.4, is possible to describe the responses of the genes belonging to each cluster at the different time point analyzed. However, table 5.4 shows a clear interpretation for the genes in each cluster at each time point analyzed.

Table 5.4 Expression pattern of the clusters to which the sequenced TDFs belong

Clusters	Time points							
	1 (8h)	2 (16h)	3 (32h)	4 (2d)	5 (3d)	6 (4d)	7 (5d)	8 (6d)
3		-	+	0	0	0	0	0
8	0	0	+	+		0		

0: expression is equal as compared with the expression in control plants, + : higher expression than in control plants, - : lower expression than in control plants. The time points where the cluster was too variable to make a statement were left empty.

As described above, the sequenced TDF 8 was placed in the cluster 3 taking into account the log ratios for R/W (symbiosis) and F/W (pathogenesis). The sequence retrieved in this TDF revealed homology with ALDH2C4. Looking at the cluster number (see Fig. 5.4) and the table 5.4, we would conclude that the gene was expressed at low level or down regulated at time point 2 and over expressed or up-regulated at time point 3 (32h) for both *Rhizobium* and *Fusarium* inoculation. For the time point 1 (8h), the clustering result was too variable to make a statement and for the other time points analyzed, expression was not different from expression in control plants. The same behavior was observed for TDF 15 in the same cluster, although in this case only for the log ratio F/W. However, in this case the sequence retrieved did not match homology with any known sequence.

The TDF 4 and the clone 4.1, with homology to the ascorbate oxidase genes in *Pisum* and *Glycine* respectively, were placed in cluster 8 for the log ratio F/W. These genes were over expressed at 32 h and 2 days after the *Fusarium* inoculation. At time points 1 (8h), 2 (16h)

and 6 (4d), the expression was not different from the expression in control plants. At time point 5 (3d), 7 (5d) and 8 (6d) the cluster result was too variable to make a statement.

The results outlined with the cDNA-AFLP protocol and the clustering performance revealed the feasibility to detect genes at early stage of the common bean interaction with symbiotic or pathogenic micro-organisms. However, our conclusions are preliminary since confirmation of the detected genes in symbiosis or pathogenesis by qRT-PCR is required.

Chapter 6

Major conclusions and perspectives

Most attention in this thesis has been directed towards some strategies to improve the *Rhizobium*-bean symbiosis since this symbiosis is known for its low rates of N fixation while common bean has an important role in integral agricultural production systems (Peoples and Ladha, 1995). In this work, combinations of *Rhizobium* and PGPR were investigated under different growth conditions to evaluate the possible contribution of phytostimulation on the performance of some common bean genotypes. Furthermore, *Rhizobium* strains isolated from Cuban soils were analyzed in symbiosis with common bean under optimal growth conditions and field conditions in order to evaluate their symbiotic phenotype and the impact of the bean genotype. The main focus has been given to the analysis of plant parameters and genes detection. However, as the *Rhizobium* and *Rhizobium*-PGPR compatibility, the genetic characteristics of the bacteria used, the ability of bacterial strains for physiological adaptation to environmental conditions are also important factors in the outcome of the symbiosis, some of these characteristics were also addressed.

The conclusions drawn form this study should be considered as support for the ongoing efforts to increase our knowledge on the interaction between common bean and beneficial microbes and to implement this knowledge in agricultural systems. The main conclusions and perspectives of our study have been formulated in correspondence with the goals set in the introduction.

OBJECTIVE 1: To determine the effect of *Rhizobium* inoculation and *Rhizobium*-PGPR co-inoculation in two common bean genotypes under different growth conditions

OBJECTIVE 2: To evaluate the host variation of the *Rhizobium* inoculation and *Rhizobium*-PGPR co-inoculation in two local Cuban bean genotypes under field conditions

Different combinations of *Rhizobium* and *Rhizobium*-PGPR were evaluated under controlled and field conditions in Cuba on local bean genotypes (chapter 2). Under pot experiment conditions, the plant growth parameters were stimulated with the *Rhizobium* (CIAT899) - *Azospirillum* (Sp7) co-inoculation at all the time points evaluated as compared with all the other treatments. The nodule number, dry weight of nodules, fresh and dry weight of roots were increased significantly with the co-inoculation of *Rhizobium* (CIAT899) - *Azospirillum* (Sp7), *Rhizobium* (CIAT899) - *Azotobacter* (isolated strain) and the single inoculation with *Azospirillum*. Under field conditions the variability among genotypes was evident. The combination of *Rhizobium* (CIAT899) and *Rhizobium* (6bIII) coinoculated with *Azospirillum* (Sp7) stimulated the nodulation, root and shoot parameters in ICA Pijao, while for BAT-304 the increase was obtained with the single inoculation of *Rhizobium* (6bIII). This study demonstrated that the growth parameters and yield can increase through the co-inoculation. The perspectives to short and also long term are focused on the conduction of more experiments under different environmental conditions to unravel the proper plant genotype-rhizobacteria combinations. The results obtained in this thesis, joint with previous reports (Remans, 2007) reinforce the implementation to short term of PGPR as commercial inoculant for co-inoculation with *Rhizobium* in local Cuban conditions to achieve the potentialities of common bean yield. The evaluation of edapho-climatic conditions, plant genotypes, influence of native strains and the signal transduction pathways that govern PGPR stimulation, specifically the contribution of bacterial hormone synthesis in the interaction, will contribute to offer integral “packages” to increase common bean N fixation and production.

OBJECTIVE 3: To characterize morphologically and genetically rhizosphere bacteria isolated from Cuban agricultural system

The genetic characterization of symbiotic bacteria has been a crucial issue to unravel the diversity of *Rhizobium* strains diversity occupying bean nodules. In our work we demonstrated the presence of both *Rhizobium etli* and *Rhizobium tropici* in common bean nodules in the field. Isolates from both species have the ability to nodulate ICA Pijao bean roots. However, there appears no statistical difference with the *Rhizobium etli* reference strain CNPAF512 for the parameters scored. Furthermore, we demonstrated the presence of *Agrobacterium tumefaciens* in bean nodules, which might negatively affect nodule performance and N fixation efficiency in common bean. It can be speculated that this could explain the low responses to *Rhizobium* inoculation observed in field condition as demonstrated in previous reports. However, more studies to short term on soil bacteria characterization need to be done. Also studies to determine the effect of plant root exudation on the bacterial rhizosphere biodiversity, the impact of physical and chemical soil characteristics, the effect of different symbiotic isolates on bean productivity and competence of isolated strain to form nodules and fix N, should be intensified. Isolation of *Rhizobium* strains well adapted to the local environment could contribute to the development of new effective inoculants. The genetic diversity between strains of a given and the influence of potential symbionts (i.e. *Ochrobactrum cytisi* isolated from soil) in interaction with legumes, should also be taken into account.

OBJECTIVE 4: To determine the influence of *Rhizobium* isolates on phenotypic parameters of two common bean genotypes under controlled growth conditions and field conditions

In the previous chapter we characterized morphologically and genetically the *Rhizobium* isolates from common bean nodules. However, as reported by Graham et al. (1991) and reviewed by Bala and Griller (2006), the *Rhizobium* phenotypic characterization still remains an essential ingredient of rhizobial classification. In chapter 4 we demonstrated the effective nodulation of common bean genotypes with the *Rhizobium* isolates. The nodulation kinetics under controlled growth conditions showed a significant increase in nodule number at early stages of common bean through the inoculation with *Rhizobium etli* RL-1, *R. tropici* RL-2 and

R. etli RL-5 isolates as compared with the reference strain CNPAF512. The nitrogenase activity showed that all the strains were able to reduce acetylene in symbiosis, although *R. etli* RL-1 had the lowest value. These results are in line with previous reports that the nodule number is not always correlated with the rates of N fixation.

The field experiments reinforce the above conclusion, where the nodulation parameters were increased with *R. etli* RL-1 in ICA Pijao, while this treatment did not affected the growth parameters or the yield. *R. tropici* RL-2 was the best strain for yield in ICA Pijao, while for BAT-304 no statistical differences were observed among the treatments. The results reported in this study evidence the ability of *R. etli* and *R. tropici* to establish a symbiotic interaction with common bean, although *R. tropici* is better adapted to the Cuban condition evaluated. Future works should focus on more detailed genetic, physiological and phenotypical analysis of rhizobia-bean variability in order to improve the N fixation and yield of bean genotypes in symbiosys with native rhizobia strains. The application of these characterized *Rhizobium* strains for common bean inoculant could be the first outcome from this study. Besides provide an effective source of new strains, it has been scientifically proved the increase in common bean growth and yield. The co-inoculation of those strains with PGPR can be another short term perspective to rich the desire increase in common bean production in low input system.

OBJECTIVE 5: To detect genes differentially expressed in common bean in interaction with *Rhizobium* using cDNA-AFLP technique

In chapter 5 we aimed for a better understanding of the molecular dialogue between common bean cv. BAT 477 and root interacting micro-organisms involved in symbiosis (*Rhizobium etli* CNPAF512) and pathogenesis (*Fusarium solani* f. sp. *phaseoli*). Using the cDNA-AFLP technique and in-house developed user's friendly software, it was possible to select a number of differentially expressed TDFs. After the cloning and sequence analysis, several genes matching homology with genes form leguminous plants were detected. In the *Rhizobium* treatment, the 14-3-3 gene homologue of *Glycine max* and the galactose kinase gene homologue of *Trifolium pratense* and *Pisum sativum* were detected. For the *Fusarium* condition, the ALDH2C4 (aldehyde dehydrogenase) gene homologue of *Arabidopsis thaliana* and the MADS domain transcription factor homologue of *Gnetum gnemon* were detected. The clustering analysis revealed the same expression pattern for respectively 11 TDFs including

the TDF 8 with homology to the ALDH2C4 genes for symbiosis (R/W) and pathogenesis (F/W) and for 3 TDFs including the TDF 4 and the clone 4.1 with homology to the ascorbate oxidase genes for pathogenesis (F/W).

Although our study revealed differentially genes expression in symbiosis and pathogenesis, the verification of the gene expression profiles through qRT-PCR is imperative to corroborate the cDNA-AFLP results.

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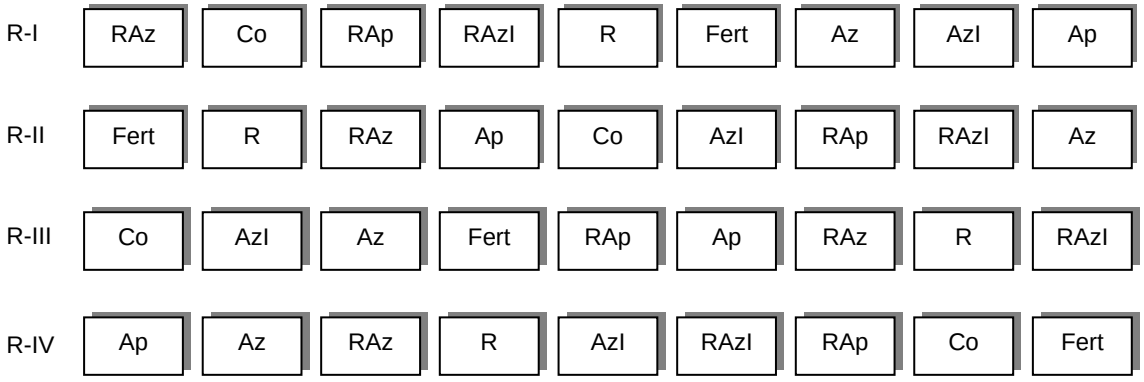
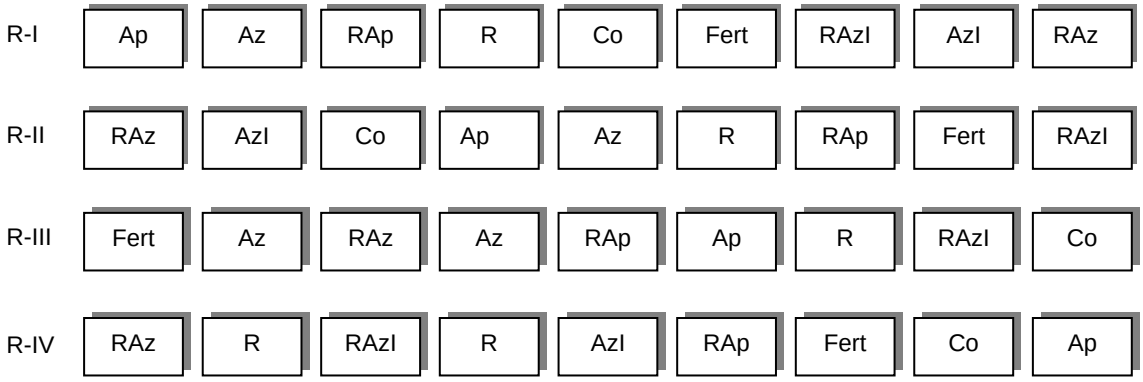
Annexes

Annex 1: Setup of randomized complete block design with 4 replicates used under controlled conditions in Santa Clara, 2005-2006 (see session 2.3.1, chapter 2).



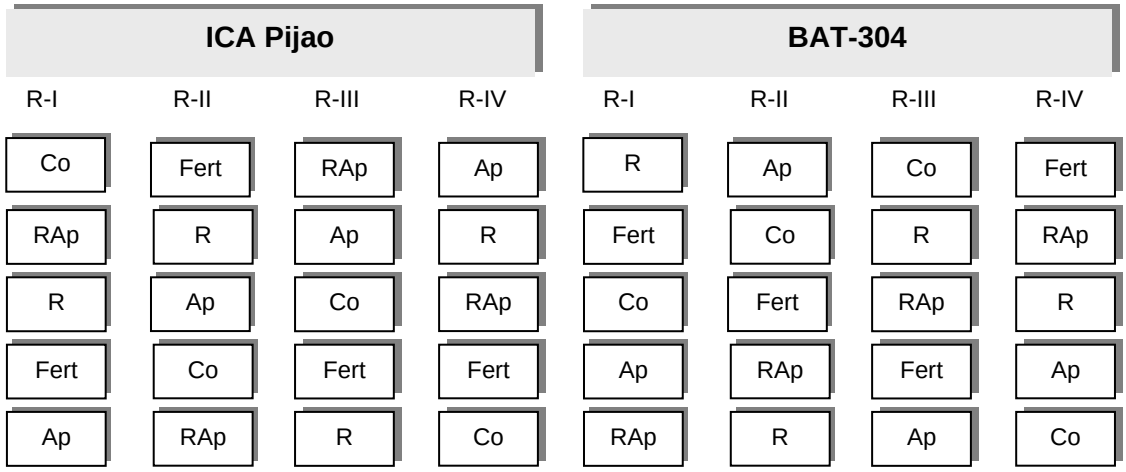
Treatments analyzed: R: inoculation with *Rhizobium* (CIAT 899); RAz: co-inoculation with *Rhizobium* and *Azotobacter* (MB-9); RAzI: co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain); RAp: co-inoculation with *Rhizobium* and *Azospirillum* (Sp7); Az: inoculation with *Azotobacter* (MB-9); AzI: inoculation with *Azotobacter* (isolated strain); Ap: inoculation with *Azospirillum* (Sp7); Fert: N fertilizer (60 kg ha^{-1}) and Co: no inoculation nor fertilizer.

Annex 2: Setup of randomized complete block design with 4 replicates used under field condition in Santo Domingo, 2005-2006 (see session 2.3.2, chapter 2).



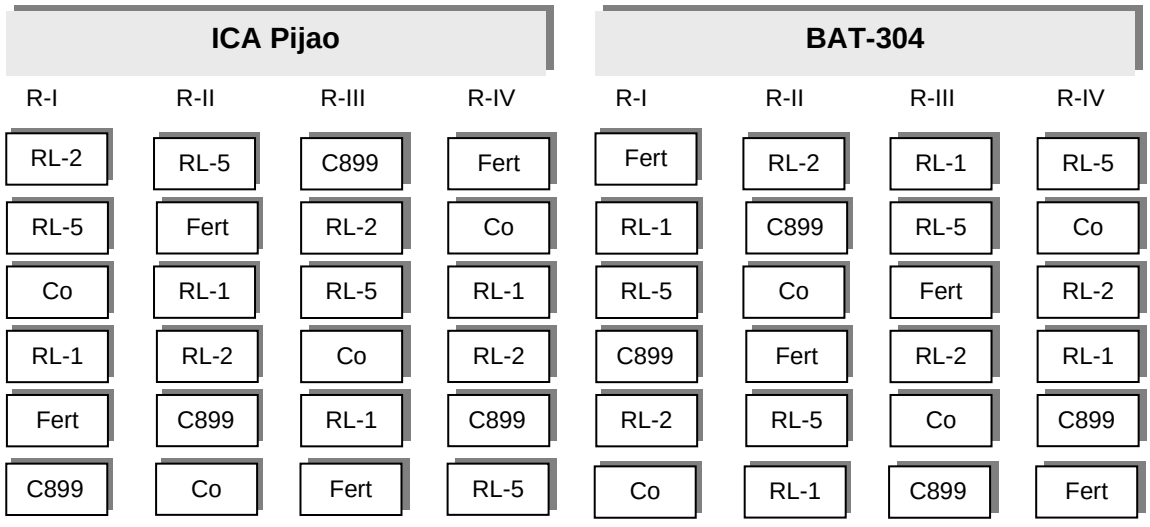
Treatments analyzed: R: inoculation with *Rhizobium* (CIAT 899); RAz: co-inoculation with *Rhizobium* and *Azotobacter* (MB-9); RAzI: co-inoculation with *Rhizobium* and *Azotobacter* (isolated strain); RAp: co-inoculation with *Rhizobium* and *Azospirillum* (Sp7); Az: inoculation with *Azotobacter* (MB-9); AzI: inoculation with *Azotobacter* (isolated strain); Ap: inoculation with *Azospirillum* (Sp7); Fert: N fertilizer (60 kg ha^{-1}) and Co: no inoculation nor fertilizer.

Annex 3: Setup of randomized complete block design with 4 replicates in Santa Clara, 2006-2007 (see session 2.3.3, chapter 2).



Treatments analyzed: R: inoculation with *Rhizobium* (6bIII); RAp: co-inoculation with *Rhizobium* and *Azospirillum* (Sp7); Ap: inoculation with *Azospirillum* (Sp7); Fert: N fertilizer (60 kg ha^{-1}) and Co: no inoculation and fertilizer.

Annex 4: Setup of randomized complete block design with 4 replicates in Quemado de Güines (see session 4.3.2, chapter 4).



Conditions analyzed: Co: no inoculation nor fertilization; C899: inoculation with *R. tropici* CIAT 899; RL-1: inoculation with *R. etli* RL-1; RL-2: inoculation with *R. tropici* RL-2; RL-5: inoculation with *R. etli* RL-5 and Fert: N fertilizer (60 kg ha^{-1}).

List of publications

Papers in international peer reviewed journals

- Remans R, Croonenborghs A, Torres Gutiérrez R, Michiels J and Vanderleyden J (2007). Effects of plant growth-promoting rhizobacteria on nodulation of *Phaseolus vulgaris* L. are dependent on plant P nutrition. Eur J Plant Pathol 19: 341-351.
- Remans R, Beebe S, Blair M, Manrique G, Tovar E, Rao I, Croonenborghs A, Torres Gutiérrez R, El-Howeity M, Michiels J and Vanderleyden J (2008). Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (*Phaseolus vulgaris* L.). Plant Soil 302: 149-161.

Papers in national reviewed journals

- Torres Gutiérrez R, Suárez N and Pérez C (2003). Influence of rhizobacteria inoculation on the germination of common bean (*Phaseolus vulgaris* L.) seeds. Centro Agrícola 3: 12-18.
- Torres Gutiérrez R, Suárez N and Pérez C (2006). Plant-Microb interaction: increments in common bean nitrogen fixation with *Rhizobium*-PGPR. Monographie. Ed. Samuel Feijo, UCLV, Santa Clara. Cuba. ISBN: 959-250-303-6. pp 82.

Reports in international reviewed newsletters

- Torres Gutiérrez, R, García J, Pérez C and Soria M (2003). Incrementos en la fijación de N_2 en el cultivo del frijol común (*Phaseolus vulgaris* L.). Ecoportal. http://www.ecoportal.net/articulos/frijol.htm
- Torres Gutiérrez, R, García J, Pérez C and Soria M (2004). Incrementos de la fijación biológica del nitrógeno mediante la inoculación combinada de bacterias fijadoras de N_2. Ilustrados. http://www.ilustrados.com/publicaciones/EpypZkykVlQGLUhfDt.php

Reports on international conferences and symposia

- Eichler B, Torres Gutiérrez R und Köppen D (2003). Einsatz von Mikroorganismen zur Verbesserung der Nährstoffversorgung von Pflanzen in den gemäßigten Breiten und in den feuchten Tropen. 46 Jahrestagung. Geissen, Germany. Book of abstracts.
- Torres Gutiérrez R, Suárez N and Pérez C (2004). Increments of biological nitrogen fixation by means of combined inoculation of atmospheric nitrogen fixation bacterias. 6th European Nitrogen Fixation Conference. Toulouse, France. Book of abstracts.

- Torres Gutiérrez R, Suárez N and Pérez C (2005). Plant microbe interaction: increments in symbiotic nitrogen fixation by means of PGPR (*Azotobacter* and *Azospirillum*) and *Rhizobium*. III International Conference on Agricultural Development and Sustainability. Santa Clara, Cuba. Book of abstracts.
- Remans R, Croonenborghs A, Torres Gutiérrez R, Michiels J and Vanderleyden J (2007). The effects of plant growth-promoting rhizobacteria on bean nodulation. Fifth Phaseomics meeting, Varenna, Italy. Book of abstracts.
- Remans R, Beebe S, Blair M, Manrique G, Tovar E, Rao I, Croonenborghs A, Torres Gutiérrez R, El-Howeity M, Michiels J and Vanderleyden J (2007). Detection of QTL affecting root responsiveness to auxin-producing plant growth-promoting rhizobacteria in common bean (*Phaseolus vulgaris* L.). 8th *Azospirillum* and related PGPR meeting. Montpellier, France. Book of abstracts. Awarded with 2nd Alan H. Gibson prize.
- Torres Gutiérrez R, Mathys J, Remans R, Hernández G, Michiels J, Cammue BPA, Vanderleyden J and De Bolle MFC (2007). Detection of genes differentially expressed in symbiosis/pathogen-*Phaseolus vulgaris* interaction using cDNA-AFLP. 6th European Conference on Grain Legume. Lisbon, Portugal. Book of abstracts.