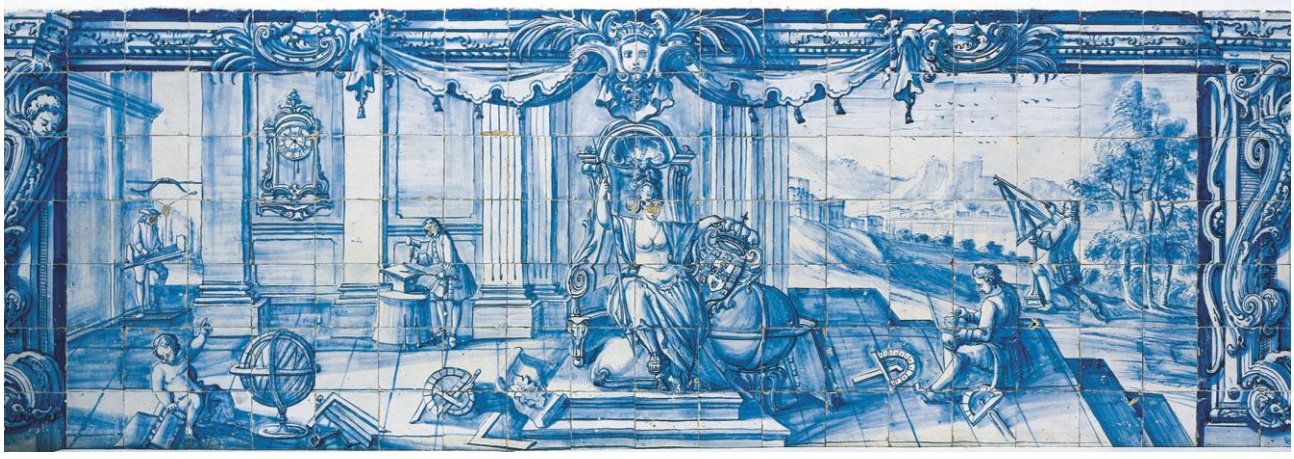
Improvement of chickpea rhizobia by genetic transformation with symbiosis genes

José Rodrigo da Silva

Thesis presented to obtain the PhD degree
in Biology by the University of Évora

SUPERVISORS: *Solange Oliveira*
Ana Alexandre

ÉVORA, MARCH 2018



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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Dona Ana, this is just for you!

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Abstract

Rhizobia are soil bacteria able to induce the formation of nodules in leguminous plants and convert atmospheric nitrogen into assimilable forms to these plants. Some *Mesorhizobium* species establish symbiosis with chickpea and can increase productivity of this culture. Rhizobia symbiosis genes, such as *nod* and *nif*, are involved in nodule development and nitrogen fixation. Nevertheless, genes involved in other molecular mechanisms, namely stress response may influence the symbiotic interaction plant-rhizobia. The objective of this study was to evaluate the effects of overexpressing symbiotic and stress response genes in the symbiotic performance of chickpea *Mesorhizobium*. *Mesorhizobium* strains were transformed with pRKnifA, pRKnodD, pRKenvZ and pRKgroEL (expression vector pRK415 with *nifA*, *nodD*, *envZ* and *groEL* genes from *M. mediterraneum* UPM-Ca36^T, respectively). From the four strains transformed with extra *nifA* copies, only V15-b was able to increase plant biomass, when compared to wild-type and empty vector strains. Among the four strains transformed with extra *nodD* copies, ST-2 and PMI-6 showed a higher symbiotic effectiveness compared to wild type and control strains. Additional copies of *envZ* led to in a higher symbiotic effectiveness when introduced in PMI-6 and EE-7. Evaluation of the symbiotic effectiveness of the four strains overexpressing *groEL* showed that only ST-2 improved, compared to wild-type and empty vector strains. For all these strains the rate of nodule formation was seen to be higher and further analysis of the infection process and nodule histological analysis were performed. Overall, this study shows that extra copies of a given gene may have different effects in the symbiotic effectiveness, depending on the modified strain. This study contributes to a better understanding of the nodulation and nitrogen fixation processes, namely regarding the contribution of non-symbiotic genes, especially *envZ*, which was to our knowledge for the first time reported to be involved in the rhizobia-legume symbiosis.

Resumo

Melhoramento de rizóbios de grão-de-bico por transformação genética com genes simbióticos

Rhizóbios são bactérias capazes de induzir a formação de nódulos em leguminosas e converter azoto atmosférico em formas assimiláveis por essas plantas. Algumas espécies de *Mesorhizobium* estabelecem simbioses com grão-de-bico e conseguem promover a produtividade desta cultura. Genes simbióticos, como *nod* e *nif*, estão envolvidos na formação dos nódulos e fixação de azoto. No entanto, genes envolvidos noutros mecanismos, nomeadamente a resposta ao stresse podem influenciar a interação simbiótica planta-rizóbio. O objetivo deste estudo foi avaliar a eficiência simbiótica de *Mesorhizobium* de grão-de-bico sobre-expressando genes simbióticos e de resposta ao stresse. Estirpes de *Mesorhizobium* foram transformadas com pRKnifA, pRKnodD, pRKenvZ e pRKgroEL (vetor de expressão pRK415 com *nifA*, *nodD*, *envZ* e *groEL* de *M. mediterraneum* UPM-Ca36^T, respetivamente). Das quatro estirpes transformadas com cópias extras de *nifA*, apenas V15-b foi capaz de produzir um aumento na biomassa das plantas inoculadas, quando comparada às estirpes selvagem e com vetor vazio. Das quatro estirpes transformadas com cópias extras de *nodD*, ST-2 e PMI-6 apresentaram maior eficiência simbiótica em comparação com as estirpes controlo. Cópias adicionais de *envZ* resultaram numa maior eficiência simbiótica quando introduzidas em PMI-6 e EE-7. A avaliação da eficiência simbiótica das quatro estirpes que sobre-expressam *groEL* mostrou que apenas a transformação de ST-2 levou a uma eficiência superior, em comparação com as estirpes selvagem e com vetor vazio. Para todas estas estirpes, a taxa de formação de nódulos também foi melhorada, pelo que análises do processo de infeção e da histologia dos nódulos foram efetuadas. Em geral, este estudo mostra que um gene introduzido pode ter efeitos diferentes na eficiência simbiótica, dependendo da estirpe modificada. Este estudo contribui para uma melhor compreensão dos processos de nodulação e fixação de azoto, nomeadamente a contribuição de genes não-simbióticos, especialmente *envZ*, que tanto quanto sabemos não foi previamente descrito como envolvido nestas simbioses.

Abbreviations

µm	micrometer
atm	atmosphere
ATP	adenosine triphosphate
AWN	average weight per nodule
BNF	biological nitrogen fixation
bp	base pair
cm	centimeter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpi	days post inoculation
EPS	exopolysaccharides
HSP	heat shock protein
kb	kilobase
min	minute
mm	millimeters
NN	number of nodules
OD	optical density
PCR	polymerase chain reaction
RDW	root dry weight

RNA	ribonucleic acid
SDW	shoot dry weight
SE	symbiotic effectiveness
TY	tryptone-yeast

Genes

16S rRNA	16S ribosomal RNA
envZ	osmolarity sensor protein
gfp	green fluorescent protein
groEL	Chaperone GroEL
nifA	nif-specific transcriptional activator NifA
nifD	nitrogenase molybdenum-iron-protein α-subunit
nifH	nitrogenase iron-protein
nifK	nitrogenase Molybdenum-Iron-protein β-subunit
nodC	NodC N-acetylglucosaminyltransferase
nodD	transcriptional regulation NodD
ompC	outer membrane porin protein C
ompF	outer membrane porin protein F
ompR	osmolarity response regulator
rfp	red fluorescent protein

Nucleotide Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

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Chapter 1

State of the art

1.1. Biological Nitrogen Fixation

It has estimated that by 2050 the world's population will increase 34 %, reaching 9.1 billion of people. This increase will occur mainly in developing countries. Urbanization will continue at an accelerated pace, and about 70 % of the world's population will be urban (compared to 49 % today). Feeding this larger, more urban and richer population, requires an increase in food production of 70 %. Annual cereal production will need to rise to about 3 billion tonnes from 2.1 billion today and annual meat production will need to rise by over 200 million tonnes to reach 470 million tonnes. It means that there will be an increase in cultivated areas, leading to an increased demand of fertilizers and research aimed to improve varieties of plants to increase their yield (FAO 2009).

After carbon (C) and hydrogen (H), nitrogen (N) is the most important element in the constitution of organic matter, representing 8-16 % (Vance 2001). Nitrogen participates in the formation of key molecules in many biological processes such as the production of nucleic acids and proteins. Despite its abundance in the atmosphere as N_2 (79 %), N is along with water, the main limiting factor of global agricultural production, because no animal or plant is able to use it directly (Hoffman et al., 2014). The main supply sources of nitrogen used for plant growth are: 1) derived from the decomposition of organic matter and rocks, 2) provided by chemical nitrogen fertilizers, 3) nitrogen supplied by the process of biological nitrogen fixation (BNF). There is also a small contribution by the reaction of electrical discharges with N_2, resulting in nitrate, which is added to the soil and represents about 4 % of positive entries in N balance on Earth (Hoffman et al. 2014).

The synthesis of nitrogen fertilizers began in the first decade of the twentieth century when Fritz Haber and Carl Bosch discovered the process that converts atmospheric N_2 into ammonia. The requirements for such chemical synthesis are: 1) hydrogen, 2) a catalyst containing iron, 3) high temperature (300° C to 600° C), 4) high pressure (200 atm to 800 atm) (Hoffman et al. 2014). Consequently, the economic cost for the chemical synthesis of nitrogen fertilizers is high, resulting mainly from the need of spending of fossil fuels, which are non-renewable energy (Hydroworld 2009). Alternative ways of supplying N to agricultural crops that to reduce these costs are urgent (Biswas and Gresshoff 2014).

Another aggravating factor in the use of nitrogen fertilizers is the low efficiency of its utilization by plants, rarely exceeding 50%. One should also consider that the indiscriminate use of nitrogenous fertilizers results in environmental pollution since the N leaching and runoff from the soil surface leads to accumulation of these compounds in the waters of rivers, lakes and groundwater, reaching toxic levels for fish and animal (Hungria et al. 2001; Jensen et al. 2012; Philippot and Hallin 2011; Richardson et al. 2009).

Some soil bacteria, designated rhizobia, can establish symbiotic associations with

legume plants, and fix atmospheric nitrogen, converting it into an assimilable form to the plant. In exchange, the legume plants provide carbohydrates from the photosynthesis process to rhizobia. This process, known as biological nitrogen fixation (BNF), can reduce the use of chemical N-fertilizers and thus decrease the environmental pollution (Oldroyd 2013) and contribute for more sustainable agricultural practices.

Considering the low cost of the bacterial inoculants compared with nitrogen fertilizers, the cost to produce legume grain could reduce, representing a great economy, which could be directed to other aspects of productivity improvement in agriculture (Vitousek et al. 2013).

The contribution of the biological nitrogen fixation to the total of produced nitrogen has been estimated to approximately 200 million tonnes annually and for every tonne of shoot dry matter produced by crop legumes, the symbiosis with rhizobia is responsible for fixing the equivalent of 30-40 kg of nitrogen (N) (Graham and Vance 2003; Peoples et al. 2009).

1.2. Rhizobia

Bacteria capable of fixing atmospheric nitrogen are called diazotrophs. These bacteria exist as free-living soil bacteria or associated with plant species, both in the rhizosphere, as endophyte or as symbionts of legume species. This latter group, more currently under study, is generically called rhizobia.

These microorganisms are able to metabolize atmospheric nitrogen and convert it into nitrogen compounds that can be absorbed by the plant. This process takes place in specialized structures on plant roots called nodules. In return, the rhizobia benefit from carbon substrates derived from photosynthesis in the plant. Contrary to the supply of nitrogen fertilizer to a crop in the form of nitrate, which represents a significant cost to both farmer and the environment, the inoculation of plants with rhizobia has environmental and economic benefits, such as use of native soils bacteria instead N fertilizer that may cause eutrophication in lakes and rivers, low cost of acquisition of the inoculant and crops rotation that keep the N accumulated from one culture to the next one (Herridge et al. 2008).

Rhizobia can live either in the soil as free-living bacteria or within the root nodules of host legumes. Within the nodules, rhizobia convert atmospheric dinitrogen (N_2) into ammonia as a result of the nitrogenase enzyme complex activity, in an ATP-dependent manner. Ammonia can be assimilated by the host, resulting in improved plant growth and productivity (Perret et al. 2000). The pairing rhizobia-legume host occurs after a complex molecular crosstalk between both partners (Downie 1998; Oldroyd et al. 2011),

which often requires cell-to-cell communication. This means that the entry into root cells requires appropriate recognition of specific chemical signals by the host plant, namely the rhizobial Nod factor signaling molecule (Oldroyd 2013). The recognition of these molecules triggers the curling of root hairs, allowing the entry of rhizobia in the plant (Oldroyd 2013). Rhizobia then induce the formation of an infection thread, which is an invagination of the plant membrane at the infection focus of the root hair. Through the deformed root hair, these bacteria enter the host plant cells and grow down to the cortical cell layers into the nodule meristem (Sprent 2009). In some cases, these bacteria enter the root by crack entry , i.e. insert themselves in cracks on the root cells (Sprent 2009). Rhizobia can also enter the root through epidermal intercellular spaces. A successful infection process ends with the formation of an effective nodule (Oldroyd and Downie 2008), which begins with the reinitiation of cell division in the root cortex, where rhizobial cells will be allocated and will initiate nitrogen fixation in exchange for carbon from the legume host (Oldroyd 2013).

In 1932, bacteria able to nodulate legumes were classified in the genus *Rhizobium* and subdivided by a criterion based mainly on host plant range (Fred 1932). In terms of taxonomy, rhizobia are currently assigned to 14 different genera. Most of them, including the agriculturally important nitrogen-fixing genera, belong to the *Alphaproteobacteria* class. Only a few genera belong to the *Betaproteobacteria* class (Chen et al. 2003; Moulin et al. 2014; Peix et al. 2015) (http://www.rhizobia.co.nz/taxonomy/rhizobia). There are approx. 89 genomes completely sequenced and annotated, including different strains and symbiovars from the same species. The size of most rhizobial genomes ranges between 6.5 and 9 Mb, and may include plasmids larger than 2 Mb (Alexandre and Oliveira 2016).

1.3. Nodulation and nitrogen fixation genes

Rhizobia genomes include two major components: the core genome (higher GC content) that comprises the housekeeping genes, which are responsible for the functioning of the cell, as well as other genes also involved in its essential maintenance (Crossman et al. 2008), and the accessory genome, which is located on plasmids or chromosomal islands (lower GC) and is composed of genes that confer special characteristics to these organisms, such as antibiotics resistance and symbiosis genes (MacLean et al. 2007; Young et al. 2006).

There are two main groups of genes responsible for the symbiosis process in rhizobia, namely genes involved in the nodulation process and those responsible for nitrogen fixation (Downie 1998; Kaminski et al. 1998). Nodulation genes (e.g. *nodABC*)

encode enzymes responsible for the biosynthesis and secretion of Nod factors, which are lipochitooligosaccharides (LCOs) that interact with plant flavonoids, thus important for determining the rhizobium-legume pairing (Oldroyd 2013; Via et al. 2016). Different rhizobia species can have different *nod* genes, therefore producing LCOs with varied structures (Limpens et al. 2015). Genes involved in the nitrogen fixation process include those that encode the nitrogenase enzyme (*nifHDK*), responsible for the capture and conversion of atmospheric nitrogen into ammonia (Kaminski et al. 1998). In addition to these major groups of genes, there are many others with important functions, both in nodulation and nitrogen fixation. For example, *nodPQ*, *nodX*, *nodEF* and *noe* genes are involved in the synthesis of Nod factors substituents (D'Haeze and Holsters 2002; Geurts and Bisseling 2002), while *nifA*, *fixLJ*, *fixK* encode transcriptional regulators and *fixABCX* are involved in the electron transport chain to nitrogenase (Dixon and Kahn 2004). Table 1 shows the most important genes involved in the symbiosis.

Table 1. Most common rhizobia genes involved in symbiosis (from Laranjo et al. 2014).

Genes	Function of gene product
Nodulation genes	
nodA	Acyltransferase
nodB	Chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator of common nod genes
nodIJ	Nod factors transport
nodPQ	Synthesis of Nod factors substituents
nodX	Synthesis of Nod factors substituents
nofEF	Synthesis of Nod factors substituents
other *nod* genes	Several functions in synthesis of Nod factors
nol genes	Several functions in synthesis of Nod factors substituents and secretion
NOE genes	Synthesis of Nod factors substituents
Nitrogen fixation	
genes *nifHDK*	Nitrogenase
nifA	Transcriptional regulator
nifBEN	Biosynthesis of the Fe-Mo cofactor
fixABCX	Electron transport chain to nitrogenase
fixNOPQ	Cytochrome oxidase
fixLJ	Transcriptional regulators
fixK	Transcriptional regulator
fixGHIS	Copper uptake and metabolism
fdxN	Ferredoxin
Other genes	
exo	Exopolyssacharide production
hup	Hydrogen uptake
gln	Glutamine synthase
dct	Dicarboxylate transport
nfe	Nodulation efficiency and competitiveness
ndv	β-1,2 glucans synthesis
lps	Lipopolysaccharide production

1.3.1. Nodulation process

What distinguishes essentially symbiotic and associative bacterial infection is that the former occurs in the nodule. This is a remarkable feature since the endocellular colonization, including by pathogens is rare in bacteria associated with plants. The biological nitrogen fixation involves a series of processes that begin with the colonization

of the host plant by the bacterium and culminate in the fixation of atmospheric N_2. (Oldroyd 2013).

The legume-rhizobia interaction occurs when plants release signals, such as flavonoids or isoflavonoids, which are recognize by bacteria using a positively acting transcription factor, usually encoded by *nodD* (Downie 2010; Recourt et al. 1989) (Fig 1). The type of secreted signals, as well as the NodD protein usually differ depending of the plant or bacteria, respectively (Downie 2010). Following NodD and flavonoids interaction, NodD binds to highly conserved bacterial promoters called *nod* boxes and induces the expression of several genes directly related with the nodulation process (as those involved in the synthesis of Nod factors) (Fig 1).

The NodD regulator belongs to the LysR family of transcriptor regulators. Constitutively expressed, NodD is responsible for the transcription of other nodulation genes (e.g. *nodABC*) in the presence of compatible flavonoids released by legume plants, consequently initiating the nodulation process (Kondorosi et al. 1989; Oldroyd 2013; Spaink 2000). In addition, NodD also regulates directly or indirectly several other symbiotic phenotypes in rhizobia, such as polysaccharide production, phytohormone synthesis, motility, quorum-sensing and the activation of the type-III secretion system (Krause et al. 2002; Lopez-Baena et al. 2008; Perez-Montano et al. 2011; Pérez-Montaño et al. 2014; Theunis et al. 2004; Vinardell et al. 2004). The number of *nodD* copies can vary depending on the rhizobial species and accordingly it was found that species harboring only one copy have the nodulation completely aborted when this gene is mutated, while species with multiple *nodD* copies, a complex interaction between the *nodD* genes seems to occur and the nodulation is not totally suppressed (Broughton et al. 2000; Garcia et al. 1996).

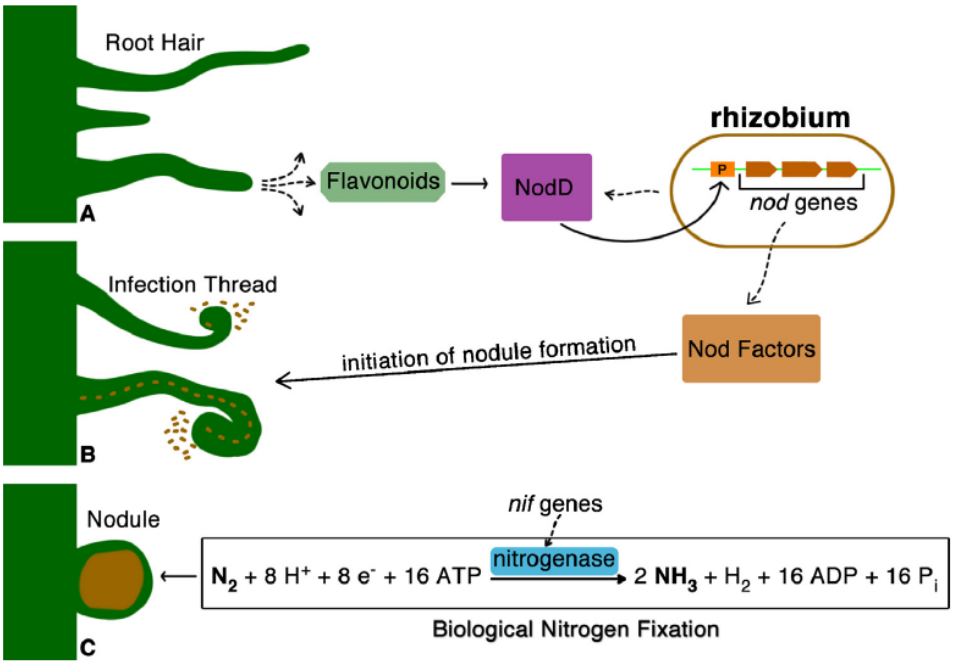

Figure 1. Schematic overview of the nodulation process and biological nitrogen fixation (from Laranjo et al. 2014).

Besides determining which legumes the bacteria is able to nodulate (Downie 1998; Perret et al. 2000), the Nod factors are responsible for root hair curl, infection thread formation, induction of cell division and gene expression in the root cortex and pericycle, nodule development and the number of nodules (Garg and Geetanjali 2007; Laranjo et al. 2014; Oldroyd 2013; van Brussel et al. 2002). The Fig 2 shows the colonization on chickpea root hair tips by green fluorescent protein–tagged rhizobia.

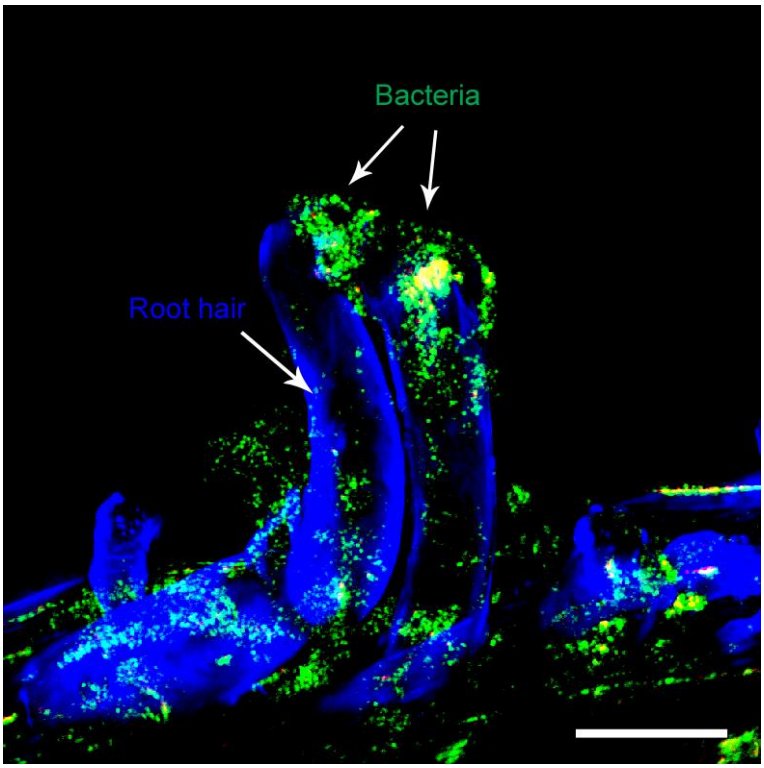

Figure 2. Confocal laser scanning micrographs showing the colonization process on chickpea root hairs inoculated with green fluorescent protein–tagged rhizobia. Scale bar: 25 μm.

The mechanisms by which rhizobia and legumes choose their partners is not completely clear yet. Some authors suggest that the evolution of rhizobia-legumes associations may be host driven (Kiers et al. 2003; Sachs et al. 2011). However, legumes can be nodulated by rhizobia even if the association results in low symbiotic nitrogen fixation ((Den Herder and Parniske 2009). Most legumes are promiscuous meaning that they can be nodulated by different rhizobia species and particularly by broad host range rhizobia strains (Zhao et al. 2008).

Upon contact of the plant cells with the released nodulation factors, curling of root hairs is initiated. The infection channel is formed within the root hair, while in the pericycle starts the microtubular cytoskeleton rearrangement. Cell activation subsequently occurs in the inner part of the cortex, which is divided forming a nodule primordium. Cell activation gradually extends to the middle and the outermost part of the cortex in accordance with two gradients of cellular differentiation resulting in the formation of a nodular primordium (Gerahty et al. 1992).

To better understand how the growth and development of the nodule occurs Gerahty et al. (1992) proposed, chronologically, different stages, according to the anatomical changes in soybean roots after infection, when cell divisions are initiated in

and out of the root cortex generating meristematic nodules where successive mitotic divisions occur. These processes were explained chronologically through stages of growth and nodule development: stage 0 corresponds to uninfected root; stage I - early infection; stage II - external cortical cells begin to divide; stage III - the division is evident in some inner cortex and outer cortex cells; stage IV - the cells are more isodiametric and have some externally and internally oblique divisions in the cortex, forming a nodule meristem; stage-V the meristem is increased; and stage VI - nodule emergency.

During the growth of the nodule, internalized bacteria are released in the cytoplasm, they begin to divide and the membrane that surrounds the cell surface increases to accommodate this growth by merging with smaller vesicles (Gerahty et al. 1992). In the growth stage V, the expansion of the nodule is controlled, which is considered a mechanism of self-regulation, a situation that leads to maturation of the nodule. In this phase, bacteria stop dividing and begin to increase in size and differentiate into endosymbiotic organelles termed N-fixing bacteroids (Taíz and Zieger 2004).

Depending on the site and depth of initial cortical cell division and the time of active meristem termination (nodule growth), nodules formed by legume may be either determinate (e.g., on soybean and *L. japonicus*) or indeterminate (e.g., on *M. truncatula* and chickpea) (Biswas and Gresshoff 2014). Determinate nodules are usually initiated sub-epidermally in the outer cortex, and nodular meristematic activity is early terminated, giving rise to spherical nodules. In indeterminate nodules, cell division initially occurs anticlinally in the inner cortex, followed by periclinal divisions in the pericycle; meristems are persistent for a longer time, resulting in cylindrical nodules (B G Rolfe and Gresshoff 1988; Gresshoff and Delves 1986; Newcomb et al. 1979; Terpolilli et al. 2012).

1.3.2. Mechanisms of Biological Nitrogen Fixation

To convert atmospheric nitrogen (N_2) to ammonia (NH_3) by biological nitrogen fixation process, high amounts of energy is spent by the plants (E. Newton 2007). This process takes place inside the nodule by the bacteroids through the activity of the nitrogenase enzyme complex (Terpolilli et al. 2012). The biological nitrogen fixation can be represented by the following equation:

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16Pi}$$

The nitrogenase enzyme complex formed by two protein units (Fig. 3), the iron - protein (encoded by *nifH*) and the Molybdenum-Iron-protein (α-subunit encoded by *nifD* and β-subunit by *nifK* genes), is responsible for nitrogen fixation in the nodule (E. Newton

2007; Rubio and Ludden 2008):

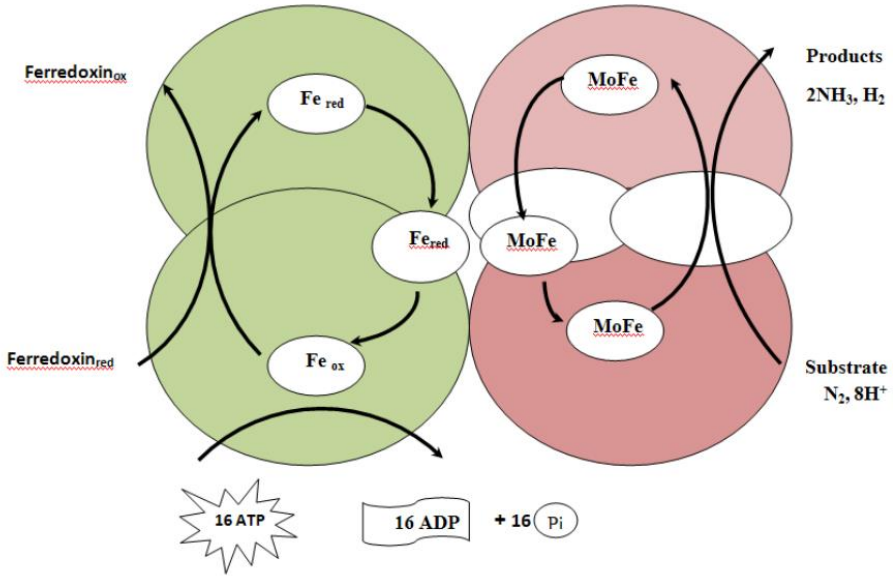

Figure 3. The structure of Nitrogenase (from Issa et al. 2014)

For biological nitrogen fixation to occur it is necessary that the nitrogenase is in low oxygen conditions (Biswas and Gresshoff 2014; Kuzma et al. 1993). The nodules express a plant protein called leghemoglobin that binds oxygen when it is present in high concentrations (Minchin et al. 2008). Both leghemoglobin and the oxygen diffusion barrier in the nodule are important regulators of oxygen tension in the nodule, protecting the nitrogenase enzyme complex that is irreversibly inactivated by oxygen (Dixon and Kahn 2004). According to Deninson and Harter (1995), storing enough oxygen to maintain bacteroid respiration.

In rhizobia, the most important regulator of N_2 fixation is the NifA protein. Together with the sigma factor *σ54*, it activates the transcription of a series of *nif* and *fix* genes that are essential for N_2 fixation (Dixon and Kahn 2004; Fischer 1994; Terpolilli et al. 2012).

Figure 4 shows a comparison of regulatory cascades that control the transcription of *nif* genes in free-living diazotrophic bacterium *Klebsiella pneumoniae* and symbiotic diazotrophs *Sinorhizobium meliloti* and *Bradyrhizobium japonicum*.

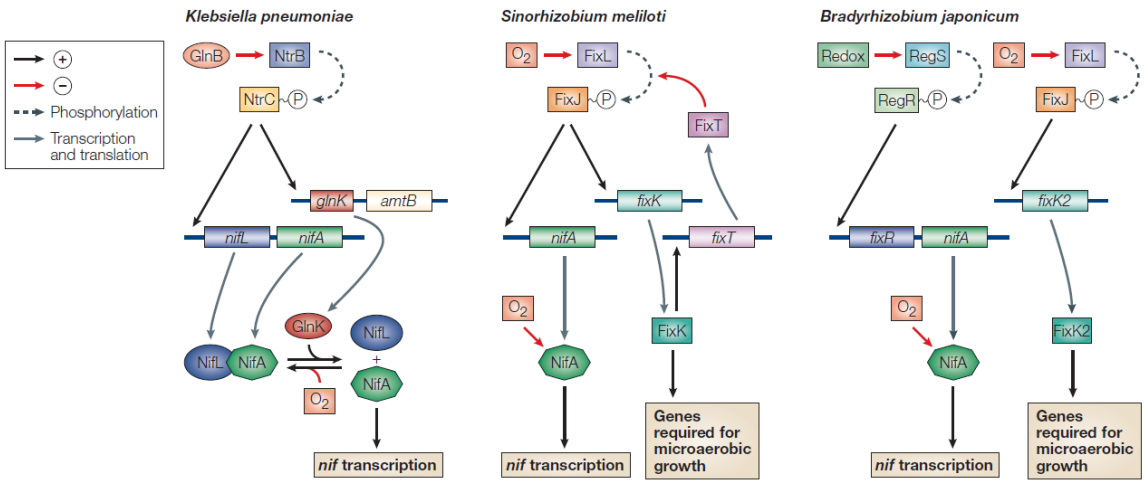

Figure 4. Scheme comparing the transcription regulation of *nif* genes in *Klebsiella pneumoniae*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* (from Dixon and Kahn 2004).

As mentioned above, the limitation of oxygen concentration is a key factor in the expression of *nif* and *fix* genes (Soupène et al. 1995). The *fixL* and *fixJ* genes encode a regulating system in which the two components-oxygen sensor FixL transfers phosphate to the response regulator FixJ. In *Sinorhizobium meliloti* the phosphorylated FixJ positively controls the transcription of *nifA* and *fixK* (de Philip et al. 1990; Gilles-Gonzalez et al. 1991; Hertig et al. 1989). FixK induces the expression of *fixNOQP* and negatively affects the expression of *nifA* (Batut et al. 1989). *nifA* is required for the transcription of *fixABCX*, and *nifN nifB* as well as for the transcription of the operon *nifHDK*, encoding the subunits of nitrogenase. The operon *fixNOQP* encode the cytochrome terminal oxidase *cbb3* protein complex which is involved in the respiratory chain with a high affinity for O_2 responsible for ATP production needed to symbiosis, while *fixABCX* is involved in the regulation of gene transcription under low oxygen concentrations (Black et al. 2012; Lopez et al. 2001). Moreover, NifA in bacteria such as *Sinorhizobium meliloti*, also controls some other genes that are not directly involved in nitrogen fixation, such as those related to competitiveness in nodulation genes, the development of nodules and bacteroid persistence. In *Rhizobium leguminosarum*, *nifA* is autoregulated (Martinez et al. 2004).

Regarding the number of copies of *nifA*, in species like *Sinorhizobium meliloti*, *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* there is only one copy (Barnett et al. 2001; Fischer et al. 1986; Schetgens et al. 1985). In *Mesorhizobium loti*, on the other hand, it contains two copies, *nifA1* and *nifA2* genes, both encoded in the symbiotic island. The *nifA1* gene is more similar to *nifA* from *Rhizobium etli, R.*

leguminosarum, Sinor*hizobium fredii* strain NGR234 and *Sinorhizobium meliloti* (Albright et al. 1989; Alvarez-Morales et al. 1986; Alvarez-Morales and Hennecke 1985). In contrast, *nifA2* is more similar to *nifA* from *Bradyrhizobium japonicum* (Sullivan et al. 2013). The two genes do not perform the same function, while mutant *M. loti nifA2* forms Fix$^-$ nodules, *nifA1* mutants are not affected symbiotically (Nukui et al. 2006; Sullivan et al. 2013; Sullivan et al. 2001). In *M. japonicum* MAFF303099 *nifA2* also regulates the gene encoding the enzyme 1- aminocyclopropane -1 -carboxylic acid (ACC) deaminase, responsible for degradation of ACC, the precursor of the phytohormone ethylene (Nukui et al. 2006).

Although there are few studies reporting the effects of overexpression of *nifA* in diazotrophic, it is known that multiple copies of this gene may increase the nodulation competitiveness (Sanjuan and Olivares 1991). Studies by Jieping et al. (Jieping et al. 2002) showed that an extra *nifA* copies in *Sinorhizobium fredii* enhanced nodulation and nodulation competitiveness in soybean.

1.4. Stress response genes

Survival under non-optimal conditions requires, first of all, the ability to sense these fluctuations in the immediate surroundings and secondly, the ability to modulate gene expression in order to adjust bacterial physiology to new conditions. Sensing an extracellular change might involve periplasmic protein sensors, as for example sensing envelope stress including pH or salt (Tschauner et al. 2014), or *cis*-acting RNA elements, as in the case of temperature-sensing (Narberhaus 2010).

The heat shock response is particularly well studied in many bacteria (Schumann 2016), including rhizobia. The heat shock proteins (HSPs) are encoded by genes induced after a sudden increase of temperature. There are two major classes of HSPs involved in protecting cells from protein denaturation caused by temperature upshift: chaperones and proteases. Chaperones systems, such as GroESL and DnaKJ, have the important role of rescuing misfolded proteins and allowing their refolding into the native and functional conformation. Proteases, as for example FtsH and ClpXP, are involved in the degradation of protein aggregates (misfolded proteins that are no longer able to acquire their native conformation). It is noteworthy that these HSPs are often involved in the response to other stressors and moreover, chaperones and proteases are also important under normal conditions, namely for the correct folding of newly synthesized polypeptides.

1.4.1. Stress response genes in rhizobia

Besides the symbiosis genes, which are crucial for the interaction with the legume host, rhizobia genomes harbor other genes important for the rhizobia lifestyle, such as stress response genes that allow these bacteria to survive in the soil challenging conditions as well as inside the root nodules.

The study of the molecular bases of stress response in rhizobia is particular interesting since these bacteria are exposed not only to the soil conditions, but also to the endosymbiotic lifestyle, inside the host plant root or shoot nodules. On a more applied perspective, development of highly effective rhizobia strains to be used as field inoculants must not disregard the importance of stress tolerance. If the inoculant formulation is not able to survive to abiotic stresses, its successful performance in the field is greatly compromised.

Functional studies that focus on a given stress response gene or operon also represent important contributions to our understanding of the molecular bases of stress response. Rhizobial genomes typically encode several copies of the major chaperone system GroESL and these genes were known to be essential for *E. coli* viability (Fayet et al. 1989) as well as determinant for the limit temperature for growth (Ferrer et al. 2003). Their study in rhizobia showed that there is some functional redundancy among different copies, although different regulatory mechanisms of these operons can be found in the same strain. The differential regulation of these operons allows a fine tune of the GroESL pool under different conditions, including inside the nodule, during symbiosis with the host plant (Alexandre and Oliveira 2013). For example, in *S. meliloti* and *Bradyrhizobium japonicum*, both with five *groEL* copies, all *groEL* single mutants are viable (Bittner et al. 2007; Fischer et al. 1993). Contrary to this, one of the three *groEL* copies of *R. leguminosarum* is essential for growth (Rodriguez-Quinones et al. 2005). In terms of heat tolerance phenotype, a *S. meliloti* strain with mutations in $groEL_1$ and $groEL_5$ showed a slower growth compared to the wild-type, especially under higher temperatures (Bittner et al. 2007).

Several studies showed that some genes may be involved in the tolerance to several stress conditions. For example, genes involved in trehalose biosynthesis (*otsAB*, *treS* and *treZY* genes) have been associated to rhizobial tolerance to desiccation, salinity and heat (Moussaid et al. 2015; Reina-Bueno et al. 2012). Besides its action as stress protectant, in free-living rhizobia, trehalose may be used as carbon and energy source. Trehalose has also been detected in bacteroids, however it seems that trehalose synthesis has different pathways under free-living or symbiotic conditions (Reina-Bueno et al. 2012).

1.4.2. The role of stress response genes in the symbiosis

Since early events in the symbiosis process such as molecular signalling and rhizobial attachment, are particularly sensitive to high temperatures, salinity, acidity and other environmental stresses (Hungria and Stacey 1997; Hungria and Vargas 2000; Zhang and Smith 1996), rhizobia have to be able to physiologically adapt to environmental conditions, in order to ensure a successful symbiosis with its legume partner. These stresses that negatively affect the microsymbiont in free-living conditions as well as during the symbiotic relationship can lead to a delay in infection and nodule formation, development of non-fixing nodules or even to failure of the nodulation process (Zahran 1999). Moreover, during infection, rhizobia also have to deal with adverse conditions within the host cells and with the plant innate immunity that induces physiological stress responses, which may interfere with symbiosis (Soto et al. 2009). In fact, it was suggested that among the genes required for bacteroid formation, some are specific for symbiosis and others are involved in physiological adaptation to the environmental conditions within and outside the nodule (Oke and Long 1999).

Transcriptomic and proteomic analyses of rhizobia in symbiosis with their host legumes suggest the involvement of stress response genes, mainly heat shock proteins such as ClpB and GroESL, in the symbiotic process. For example, overexpression of the ClpB and GroEL/ES proteins was detected in nodules formed by *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* strains (Djordjevic 2004; Djordjevic et al. 2003b; Nomura et al. 2010b; Sarma and Emerich 2005; Sarma and Emerich 2006). These findings are reinforced through transcriptomic analyses where up-regulation of these genes was observed in root nodules (Karunakaran et al. 2009; Pessi et al. 2007a; Uchiumi et al. 2004).

The most studied molecular chaperone in terms of its involvement in the symbiosis is GroEL. Particular copies of this chaperone gene, usually upregulated in the bacteroids, seem to play a fundamental role in the formation of functional NodD and nitrogenase complex (Fischer et al. 1999; Ogawa and Long 1995). For example, among the five *groESL* operons in the *S. meliloti* genome only one operon (*groEL*$_1$) was found to be involved in symbiosis (Ogawa and Long 1995). Fischer et al. (Fischer et al. 1993) found a co-regulation between *groESL*$_3$ and nitrogen fixation genes in *B. japonicum*, yet none of the *B. japonicum* mutants that individually lack one *groEL* gene were depleted in their symbiotic phenotype (Bittner et al. 2007; Fischer et al. 1999). However, double mutation on *groEL*$_3$ and *groEL*$_4$ genes in *B. japonicum* affects the symbiotic performance, since these copies are required for the formation of a functional nitrogenase (Fischer et al. 1999). These two copies are the most abundant in the GroEL pool in bacteroids (Fischer

et al. 1993). Studies on the symbiotic performance of strains mutated in the *dnaJ* gene also revealed distinct results, using different rhizobia species. For example, a *B. japonicum dnaJ* mutant strain was able to establish fully effective symbiosis with soybeans (Minder et al. 1997). In contrast, Nogales et al. (2002) found that a *dnaJ* mutant of *Rhizobium tropici* was able to form nodules in *Phaseolus vulgaris*, however this mutant showed low nitrogenase activity, which was also evident in the reduced plant growth and in the reduction of the nitrogen content of the plant shoots. Similarly, *dnaJ* is required for effective symbiosis of *R. leguminosarum* bv. *phaseoli* (Labidi et al. 2000). On the other hand, the DnaK chaperone, another protein that constitutes the DnaK-DnaJ chaperone system, is required for optimum symbiotic function in *S. meliloti* (Summers et al. 1998). More recently, the involvement of the ClpB chaperone in the symbiotic process was evaluated. Although a *Mesorhizobium clpB* mutant strain was able to establish symbiosis with chickpea plants, the ClpB absence caused a delay in nodule formation and development (Brígido et al. 2012b), indicating its involvement in the symbiotic process.

Other genes involved in stress response, namely major regulators of the heat shock response, have been implicated in the symbiosis. For instance, *S. meliloti rpoH1* mutants have been shown to have defective symbiotic phenotypes, showing poor colonization and survival in bacteroids and do not fix nitrogen (Mitsui et al. 2004; Oke et al. 2001). In contrast, a *rpoH2* mutant showed a symbiotic phenotype similar to the wild-type (Mitsui et al. 2004; Oke et al. 2001; Ono et al. 2001). Nevertheless, *rpoH1 rpoH2* double mutants exhibited a more severe symbiotic phenotype than the *rpoH1* mutant (Bittner and Oke 2006). Similar results were obtained by Martinez-Salazar et al. (2009) where *R. etli rpoH1* and *rpoH2 rpoH1* mutants exhibited reduced nitrogenase activity and bacterial viability in early and late symbiosis, compared with nodules formed by the *rpoH2* mutant and wild-type.

Despite the fact that functional studies showed results that vary with the rhizobia species analysed, stress response genes seem to be implicated in rhizobial infection and nitrogen-fixation. The lower symbiotic performance obtained with most of the chaperone mutants, suggests that the role of chaperones is important for bacterial cells to achieve an efficient and effective symbiotic interaction with their legume hosts. The negative effects in their symbiotic phenotypes, due to the loss of specific chaperone genes, is most likely due to the role of these proteins in the folding of newly synthesized polypeptides, refolding of denatured proteins and disaggregation of proteins involved in the symbiosis. Altogether, the major chaperone genes seem to be involved in the symbiotic process between rhizobia and legume hosts, not only due to their role in the folding of important symbiosis proteins, but also due to their direct involvement in response to stressful conditions found in the rhizosphere and within the root cells.

However, further studies are required to elucidate in which step of the symbiotic interaction these and other stress response genes are particularly important.

1.4.3. The EnvZ/OmpR two-component regulatory systems

The two-component regulatory systems (TCRS) histidine kinase (HK) and its cognate response regulator (RR) may mediate responses to environmental stresses in bacteria. They are essential in the adaptation of bacteria and fungi to changing environmental conditions (Foo et al. 2015; Wang et al. 2012). The environmental stress is detected by the HK that allows the autophosphorylation of the conserved histidine residue (Wang et al. 2012), and this phosphoryl group is then transferred by the conserved aspartic acid residue of the response regulator. When the response regulator is phosphorylated, it binds to DNA target sequences and stimulates the transcription of genes targets (Gao and Stock 2009; Yoshida et al. 2002).

Among the TCRS, the EnvZ/OmpR system, present in *E. coli*, is one of the best-characterized. It is activated in osmotic stress, regulating the expression of outer membrane porins OmpF and OmpC (Alphen and Lugtenberg 1977; Wang et al. 2012). At high osmolarity EnvZ is autophosphorylated and transfers the phosphoryl group to OmpR, which in turn binds to the promoter regions of outer membrane porin genes *ompF* and *ompC* (Yoshida et al. 2002; Yuan et al. 2011). The cellular OmpR-P levels control the transcription of *ompF* and *ompC*. Low medium osmolarity reduce the level of OmpR-P and favors the transcription of *ompF*. At high medium osmolarity an elevated OmpR-P level, resulting from the increased kinase/phosphatase ratio of EnvZ, allows the activation of *ompC* transcription. On the other hand, more OmpR-P molecules bind to the *ompF* promoter upstream region causing repression of *ompF* expression (Cai and Inouye 2002). Both porins act in the nutrient exchange allowing the passive diffusion of small hydrophilic molecules across the membrane. (Aiba et al. 1989; Nikaido 2003; Wang et al. 2012). EnvZ also acts as a phosphatase, which dephosphorylates the OmpR-P when the osmotic stress disappears (Aiba et al. 1989; Mattison and Kenney 2002).

EnvZ is an integral membrane protein that has all of the conserved motifs common to the histidine kinase family: H, N, G1, D/F, and G2 boxes (Parkinson and Kofoid 1992). Three enzymatic activities have been shown to be associated with the carboxy-terminal cytoplasmic domain of EnvZ: autokinase, OmpR kinase, and OmpR-phosphate (OmpR-P) phosphatase (Hsing and Silhavy 1997).

EnvZ, a protein with 450 amino acid residues, exists as a dimer located in the inner cytoplasmic membrane of *E. coli*. EnvZc, the cytoplasmic domain of EnvZ, also exists as

a dimer and consists of the linker region, domain A and domain B. It contains both kinase and phosphatase activities similar to the intact EnvZ (Dutta et al. 1999; Park et al. 1998). The linker region is responsible for transducing the signal from the periplasmic receptor domain to the cytoplasmic catalytic domain (Park and Inouye 1997). It was demonstrated that domain A by itself can serve as the OmpR-P phosphatase (Zhu et al. 2000).

OmpR, the response regulator, consists of an N-terminal CheY-like receiver domain possessing the highly conserved Asp-55 phosphorylation site and a C-terminal DNA-binding domain (Kato et al. 1989). The phosphorylation of OmpR at Asp-55 enhances its binding affinity for the regulatory sequences upstream of the *ompF* and *ompC* promoters (Aiba et al. 1989; Forst et al. 1989; Head et al. 1998; Huang and Igo 1996). OmpR-P binds to the -100 to -38 region of *ompC* and the -380 to -361 region as well as the -100 to -39 region of *ompF* (Bergstrom et al. 1998; Huang and Igo 1996; Rampersaud et al. 1994; Tsung et al. 1989). These OmpR binding sites consist of 20 base pairs each sharing a consensus sequence (Harlocker et al. 1995). It has been shown that two OmpR-P molecules bind to each site in a head-to-tail manner (Harlocker et al. 1995).

ompR and *envZ* as part of the *ompB* operon of *E. coli*, are co-transcribed as a polycistronic mRNA from a promoter upstream the *ompR* gene (Hall and Silhavy 1981). In a cell, significantly fewer EnvZ molecules are produced compared with OmpR molecules. It has been estimated that there are about 1000 molecules of OmpR whereas there are only 10 molecules of EnvZ per cell (Dziejman and Mekalanos 1995). Cai and Inouye (2002) showed that the OmpR and EnvZ levels in *E. coli* cells are approximately 3500 and 100 molecules per cell, respectively.

It was reported that mutation in *envZ*/*ompR* of *E. coli* alter, directly or indirectly, the expression of more than 100 genes, including genes related to amino acid biosynthesis, such as isoleucine and cysteine, iron and maltose transport, and flagellar synthesis (Oshima et al. 2002). A single mutation in *ompR* of *Yersinia pestis* affected the expression of 224 genes, indicating a global regulatory role (Gao et al. 2011). In addition to its role as osmosensor, the EnvZ/OmpR system has also been associated with other functions, such as virulence in pathogens, fatty acid uptake, exopolysaccharide (EPS) production, peptide transport and flagella production (Bernardini et al. 1990; Feng et al. 2003; Li et al. 2014; Mills et al. 1998; Pickard et al. 1994; Shin and Park 1995; Vidal et al. 1998; Yuan et al. 2011). Furthermore, EnvZ/OmpR was also seen to be involved in the regulation of the type III secretion system genes in pathogenic bacteria, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Erwinia amylovora* and *Pseudomonas syringae* (Brzostek et al. 2007; Feng et al. 2003; Feng et al. 2004; Li and Zhao 2011).

1.5. The chickpea-rhizobia symbiosis

The *Mesorhizobium* genus is one of the least studied among rhizobia, however this group represents an outstanding model to study the evolution of symbiosis due to its wide geographic spread, diverse organization of symbiotic genes and growing number of genomes available (Laranjo et al. 2014). Bacteria from this genus have a host range that can be very narrow, as appears to be the case of the type strains of *M. mediterraneum* and *M. ciceri* that are known to nodulate only *Cicer* species (Nour et al. 1995; Nour et al. 1994), or may be wider, for example *M. plurifarium* and *M. tianshanense* nodulating legumes of different genera (Chen et al. 1995; de Lajudie et al. 1998). *Mesorhizobium* species establish symbiotic relationships with legumes in temperate, tropical, subtropical clime and even in arctic areas (Chen et al. 2005), and can also colonize the host plant as endophytes (Wei et al. 2007).

Studies with diazotrophs typically have the goal of using knowledge generated in the production of inoculants for crops of agronomic interest. For example, *Mesorhizobium* species are able to associate symbiotically with chickpea (*Cicer arietinum* L.), an important grain used for feed or with biserrula (*Biserrula pelecinus* L.), a forage used for pasture (Laranjo et al. 2014).

There are currently 43 species of *Mesorhizobium* standing on the List of bacterial names standing in nomenclature (http://www.bacterio.net), however this number is increasing mostly due to the identification of microsymbionts from newly studied wild legumes (Euzéby 1997). The main gene used for phylogenetic purposes is the 16S rRNA, but other genes have been also used for this purposes as *dnaK* (Stepkowski et al. 2003), *recA* and *atpD* (Vinuesa et al. 2005) and the *dnaJ* gene (Alexandre et al. 2008).

Chickpea (*Cicer arietinum*, L.) is an agronomically important crop belonging to the Fabaceae family cultivated for over 10,000 years (Albala 2007). It is the third most important grain legume cultivated in several areas, such as Asia, Mediterranean regions, Australia, Canada, the USA and Africa (Acharjee and Sarmah 2013). Worldwide cultivated in more than 50 countries, the chickpea production covers an area of 14 million hectares and its production is approximately 13 million tonnes. Asia is largest producer of chickpeas in the world (76% of the total production) followed by Middle East and northern Africa. In Portugal the last production data vary from 500 to 600 tonnes, whereas Europe as a whole produced approximately 164 000 tonnes (FAOSTAT 2014).

The importance of this crop is mainly due its rich nutritional value, with a total protein content ranging from 17 to 30 % (Jukanti et al. 2012). Therefore, chickpea is a low-price alternative to replace the lack of other protein source in developing countries. On average the estimated amounts of N fixed by chickpeas under regular precipitation and drought stress conditions are 60 kg/ha (Unkovich and Pate 2000) and 19–24 kg/ha (Carranca et al. 1999),

respectively.

Currently three species of the *Mesorhizobium* genus were described as chickpea microsymbiont, namely *Mesorhizobium ciceri* (Nour et al. 1994), *Mesorhizobium mediterraneum* (Nour et al. 1995) and more recently *Mesorhizobium muleiense* (Zhang et al. 2012). However, several studies have shown that other *Mesorhizobium* species are able to nodulate and fix nitrogen in symbiosis with chickpea (Alexandre et al. 2009; Laranjo et al. 2004; Laranjo et al. 2012; Nandwani and Dudeja 2009; Rivas et al. 2007; Tena et al. 2017).

The main aim of the present study is to improve the symbiotic performance of chickpea *Mesorhizobium* strains, through the overexpression of symbiotic and stress response genes. Two symbiosis genes encoding important regulator were selected, namely *nifA*, which is involved in the nitrogen fixation process, and *nodD*, which is involved in the nodulation process. In addition, two stress response genes were analysed, namely the *groEL* gene, which encodes an important chaperone and seems to be indirectly involved in the symbiosis, and the *envZ* gene, which encodes one protein of the two-component system EnvZ/OmpR, known as an osmosensor complex in *E. coli*.

1.6. Reference

Acharjee, S., & Sarmah, B. K. (2013). Biotechnologically generating 'super chickpea' for food and nutritional security. *Plant Sci, 207*, 108-116. doi: 10.1016/j.plantsci.2013.02.003

Aiba, H., Nakasai, F., Mizushima, S., & Mizuno, T. (1989). Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in *Escherichia coli*. *Journal of Biological Chemistry, 264*(24), 14090-14094.

Albala, K. (2007). *Beans: A History*. New York, NY, USA: Berg Publishers.

Albright, L. M., Ronson, C. W., Nixon, B. T., & Ausubel, F. M. (1989). Identification of a gene linked to *Rhizobium meliloti ntrA* whose product is homologous to a family to ATP-binding proteins. *J Bacteriol, 171*(4), 1932-1941.

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microbial Ecology, 58*, 930-941.

Alexandre, A., Laranjo, M., Young, J. P. W., & Oliveira, S. (2008). *dnaJ* is a useful phylogenetic marker for alphaproteobacteria *Int J Syst Evol Microbiol, 58*(12), 2839-2849.

Alexandre, A., & Oliveira, S. (2013). Response to temperature stress in rhizobia. *Critical Reviews in Microbiology, 39*(3), 219-228.

Alexandre, A., & Oliveira, S. (2016). Heat shock response in bacteria with large genomes: lessons from rhizobia. In F. J. de Bruijn (Ed.), *Stress and Environmental Control of Gene Expression in Bacteria*: Wiley-Blackwell Publishers.

Alphen, W. V., & Lugtenberg, B. (1977). Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J Bacteriol, 131*(2), 623-630.

Alvarez-Morales, A., Betancourt-Alvarez, M., Kaluza, K., & Hennecke, H. (1986). Activation of the *Bradyrhizobium japonicum nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences. *Nucleic Acids Research, 14*(10), 4207-4227.

Alvarez-Morales, A., & Hennecke, H. (1985). Expression of *Rhizobium japonicum nifH* and *nifDK* operons can be activated by the *Klebsiella pneumonia nifA* protein but not by the product of *ntrC*. *Molecular & General Genetics : Mgg, 199*(2), 306-314.

B G Rolfe, a., & Gresshoff, P. M. (1988). Genetic Analysis of Legume Nodule Initiation. *Annual Review of Plant Physiology and Plant Molecular Biology, 39*(1), 297-319. doi: 10.1146/annurev.pp.39.060188.001501

Barnett, M. J., Fisher, R. F., Jones, T., Komp, C., Abola, A. P., Barloy-Hubler, F., . . . Long, S. R. (2001). Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* p*Sym*A megaplasmid. *Proceedings of the National Academy of Sciences of the United States of America, 98*(17), 9883-9888.

Batut, J., Daveran-Mingot, M. L., David, M., Jacobs, J., Garnerone, A. M., & Kahn, D. (1989). *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *Embo j, 8*(4), 1279-1286.

Bergstrom, L. C., Qin, L., Harlocker, S. L., Egger, L. A., & Inouye, M. (1998). Hierarchical and co-operative binding of OmpR to a fusion construct containing the *ompC* and *ompF* upstream regulatory sequences of *Escherichia coli*. *Genes Cells, 3*(12), 777-788.

Bernardini, M. L., Fontaine, A., & Sansonetti, P. J. (1990). The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. *J Bacteriol, 172*(11), 6274-6281.

Biswas, B., & Gresshoff, P. (2014). The Role of Symbiotic Nitrogen Fixation in Sustainable Production of Biofuels. *International Journal of Molecular Sciences, 15*(5), 7380.

Bittner, A. N., Foltz, A., & Oke, V. (2007). Only one of five *groEL* genes is required for viability and successful symbiosis in *Sinorhizobium meliloti*. *J. Bacteriol., 189*(5), 1884-1889. doi: 10.1128/jb.01542-06

Bittner, A. N., & Oke, V. (2006). Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. *J. Bacteriol., 188*(10), 3507-3515.

Black, M., Moolhuijzen, P., Chapman, B., Barrero, R., Howieson, J., Hungria, M., & Bellgard, M. (2012). The Genetics of Symbiotic Nitrogen Fixation: Comparative Genomics of 14 Rhizobia Strains by Resolution of Protein Clusters. *Genes, 3*(1), 138.

Brígido, C., Robledo, M., Menendez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604. doi: 10.1094/mpmi-05-12-0140-r

Broughton, W. J., Jabbouri, S., & Perret, X. (2000). Keys to symbiotic harmony. *J Bacteriol, 182*(20), 5641-5652.

Brzostek, K., Brzóstkowska, M., Bukowska, I., Karwicka, E., & Raczkowska, A. (2007). OmpR negatively regulates expression of invasin in *Yersinia enterocolitica*. *Microbiology, 153*(8), 2416-2425. doi: doi:10.1099/mic.0.2006/003202-0

Cai, S. J., & Inouye, M. (2002). EnvZ-OmpR Interaction and Osmoregulation in *Escherichia coli*. *Journal of Biological Chemistry, 277*(27), 24155-24161. doi: 10.1074/jbc.M110715200

Carranca, C., de Varennes, A., & Rolston, D. (1999). Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the N-15 isotope dilution technique. *European Journal of Agronomy, 10*(1), 49-56.

Chen, W.-M., Moulin, L., Bontemps, C., Vandamme, P., Béna, G., & Boivin-Masson, C. (2003). Legume Symbiotic Nitrogen Fixation by beta-Proteobacteria Is Widespread in Nature. *J Bacteriol, 185*(24), 7266-7272.

Chen, W. X., Wang, E., Wang, S. Y., Li, Y. B., & Chen, X. Q. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root-nodule bacterium isolated from an arid saline environment in Xinjiang, Peoples-Republic-of-China. *International Journal of Systematic Bacteriology, 45*(1), 153-159.

Chen, W. X., Wang, E. T., & Kuykendall, D. (2005). Genus VI. *Mesorhizobium*. *Bergeys Manual of Systematic Bacteriology* (Vol. vol. 2 (The Proteobacteria), pp. pp. 403-408). New York: Springer.

Crossman, L. C., Castillo-Ramírez, S., McAnnula, C., Lozano, L., Vernikos, G. S., Acosta, J. L., . . . González, V. (2008). A Common Genomic Framework for a Diverse Assembly of Plasmids in the Symbiotic Nitrogen Fixing Bacteria. *PLoS One, 3*(7), e2567. doi: 10.1371/journal.pone.0002567

D'Haeze, W., & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology, 12*(6), 79R-105R.

de Lajudie, P., Willems, A., Nick, G., Moreira, F., Molouba, F., Hoste, B., . . . Gillis, M. (1998). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *International Journal of Systematic Bacteriology, 48*(2), 369-382.

de Philip, P., Batut, J., & Boistard, P. (1990). *Rhizobium meliloti* Fix L is an oxygen sensor and regulates *R. meliloti nifA* and *fixK* genes differently in *Escherichia coli*. *J Bacteriol, 172*(8), 4255-4262.

Den Herder, G., & Parniske, M. (2009). The unbearable naivety of legumes in symbiosis. *Curr Opin Plant Biol, 12*(4), 491-499. doi: 10.1016/j.pbi.2009.05.010

Denison, R. F., & Harter, B. L. (1995). Nitrate Effects on Nodule Oxygen Permeability and Leghemoglobin (Nodule Oximetry and Computer Modeling). *Plant Physiol, 107*(4), 1355-1364.

Dixon, R., & Kahn, D. (2004). Genetic Regulation of Biological Nitrogen Fixation. *Nature Reviews Microbiology, 2*(8), 621-631.

Djordjevic, M. A. (2004). *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. *PROTEOMICS, 4*(7), 1859-1872.

Djordjevic, M. A., Chen, H. C., Natera, S., van Noorden, G., Menzel, C., Taylor, S., . . . Weiller, G. F. (2003). A global analysis of protein expression profiles in *Sinorhizobium meliloti*: Discovery of new genes for nodule occupancy and stress adaptation. *Molecular Plant-Microbe Interactions, 16*(6), 508-524.

Downie, J. A. (1998). Functions of rhizobial nodulation genes. In H. P. Spaink, A. Kondorosi & P. J. J. Hooykaas (Eds.), *The Rhizobiaceae Molecular Biology of Model Plant-Associated Bacteria* (pp. 387-402). Dordrecht: Kluwer Academic Publishers.

Downie, J. A. (2010). The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *Fems Microbiology Reviews, 34*(2), 150-170. doi: 10.1111/j.1574-6976.2009.00205.x

Dutta, R., Qin, L., & Inouye, M. (1999). Histidine kinases: diversity of domain organization. *Molecular Microbiology, 34*(4), 633-640. doi: 10.1046/j.1365-2958.1999.01646.x

Dziejman, M., & Mekalanos, J. J. (1995). Two-Component Signal Transduction and Its Role in the Expression of Bacterial Virulence Factors *Two-Component Signal Transduction*: American Society of Microbiology.

E. Newton, W. (2007). *Physiology, Biochemistry, and Molecular Biology of Nitrogen Fixation*.

Euzéby, J. P. (1997). List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *Int. J. Syst. Bacteriol*(47), 590-592.

FAO. (2009). How to feed the world 2050 (pp. 35). Rome, Italy: Food and Agriculture Organization.

FAOSTAT. (2014). Food and Agriculture Organization. *FAO Statistical Year Book.* Retrieved 18/09/2017, 2017, from http://www.fao.org/faostat/en/#data/QC

Fayet, O., Ziegelhoffer, T., & Georgopoulos, C. (1989). The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J Bacteriol, 171*(3), 1379-1385.

Feng, X., Oropeza, R., & Kenney, L. J. (2003). Dual regulation by phospho-OmpR of *ssrA*/*B* gene expression in *Salmonella* pathogenicity island 2. *Mol Microbiol, 48*(4), 1131-1143.

Feng, X., Walthers, D., Oropeza, R., & Kenney, L. J. (2004). The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. *Mol Microbiol, 54*(3), 823-835. doi: 10.1111/j.1365-2958.2004.04317.x

Ferrer, M., Chernikova, T. N., Yakimov, M. M., Golyshin, P. N., & Timmis, K. N. (2003). Chaperonins govern growth of *Escherichia coli* at low temperatures. *Nature Biotechnology, 21*(11), 1266-1267.

Fischer, H. M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiological Reviews, 58*(3), 352-386.

Fischer, H. M., Alvarez-Morales, A., & Hennecke, H. (1986). The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of nif gene expression and formation of determinate symbiosis. *Embo j, 5*(6), 1165-1173.

Fischer, H. M., Babst, M., Kaspar, T., Acuña, G., Arigoni, F., & Hennecke, H. (1993). One member of a *gro*ESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *The EMBO Journal, 12*(7), 2901-2912.

Fischer, H. M., Schneider, K., Babst, M., & Hennecke, H. (1999). GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum*. *Arch Microbiol, 171*(4), 279-289.

Foo, Y. H., Gao, Y., Zhang, H., & Kenney, L. J. (2015). Cytoplasmic sensing by the inner membrane histidine kinase EnvZ. *Prog Biophys Mol Biol, 118*(3), 119-129. doi: 10.1016/j.pbiomolbio.2015.04.005

Forst, S. A., Delgado, J., & Inouye, M. (1989). DNA-binding properties of the transcription activator (OmpR) for the upstream sequences of *ompF* in *Escherichia coli* are altered by *envZ* mutations and medium osmolarity. *J Bacteriol, 171*(6), 2949-2955. doi: 10.1128/jb.171.6.2949-2955.1989

Fred, E. B. (1932). *Root nodule bacteria and leguminous plants.* Madison.USA.: University of Wisconsin Press.

Gao, H., Zhang, Y., Han, Y., Yang, L., Liu, X., Guo, Z., . . . Yang, R. (2011). Phenotypic and transcriptional analysis of the osmotic regulator OmpR in *Yersinia pestis*. *BMC Microbiol, 11*, 39-39. doi: 10.1186/1471-2180-11-39

Gao, R., & Stock, A. M. (2009). Biological insights from structures of two-component proteins. *Annu Rev Microbiol, 63*, 133-154. doi: 10.1146/annurev.micro.091208.073214

Garcia, M., Dunlap, J., Loh, J., & Stacey, G. (1996). Phenotypic characterization and regulation of the *nolA* gene of *Bradyrhizobium japonicum*. *Mol Plant Microbe Interact, 9*(7), 625-636.

Garg, N., & Geetanjali. (2007). Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. *Agron Sustain Dev, 27*. doi: 10.1051/agro:2006030

Gerahty, N., Caetano-Anollés, G., Joshi, P. A., & Gresshoff, P. M. (1992). Anatomical analysis of nodule development in soybean reveals an additional autoregulatory control point. *Plant Science, 85*(1), 1-7. doi: https://doi.org/10.1016/0168-9452(92)90087-3

Geurts, R., & Bisseling, T. (2002). Rhizobium Nod Factor Perception and Signalling. *The Plant Cell, 14*(Suppl), s239-s249. doi: 10.1105/tpc.002451

Gilles-Gonzalez, M. A., Ditta, G. S., & Helinski, D. R. (1991). A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature, 350*(6314), 170-172. doi: 10.1038/350170a0

Graham, P. H., & Vance, C. P. (2003). Legumes: importance and constraints to greater use. *Plant Physiol, 131*(3), 872-877. doi: 10.1104/pp.017004

Gresshoff, P. M., & Delves, A. C. (1986). Plant Genetic Approaches to Symbiotic Nodulation and Nitrogen Fixation in Legumes. In A. D. Blonstein & P. J. King (Eds.), *A Genetic Approach to Plant Biochemistry* (pp. 159-206). Vienna: Springer Vienna.

Hall, M. N., & Silhavy, T. J. (1981). Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. *J Mol Biol, 151*(1), 1-15.

Harlocker, S. L., Bergstrom, L., & Inouye, M. (1995). Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. *J Biol Chem, 270*(45), 26849-26856.

Head, C. G., Tardy, A., & Kenney, L. J. (1998). Relative binding affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* regulatory sites. *J Mol Biol, 281*(5), 857-870. doi: 10.1006/jmbi.1998.1985

Herridge, D. F., Peoples, M. B., & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil, 311*(1-2), 1-18. doi: 10.1007/s11104-008-9668-3

Hertig, C., Li, R. Y., Louarn, A. M., Garnerone, A. M., David, M., Batut, J., . . . Boistard, P. (1989). *Rhizobium meliloti* regulatory gene *fixJ* activates transcription of *R. meliloti nifA* and *fixK* genes in *Escherichia coli*. *J Bacteriol, 171*(3), 1736-1738.

Hoffman, B. M., Lukoyanov, D., Yang, Z. Y., Dean, D. R., & Seefeldt, L. C. (2014). Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem Rev, 114*(8), 4041-4062. doi: 10.1021/cr400641x

Hsing, W., & Silhavy, T. J. (1997). Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. *J Bacteriol, 179*(11), 3729-3735. doi: 10.1128/jb.179.11.3729-3735.1997

Huang, K. J., & Igo, M. M. (1996). Identification of the bases in the *ompF* regulatory region, which interact with the transcription factor OmpR. *J Mol Biol, 262*(5), 615-628. doi: 10.1006/jmbi.1996.0540

Hungria, M., Campo, R., Chueire, L., Grange, L., & Megías, M. (2001). Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. *Biology and Fertility of Soils, 33*(5), 387-394. doi: 10.1007/s003740100338

Hungria, M., & Stacey, G. (1997). Molecular signals exchanged between host plants and rhizobia: Basic aspects and potential application in agriculture. *Soil Biology and Biochemistry, 29*(5), 819-830. doi: http://dx.doi.org/10.1016/S0038-0717(96)00239-8

Hungria, M., & Vargas, M. A. T. (2000). Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research, 65*(2-3), 151-164.

Hydroworld. (2009). Renewable Fuels: Manufacturing Ammonia from Hydropower. Retrieved 28/09/2017, 2017, from http://www.hydroworld.com/articles/hr/print/volume-28/issue-7/articles/renewable-fuels-manufacturing.html

Issa, A. A., Abd-Alla, M. H., & Ohyama, T. (2014). Nitrogen Fixing Cyanobacteria: Future Prospect. In T. Ohyama (Ed.), *Advances in Biology and Ecology of Nitrogen Fixation* (pp. Ch. 02). Rijeka: InTech.

Jensen, E. S., Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Hauggaard-Nielsen, H., Alves, B. J. R., & Morrison, M. J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for Sustainable Development, 32*(2), 329-364. doi: 10.1007/s13593-011-0056-7

Jieping, Z., Xiaomi, D., Ling, X., Jiabi, Z., Shanjiong, S., & Guanqiao, Y. (2002). Extra-copy *nifA* enhances the nodulation efficiency of *Sinorhizobium fredii*. *Chinese Science Bulletin, 47*(7), 565-567. doi: 10.1360/02tb9130

Jukanti, A. K., Gaur, P. M., Gowda, C. L., & Chibbar, R. N. (2012). Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *Br J Nutr, 108 Suppl 1*, S11-26. doi: 10.1017/s0007114512000797

Kaminski, P. A., Batut, J., & Boistard, P. (1998). A Survey of Symbiotic Nitrogen Fixation by Rhizobia. In H. P. Spaink, A. Kondorosi & P. J. J. Hooykaas (Eds.), *The Rhizobiaceae: Molecular Biology of Model Plant-Associated Bacteria* (pp. 432-460). Dordrecht: Kluwer Academic Publishers.

Karunakaran, R., Ramachandran, V. K., Seaman, J. C., East, A. K., Mouhsine, B., Mauchline, T. H., . . . Poole, P. S. (2009). Transcriptomic Analysis of *Rhizobium leguminosarum* Biovar *viciae* in Symbiosis with Host Plants *Pisum sativum* and *Vicia cracca*. *J Bacteriol, 191*(12), 4002-4014. doi: 10.1128/JB.00165-09

Kato, M., Aiba, H., Tate, S., Nishimura, Y., & Mizuno, T. (1989). Location of phosphorylation site and DNA-binding site of a positive regulator, OmpR, involved in activation of the osmoregulatory genes of *Escherichia coli*. *FEBS Lett, 249*(2), 168-172.

Kiers, E. T., Rousseau, R. A., West, S. A., & Denison, R. F. (2003). Host sanctions and the legume-rhizobium mutualism. *Nature, 425*(6953), 78-81.

Kondorosi, E., Gyuris, J., Schmidt, J., John, M., Duda, E., Hoffmann, B., . . . Kondorosi, A. (1989). Positive and negative control of nod gene expression in *Rhizobium meliloti* is required for optimal nodulation. *The EMBO Journal, 8*(5), 1331-1340.

Krause, A., Doerfel, A., & Gottfert, M. (2002). Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. *Mol Plant Microbe Interact, 15*(12), 1228-1235. doi: 10.1094/mpmi.2002.15.12.1228

Kuzma, M. M., Hunt, S., & Layzell, D. B. (1993). Role of Oxygen in the Limitation and Inhibition of Nitrogenase Activity and Respiration Rate in Individual Soybean Nodules. *Plant Physiol, 101*(1), 161-169.

Labidi, M., Laberge, S., Vezina, L. P., & Antoun, H. (2000). The *dnaJ* (hsp40) locus in *Rhizobium leguminosarum* bv. *phaseoli* is required for the establishment of an effective symbiosis with *Phaseolus vulgaris*. *Molecular Plant-Microbe Interactions, 13*(11), 1271-1274.

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol Res, 169*(1), 2-17. doi: 10.1016/j.micres.2013.09.012

Laranjo, M., Machado, J., Young, J. P. W., & Oliveira, S. (2004). High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiology Ecology, 48*(1), 101-107.

Laranjo, M., Young, J. P. W., & Oliveira, S. (2012). Multilocus sequence analysis reveals multiple symbiovars within *Mesorhizobium* species. *Systematic and Applied Microbiology, 35*(6), 359-367. doi: 10.1016/j.syapm.2012.06.002

Li, W., Ancona, V., & Zhao, Y. (2014). Co-regulation of polysaccharide production, motility, and expression of type III secretion genes by EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora*. *Mol Genet Genomics, 289*(1), 63-75. doi: 10.1007/s00438-013-0790-4

Li, W., & Zhao, Y. (2011). Effect of EnvZ/OmpR and GrrS/GrrA systems on *Erwinia amylovora* virulence. *Phytopathology, 101*(6), S102-S102.

Limpens, E., van Zeijl, A., & Geurts, R. (2015). Lipochitooligosaccharides modulate plant host immunity to enable endosymbioses. *Annu Rev Phytopathol, 53*, 311-334. doi: 10.1146/annurev-phyto-080614-120149

Lopez-Baena, F. J., Vinardell, J. M., Perez-Montano, F., Crespo-Rivas, J. C., Bellogin, R. A., Espuny Mdel, R., & Ollero, F. J. (2008). Regulation and symbiotic significance of nodulation outer proteins secretion in *Sinorhizobium fredii* HH103. *Microbiology, 154*(Pt 6), 1825-1836. doi: 10.1099/mic.0.2007/016337-0

Lopez, O., Morera, C., Miranda_Rios, J., Girard, L., Romero, D., & Soberon, M. (2001). Regulation of gene expression in response to oxygen in *Rhizobium etli*: role of *FnrN* in *fixNOQP* expression and in symbiotic nitrogen fixation. *J Bacteriol, 183*(24), 6999-7006.

MacLean, A. M., Finan, T. M., & Sadowsky, M. J. (2007). Genomes of the symbiotic nitrogen-fixing bacteria of legumes. *Plant Physiol, 144*(2), 615-622. doi: 10.1104/pp.107.101634

Martínez-Salazar, J. M., Sandoval-Calderon, M., Guo, X. W., Castillo-Ramirez, S., Reyes, A., Loza, M. G., . . . Ramirez-Romero, M. A. (2009). The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses. *Microbiology, 155*, 386-397. doi: 10.1099/mic.0.021428-0

Martinez, M., Palacios, J. M., Imperial, J., & Ruiz-Argueso, T. (2004). Symbiotic autoregulation of *nifA* expression in *Rhizobium leguminosarum* bv. *viciae*. *J Bacteriol, 186*(19), 6586-6594.

Mattison, K., & Kenney, L. J. (2002). Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *J Biol Chem, 277*(13), 11143-11148. doi: 10.1074/jbc.M111128200

Mills, S. D., Ruschkowski, S. R., Stein, M. A., & Finlay, B. B. (1998). Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *ompR* and *envZ* mutants are defective for the formation of *Salmonella*-induced filaments. *Infect Immun, 66*(4), 1806-1811.

Minchin, F. R., James, E. K., & Becana, M. (2008). Oxygen Diffusion, Production Of Reactive Oxygen And Nitrogen Species, And Antioxidants In Legume Nodules. In M. J. Dilworth, E. K. James, J. I. Sprent & W. E. Newton (Eds.), *Nitrogen-fixing Leguminous Symbioses* (pp. 321-362). Dordrecht: Springer Netherlands.

Minder, A. C., Narberhaus, F., Babst, M., Hennecke, H., & Fischer, H. M. (1997). The *dnaKJ* operon belongs to the sigma(32)-dependent class of heat shock genes in *Bradyrhizobium japonicum*. *Molecular & General Genetics, 254*(2), 195-206.

Mitsui, H., Sato, T., Sato, Y., Ito, N., & Minamisawa, K. (2004). *Sinorhizobium meliloti* RpoH(1) is required for effective nitrogen-fixing symbiosis with alfalfa. *Molecular Genetics and Genomics, 271*(4), 416-425.

Moulin, L., Klonowska, A., Caroline, B., Booth, K., Vriezen, J. A., Melkonian, R., . . . Riley, M. (2014). Complete Genome sequence of *Burkholderia phymatum* STM815(T), a broad host range and efficient nitrogen-fixing symbiont of Mimosa species. *Stand Genomic Sci, 9*(3), 763-774. doi: 10.4056/sigs.4861021

Moussaid, S., Domínguez-Ferreras, A., Muñoz, S., Aurag, J., Berraho, E. B., & Sanjuán, J. (2015). Increased trehalose biosynthesis improves *Mesorhizobium ciceri* growth and symbiosis establishment in saline conditions. *Symbiosis, 67*(1), 103-111. doi: 10.1007/s13199-015-0338-y

Nandwani, R., & Dudeja, S. S. (2009). Molecular diversity of a native mesorhizobial population of nodulating chickpea (*Cicer arietinum* L.) in Indian soils. *J Basic Microbiol, 49*(5), 463-470. doi: 10.1002/jobm.200800355

Narberhaus, F. (2010). Translational control of bacterial heat shock and virulence genes by temperature-sensing mRNAs. *Rna Biology, 7*(1), 84-89.

Newcomb, W., Sippell, D., & Peterson, R. L. (1979). The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Canadian Journal of Botany, 57*(23), 2603-2616. doi: 10.1139/b79-309

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev, 67*(4), 593-656.

Nogales, J., Campos, R., BenAbdelkhalek, H., Olivares, J., Lluch, C., & Sanjuan, J. (2002). *Rhizobium tropici* genes involved in free-living salt tolerance are required for the establishment of efficient nitrogen-fixing symbiosis with *Phaseolus vulgaris*. *Molecular Plant-Microbe Interactions, 15*(3), 225-232.

Nomura, M., Arunothayanan, H., Van Dao, T., Le, H. T.-P., Kaneko, T., Sato, S., . . . Tajima, S. (2010). Differential protein profiles of *Bradyrhizobium japonicum* USDA110 bacteroid during soybean nodule development. *Soil Science & Plant Nutrition, 56*(4), 579-590. doi: 10.1111/j.1747-0765.2010.00500.x

Nour, S. M., Cleyet-Marel, J.-C., Normand, P., & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology, 45*(4), 640-648.

Nour, S. M., Fernandez, M. P., Normand, P., & Cleyet-Marel, J.-C. (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *International Journal of Systematic Bacteriology, 44*(3), 511-522.

Nukui, N., Minamisawa, K., Ayabe, S.-I., & Aoki, T. (2006). Expression of the 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Gene Requires Symbiotic Nitrogen-Fixing Regulator Gene *nifA2* in *Mesorhizobium loti* MAFF303099. *Appl. Environ. Microbiol., 72*(7), 4964-4969.

Ogawa, J., & Long, S. R. (1995). The *Rhizobium meliloti groELc* locus is required for regulation of early nod genes by the transcription activator *nodD*. *Genes & Development, 9*(6), 714-729.

Oke, V., & Long, S. R. (1999). Bacteroid formation in the *Rhizobium*-legume symbiosis. *Curr Opin Microbiol, 2*(6), 641-646.

Oke, V., Rushing, B. G., Fisher, E. J., Moghadam-Tabrizi, M., & Long, S. R. (2001). Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*. *Microbiology, 147*(Pt 9), 2399-2408. doi: 10.1099/00221287-147-9-2399

Oldroyd, G. E., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet, 45*, 119-144. doi: 10.1146/annurev-genet-110410-132549

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology, 11*(4), 252-263. doi: 10.1038/nrmicro2990

Oldroyd, G. E. D., & Downie, J. M. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes *Annual Review of Plant Biology* (Vol. 59, pp. 519-546).

Ono, Y., Mitsui, H., Sato, T., & Minamisawa, K. (2001). Two RpoH homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti*. *Molecular and General Genetics, 264*(6), 902-912.

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., . . . Mizuno, T. (2002). Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol, 46*(1), 281-291.

Park, H., & Inouye, M. (1997). Mutational analysis of the linker region of EnvZ, an osmosensor in *Escherichia coli*. *J Bacteriol, 179*(13), 4382-4390.

Park, H., Saha, S. K., & Inouye, M. (1998). Two-domain reconstitution of a functional protein histidine kinase. *Proceedings of the National Academy of Sciences of the United States of America, 95*(12), 6728-6732.

Parkinson, J. S., & Kofoid, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu Rev Genet, 26*, 71-112. doi: 10.1146/annurev.ge.26.120192.000443

Peix, A., Ramírez-Bahena, M. H., Velázquez, E., & Bedmar, E. J. (2015). Bacterial Associations with Legumes. *Critical Reviews in Plant Sciences, 34*(1-3), 17-42. doi: 10.1080/07352689.2014.897899

Peoples, M. B., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., . . . Jensen, E. S. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis, 48*(1), 1-17. doi: 10.1007/BF03179980

Perez-Montano, F., Guasch-Vidal, B., Gonzalez-Barroso, S., Lopez-Baena, F. J., Cubo, T., Ollero, F. J., . . . Espuny, M. R. (2011). Nodulation-gene-inducing flavonoids increase overall production of autoinducers and expression of N-acyl homoserine lactone synthesis genes in rhizobia. *Res Microbiol, 162*(7), 715-723. doi: 10.1016/j.resmic.2011.05.002

Pérez-Montaño, F., Jiménez-Guerrero, I., Del Cerro, P., Baena-Ropero, I., López-Baena, F. J., Ollero, F. J., . . . Espuny, R. (2014). The Symbiotic Biofilm of *Sinorhizobium fredii* SMH12, Necessary for Successful Colonization and Symbiosis of *Glycine max* cv Osumi, Is Regulated by Quorum Sensing Systems and Inducing Flavonoids via NodD1. *PLoS One, 9*(8), e105901. doi: 10.1371/journal.pone.0105901

Perret, X., Staehelin, C., & Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews, 64*(1), 180-201.

Pessi, G., Ahrens, C. H., Rehrauer, H., Lindemann, A., Hauser, F., Fischer, H.-M., & Hennecke, H. (2007). Genome-Wide Transcript Analysis of *Bradyrhizobium japonicum* Bacteroids in Soybean Root Nodules. *Molecular Plant-Microbe Interactions, 20*(11), 1353-1363. doi: 10.1094/MPMI-20-11-1353

Philippot, L., & Hallin, S. (2011). Towards food, feed and energy crops mitigating climate change. *Trends in Plant Science, 16*(9), 476-480. doi: 10.1016/j.tplants.2011.05.007

Pickard, D., Li, J., Roberts, M., Maskell, D., Hone, D., Levine, M., . . . Chatfield, S. (1994). Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. *Infection and Immunity, 62*(9), 3984-3993.

Rampersaud, A., Harlocker, S. L., & Inouye, M. (1994). The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. *J Biol Chem, 269*(17), 12559-12566.

Recourt, K., van Brussel, A. A., Driessen, A. J., & Lugtenberg, B. J. (1989). Accumulation of a nod gene inducer, the flavonoid naringenin, in the cytoplasmic membrane of *Rhizobium leguminosarum* biovar *viciae* is caused by the pH-dependent hydrophobicity of naringenin. *J Bacteriol, 171*(8), 4370-4377.

Reina-Bueno, M., Argandona, M., Nieto, J. J., Hidalgo-Garcia, A., Iglesias-Guerra, F., Delgado, M. J., & Vargas, C. (2012). Role of trehalose in heat and desiccation tolerance in the soil bacterium *Rhizobium etli*. *BMC Microbiol, 12*, 207.

Richardson, A. E., Barea, J.-M., McNeill, A. M., & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil, 321*(1), 305-339. doi: 10.1007/s11104-009-9895-2

Rivas, R., Laranjo, M., Mateos, P. F., Oliveira, S., Martinez-Molina, E., & Velázquez, E. (2007). Strains of *Mesorhizobium amorphae* and *Mesorhizobium tianshanense*, carrying symbiotic genes of common chickpea endosymbiotic species, constitute a novel biovar (*ciceri*) capable of nodulating *Cicer arietinum*. *Letters in Applied Microbiology, 44*(4), 412-418. doi: doi:10.1111/j.1472-765X.2006.02086.x

Rodriguez-Quinones, F., Maguire, M., Wallington, E. J., Gould, P. S., Yerko, V., Downie, J. A., & Lund, P. A. (2005). Two of the three *groEL* homologues in *Rhizobium leguminosarum* are dispensable for normal growth. *Arch Microbiol, 183*(4), 253-265.

Rubio, L. M., & Ludden, P. W. (2008). Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. *Annu Rev Microbiol, 62*, 93-111. doi: 10.1146/annurev.micro.62.081307.162737

Sachs, J. L., Skophammer, R. G., & Regus, J. U. (2011). Evolutionary transitions in bacterial symbiosis. *Proc Natl Acad Sci U S A, 108 Suppl 2*, 10800-10807. doi: 10.1073/pnas.1100304108

Sanjuan, J., & Olivares, J. (1991). Multicopy plasmids carrying the *Klebsiella pneumoniae nifA* enhance *Rhizobium meliloti* nodulation competitiveness on alfalfa. *Molecular Plant-Microbe Interactions, 4*(4), 365-369. doi: 10.1094/mpmi-4-365

Sarma, A. D., & Emerich, D. W. (2005). Global protein expression pattern of *Bradyrhizobium japonicum* bacteroids: A prelude to functional proteomics. *PROTEOMICS, 5*(16), 4170-4184. doi: 10.1002/pmic.200401296

Sarma, A. D., & Emerich, D. W. (2006). A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. *Proteomics, 6*(10), 3008-3028. doi: 10.1002/pmic.200500783

Schetgens, R. M. P., Hontelez, J. G. J., van den Bos, R. C., & van Kammen, A. (1985). Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. *Molecular and General Genetics MGG, 200*(3), 368-374. doi: 10.1007/BF00425719

Schumann, W. (2016). Regulation of bacterial heat shock stimulons. *Cell Stress Chaperones, 21*(6), 959-968. doi: 10.1007/s12192-016-0727-z

Shin, S., & Park, C. (1995). Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. *J Bacteriol, 177*(16), 4696-4702.

Soto, M. J., Dominguez-Ferreras, A., Perez-Mendoza, D., Sanjuan, J., & Olivares, J. (2009). Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. *Cellular Microbiology, 11*(3), 381-388. doi: 10.1111/j.1462-5822.2009.01282.x

Soupène, E., Foussard, M., Boistard, P., Truchet, G., & Batut, J. (1995). Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. *Proc Natl Acad Sci U S A, 92*(9), 3759-3763.

Spaink, H. P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol, 54*. doi: 10.1146/annurev.micro.54.1.257

Sprent, J. I. (2009). Evolution of Nodulation *Legume Nodulation* (pp. 51-63): Wiley-Blackwell.

Stepkowski, T., Czaplinska, M., Miedzinska, K., & Moulin, L. (2003). The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha Proteobacteria. *Systematic and Applied Microbiology, 26*(4), 483-494.

Sullivan, J. T., Brown, S. D., & Ronson, C. W. (2013). The NifA-RpoN Regulon of *Mesorhizobium loti* Strain R7A and Its Symbiotic Activation by a Novel LacI/GalR-Family Regulator. *PLoS One, 8*(1). doi: e53762

10.1371/journal.pone.0053762

Sullivan, J. T., Brown, S. D., Yocum, R. R., & Ronson, C. W. (2001). The bio operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. *Microbiology, 147*(5), 1315-1322.

Summers, M. L., Elkins, J. G., Elliott, B. A., & McDermott, T. R. (1998). Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions, 11*(11), 1094-1101. doi: 10.1094/mpmi.1998.11.11.1094

Taíz, L., & Zieger, E. (2004). *Plant Physiology* (E. R. SANTARÉM Ed. 3 ed.). Porto Alegre, Brazil.

Tena, W., Wolde-Meskel, E., Degefu, T., & Walley, F. (2017). Genetic and phenotypic diversity of rhizobia nodulating chickpea (*Cicer arietinum* L.) in soils from southern and central Ethiopia. *Canadian Journal of Microbiology, 63*(8), 690-707. doi: 10.1139/cjm-2016-0776

Terpolilli, J. J., Hood, G. A., & Poole, P. S. (2012). What determines the efficiency of N(2)-fixing *Rhizobium*-legume symbioses? *Adv Microb Physiol, 60*, 325-389. doi: 10.1016/B978-0-12-398264-3.00005-X

Theunis, M., Kobayashi, H., Broughton, W. J., & Prinsen, E. (2004). Flavonoids, NodD1, NodD2, and *nod*-box NB15 modulate expression of the *y4wEFG* locus that is required for indole-3-acetic acid synthesis in *Rhizobium* sp. strain NGR234. *Mol Plant Microbe Interact, 17*(10), 1153-1161. doi: 10.1094/mpmi.2004.17.10.1153

Tschauner, K., Hornschemeyer, P., Muller, V. S., & Hunke, S. (2014). Dynamic interaction between the CpxA sensor kinase and the periplasmic accessory protein CpxP mediates signal recognition in E. coli. *Plos One, 9*(9), e107383. doi: 10.1371/journal.pone.0107383

Tsung, K., Brissette, R. E., & Inouye, M. (1989). Identification of the DNA-binding domain of the OmpR protein required for transcriptional activation of the *ompF* and *ompC* genes of *Escherichia coli* by in vivo DNA footprinting. *J Biol Chem, 264*(17), 10104-10109.

Uchiumi, T., Ohwada, T., Itakura, M., Mitsui, H., Nukui, N., Dawadi, P., . . . Minamisawa, K. (2004). Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J Bacteriol, 186*(8), 2439-2448.

Unkovich, M. J., & Pate, J. S. (2000). An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. *Field Crops Research, 65*(2), 211-228. doi: https://doi.org/10.1016/S0378-4290(99)00088-X

van Brussel, A. A., Tak, T., Boot, K. J., & Kijne, J. W. (2002). Autoregulation of root nodule formation: signals of both symbiotic partners studied in a split-root system of *Vicia sativa* subsp. nigra. *Mol Plant Microbe Interact, 15*(4), 341-349. doi: 10.1094/mpmi.2002.15.4.341

Vance, C. P. (2001). Symbiotic Nitrogen Fixation and Phosphorus Acquisition. Plant Nutrition in a World of Declining Renewable Resources. *Plant Physiol, 127*, 390-397. doi: http://dx.doi.org/10.1104/pp.010331

Via, V. D., Zanetti, M. E., & Blanco, F. (2016). How legumes recognize rhizobia. *Plant Signal Behav, 11*(2), e1120396. doi: 10.1080/15592324.2015.1120396

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M., & Lejeune, P. (1998). Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms

on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J Bacteriol, 180*(9), 2442-2449.

Vinardell, J. M., Lopez-Baena, F. J., Hidalgo, A., Ollero, F. J., Bellogin, R., del Rosario Espuny, M., . . . Ruiz-Sainz, J. E. (2004). The effect of FITA mutations on the symbiotic properties of *Sinorhizobium fredii* varies in a chromosomal-background-dependent manner. *Arch Microbiol, 181*(2), 144-154. doi: 10.1007/s00203-003-0635-3

Vinuesa, P., Silva, C., Lorite, M. J., Izaguirre-Mayoral, M. L., Bedmar, E. J., & Martínez-Romero, E. (2005). Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from rrs, *atpD*, *recA* and *nifH* sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. *Systematic and Applied Microbiology, 28*(8), 702-716.

Vitousek, P. M., Menge, D. N. L., Reed, S. C., & Cleveland, C. C. (2013). Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*(1621).

Wang, L. C., Morgan, L. K., Godakumbura, P., Kenney, L. J., & Anand, G. S. (2012). The inner membrane histidine kinase EnvZ senses osmolality via helix-coil transitions in the cytoplasm. *Embo j, 31*(11), 2648-2659. doi: 10.1038/emboj.2012.99

Wei, G.-H., Yang, X.-Y., Zhang, J.-W., Gao, J.-M., Ma, Y.-Q., Fu, Y.-Y., & Wang, P. (2007). Rhizobialide: A New Stearolactone Produced by *Mesorhizobium* sp. CCNWGX022, a Rhizobial Endophyte from *Glycyrrhiza uralensis*. *Chemistry & Biodiversity, 4*(5), 893-898. doi: 10.1002/cbdv.200790077

Yoshida, T., Cai, S., & Inouye, M. (2002). Interaction of EnvZ, a sensory histidine kinase, with phosphorylated OmpR, the cognate response regulator. *Mol Microbiol, 46*(5), 1283-1294.

Young, J. P., Crossman, L., Johnston, A., Thomson, N., Ghazoui, Z., Hull, K., . . . Parkhill, J. (2006). The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biology, 7*(4), R34.

Yuan, J., Wei, B., Shi, M., & Gao, H. (2011). Functional Assessment of EnvZ/OmpR Two-Component System in *Shewanella oneidensis*. *PLoS One, 6*(8), e23701. doi: 10.1371/journal.pone.0023701

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews, 63*(4), 968-989.

Zhang, F., & Smith, D. L. (1996). Genistein accumulation in soybean (Glycine max L Merr) root systems under suboptimal root zone temperatures. *Journal of Experimental Botany, 47*(299), 785-792. doi: 10.1093/jxb/47.6.785

Zhang, J. J., Liu, T. Y., Chen, W. F., Wang, E. T., Sui, X. H., Zhang, X. X., . . . Chen, W. X. (2012). *Mesorhizobium muleiense* sp. nov., nodulating with *Cicer arietinum* L. *Int J Syst Evol Microbiol, 62*(Pt 11), 2737-2742. doi: 10.1099/ijs.0.038265-0

Zhao, C. T., Wang, E. T., Chen, W. F., & Chen, W. X. (2008). Diverse genomic species and evidences of symbiotic gene lateral transfer detected among the rhizobia associated with *Astragalus* species grown in the temperate regions of China. *FEMS Microbiol Lett, 286*(2), 263-273. doi: 10.1111/j.1574-6968.2008.01282.x

Zhu, Y., Qin, L., Yoshida, T., & Inouye, M. (2000). Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain. *Proceedings of the National Academy of Sciences, 97*(14), 7808-7813. doi: 10.1073/pnas.97.14.7808

Chapter 2

Improvement of mesorhizobia strains by additional copies of *nifA*

2.1. Summary

In diazotrophic bacteria, the *nifA* gene encodes a regulatory protein that controls the expression of the nitrogenase operon *nifHDK*, among other genes. The aim of this work was to investigate the effect of overexpressing the *nifA* gene in the symbiosis between mesorhizobia and chickpea (*Cicer arietinum* L.). The *Mesorhizobium* sp. ST-2, V-15b, PMI-6 and UPM-Ca36^T strains were transformed with plasmid pRKnifA (pRK415 with the *nifA* gene from *M. mediterraneum* UPM-Ca36^T) and pRK415. The free-living phenotypes and symbiotic performance of the transformed strains were evaluated. The strains ST2pRKnifA, PMI6pRKnifA and Ca36pRKnifA did not show any improvement in the symbiotic effectiveness. On the other hand, plants inoculated with V15bpRKnifA showed a significantly higher shoot dry weight and consequently an improvement in the symbiotic effectiveness (about four times higher than V15bpRK415). Nodules from plants inoculated with V15bpRKnifA were larger and had a larger fixation zone and hydroponic assay showed that extra *nifA* copies in V-15 improved its ability to form nodules. In addition, the swimming ability was also improved in the strain V15bpRKnifA. Analyzes of root hairs of plants inoculated with V-15b, V15bpRK415 and V15bpRKnifA did not reveal any difference regarding colonization, curling or infection thread formation.

2.2. Introduction

Despite its abundance in the atmosphere, nitrogen is one of the main limiting factors of global agricultural production (Hoffman et al. 2014). Some bacteria, such as rhizobia, are able to promote plant growth by fixing atmospheric nitrogen, while in symbiosis with leguminous plants as bacteroids, in the root nodules (Haag et al. 2013).

Bacterial species of the genus *Mesorhizobium* are reported to fix atmospheric nitrogen when in symbiosis with several legumes species, some with high agronomic importance such as chickpea (*Cicer arietinum* L.), which is used for food and forage (Laranjo et al. 2014). For the establishment of an effective symbiosis two main classes of bacterial symbiosis genes are needed: nodulation and nitrogen fixation genes. Despite the fact that many studies have addressed these genes, there are few reports on the role of genes related to nodulation and nitrogen fixation in chickpea mesorhizobia.

In bacteroids, the NifA regulon includes the operon *nifHDK*, which encode the nitrogenase complex (Novichkov et al. 2013). NifA is also required for transcription of *fixABCX*, *nifN* and *nifB*. Moreover, in bacteria such as *Sinorhizobium meliloti,* NifA controls other genes that are not directly involved in nitrogen fixation, but are related to competitiveness, nodulation efficiency, development of nodules and bacteroid persistence, such as *nfe* (*nodule formation efficiency*) and *mos* (rhizopine synthesis) (Fischer 1994). Further characterization of the NifA-RpoN regulon in *Mesorhizobium loti* R7A has revealed other genes under the control of this regulator, such as a porin-encoding gene required for an effective symbiosis (Sullivan et al. 2013). In *Rhizobium leguminosarum* symbiotic *nifA* expression is under positive autoregulation by NifA and originates from a promoter (P_{nifA1}) located 4.7 kb upstream of *nifA* (Martinez et al. 2004).

The nitrogenase enzyme complex, formed by two protein units, the Iron - protein (encoded by *nifH*) and the Molybdenum-Iron-protein (encoded by *nifD* and *nifK* genes), is responsible for nitrogen fixation in the nodule (Hoffman et al. 2014). The limitation of oxygen is a key factor in the expression of *nif* and *fix* genes (Soupène et al. 1995), since the nitrogenase complex is irreversebly inactivated by O_2. The *fixL* and *fixJ* genes encode a regulatory system in which the oxygen sensor FixL transfers phosphate to the response regulator FixJ. The phosphorylated FixJ positively controls the transcription of *nifA* and *fixK* in *Sinorhizobium meliloti* (Gilles-Gonzalez et al. 1991). On the other hand, FixK induces the expression of *fixNOQP* and negatively affects the expression of *nifA* (Batut et al. 1989).

nifA mutants of *Bradyrhizobium japonicum* inoculated in soybean induced the formation of numerous nodules, although with reduced size and unable to fix nitrogen (Fischer et al. 1986). Gong et al. (2006) showed that *S. meliloti nifA* mutants induced a

different gene expression profile from wild-type strain in alfalfa nodules. Some of these genes are involved in signal communication, protein degradation, nutrient metabolism, cell growth and development.

Several reports describe the use of additional copies of *nifA* in rhizobia, nevertheless these studies are limited to the *Sinorhizobium* genus. Initial studies on the *nifA* gene from *Klebsiella pneumoniae* showed that its overexpression in *S. meliloti* increased competitiveness on alfalfa plants (Sanjuan and Olivares 1991). However, a later study by van Dillewijn and co-workers (1998) showed that the expression of *nifA* from *K. pneumoniae* does not affect the *S. meliloti* competitiveness. On the other hand, a similar study in *Sinorhizobium fredii* reported that extra copies of the *nifA* gene of *K. pneumoniae* accelerated the nodulation and increased competitiveness on soybean (Jieping et al. 2002). A more recent study described that extra copies of the *nifA* gene in *S. meliloti* 1021 improved the nitrogen-fixing efficiency in *Medicago sativa* root nodules to a greater extent than that observed upon transfer of the *Enterobacter cloacae nifA* gene (Chengtao et al. 2004). Furthermore, Bosworth et al. (1994) performed field trials with alfalfa using *S. meliloti* modified with extra *nifA* and *dctABD* copies obtaining an increased alfalfa biomass by 12.9% compared with the yield achieved with the wild-type strain.

The objective of this study was to evaluate the effect of overexpressing the *nifA* gene in the nodulation and symbiotic effectiveness (SE) of *Mesorhizobium* strains, namely to evaluate if a higher expression of this regulator gene could lead to an improvement of the symbiotic performance. Four *Mesorhizobium* strains able to nodulate chickpea were transformed with additional copies of the *nifA* gene from *M. mediterraneum* UPM-Ca36^T, cloned in the expression vector pRK415.

2.3. Material and Methods

Bacterial strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in table 1. Three chickpea mesorhizobia, isolated from Portuguese soils and previously characterized, were selected: V-15b-Viseu, PMI-6-Portimão and ST-2-Setúbal (Alexandre et al. 2009; Brígido et al. 2012a; Laranjo et al. 2008). In addition, the type strain of *Mesorhizobium mediterraneum* (strain UPM-Ca36) was also used (Nour et al. 1995).

The mesorhizobia strains were routinely grown at 28º C in tryptone-yeast (TY) medium (Beringer 1974b). The growth medium for pRK415-transformed mesorhizobia strains was supplemented with tetracycline (15 μg.ml^{-1}). The *Escherichia coli* DH5α and

MT616 strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001) at 37º C. For the *E. coli* strains containing pRK415, 15 μg.ml^{-1} of tetracycline was used, while for the strain MT616 with pRK600, the medium was supplemented with 25 μg.ml^{-1} of chloramphenicol.

Table 1 Bacterial strains and plasmids used in this work

Plasmids/Strains	Characteristics	Reference
pRK600	pRK2013 *npt*::Tn9. Cm^r	(Finan et al. 1986)
pRK415	Broad-host-range vector; Tc^r	(Keen et al. 1988)
pRKnifA	Plasmid pRK415 containing the *nifA* gene from *M. mediterraneum* UPM-$Ca36^T$; Tc^r	This work
pMRGFP	Plasmid containing the *gfp* gene; Km^r	(García-Fraile et al. 2012)
E. coli		
MT616	Strain containing the helper plasmid pRK600	(Finan et al. 1986)Finan *et al* 1986
DH5α	Competent cells	NZYTech
Mesorhizobium		
V-15b	*Mesorhizobium* sp. V-15b-Viseu isolated from chickpea root nodules	(Alexandre et al. 2009)
ST-2	*Mesorhizobium* sp. ST-2-Setubal isolated from chickpea root nodules	(Alexandre et al. 2009)
PMI-6	*Mesorhizobium* sp. PMI-6-Portimão isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
UPM-$Ca36^T$	*Mesorhizobium mediterraneum* UPM-$Ca36^T$ isolated from chickpea root nodules from Spain	(Nour et al. 1995)
V15bpRKnifA	V-15b strain harboring pRKnifA	This work
ST2pRKnifA	ST-2 strain harboring pRKnifA	This work
PMI6pRKnifA	PMI-6 strain harboring pRKnifA	This work
Ca36pRKnifA	UPM-$Ca36^T$ strain harboring pRKnifA	This work
V15bpRK415	V-15b strain harboring pRK415	This work
ST2pRK415	ST-2 strain harboring pRK415	This work
PMI6pRK415	PMI-6 strain harboring pRK415	This work
Ca36pRK415	UPM-$Ca36^T$ strain harboring pRK415	This work
V15bGFP	V-15b strain harboring pMRGFP	This work
V15bpRKnifAGFP	V-15b strain harboring pRKnifA and pMRGFP	This work
V15bpRK415GFP	V-15b strain harboring pRK415 and pMRGFP	This work

Transforming the strains with the *nifA* gene

The *nifA* gene from *Mesorhizobium mediterraneum* UPM-$Ca36^T$ (accession number KT285486) was obtained by PCR amplification. Strain UPM-$Ca36^T$ is the *M. mediterraneum* type strain that nodulates chickpea (Nour et al. 1995). The PCR was performed in 50 µl using 1 µL of DNA, 1× Buffer for KOD Hot Start DNA Polymerase, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 15 pmol of each primer and 0.02 U of KOD Hot Start DNA Polymerase (Novagen). The primers used were nifAi-F (5'-AAGCTTAATGGGCTGCCAAATGGAACG-3') and nifA-R (5'-

AAGCTTTCAGAGACGCTTGATCTCGA-3'). The amplification program was: 2 min at 95 °C, 30 cycles of 20 s at 95 ºC, 10 s at 62 °C, and 21 s at 70 ºC. The obtained *nifA* fragment of 1050 bp was cloned in pCR-Blunt™ vector (ThermoFisher Scientific), sequenced and subcloned in the expression vector pRK415, previously digested with *Hind*III.

The four chickpea mesorhizobia strains were transformed by triparental mating with the plasmid pRK415 containing the exogenous *nifA* gene under the control of the *lac* promoter (pRKnifA). *E. coli* DH5α cells containing the plasmid pRKnifA were used as the donor, mesorhizobia isolates were the recipient and *E. coli* MT616 cells, with pRK600 acted as helper, as described by Nascimento et al. (2012b). The pRKnifA-transformed strains were named V15bpRKnifA, ST2pRKnifA, PMI6pRKnifA and Ca36pRKnifA. The mesorhizobia strains were also transformed with pRK415 and named V15bpRK415, ST-2pRK415, PMI6pRK415 and Ca36pRK415.

In order to confirm the transformation of mesorhizobia cells with pRKnifA, total DNA was extracted according to Rivas et al. (2001) and used to amplify the region of the expression vector that includes the *nifA* gene. A DNA fragment of 1181 bp is expected using the universal primers M13F and M13R-pUC. The PCR reaction was performed in a final volume of 50 µL, using 5 µL of DNA, 1× reaction Green GoTaq® Flexi buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 15 pmol of each primer and 0.625U of GoTaq® G2 Flexi DNA Polymerase (Promega). The amplification program was: 2 min of initial denaturation at 95 °C, 30 cycles of 60 s at 95 ºC, 45 s at 56 °C, 71 s at 72 ºC, and a final extension of 5 min at 72 ºC.

Once the transformations were confirmed, strains harboring pRKnifA or pRK415 were transformed with pMRGFP (containing the *gfp* gene) by triparental mating, as described above. These transformations were confirmed by fluorescence microscopy observations.

Confirmation of exogenous *nifA* gene expression and analysis of *nifH* expression by semiquantitative RT-PCR

In order to confirm the expression of the *nifA* gene cloned in pRK415 and to evaluate changes in the *nifH* expression, semiquantitative RT-PCR analyses were performed (Moscatiello et al. 2009). Total RNA of V-15b transformed strains (V15bpRKnifA and V15bpRK415) was extracted using the GeneJET™ RNA Purification Kit (ThermoFisher Scientific), from bacteria grown in TY at 28 ºC for 18 hours, with a final OD_{540} of 0.4 and from nodules of *Cicer arietinum* plants 21 days after inoculation. DNase

I (Roche Diagnostics) was used to eliminate DNA contamination, followed by RNA cleanup using GeneJET™ RNA Purification Kit. Approximately 500 ng of total RNA were subjected to reverse transcription for cDNA synthesis, using the Reverted first strand cDNA synthesis kit (ThermoFisher Scientific). The cDNA obtained was used for PCR amplification of *nifA* gene (primers nifA-F 5'- CGCTTTGAGGCTGCCGACAA-3' and nifA-R 5'- AAGCTTTCAGAGACGCTTGATCTCGA-3'), which generates a fragment of 759 bp. The PCR reaction was performed in a final volume of 25 µL, using 4 µL of cDNA, 1× reaction Green GoTaq® Flexi buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 15 pmol of each primer and 0.125U of GoTaq® G2 Flexi DNA Polymerase (Promega). The amplification program was: 2 min of initial denaturation at 95 °C, 30 cycles of 60 s at 95 ºC, 45 s at 55 °C, 46 s at 72 ºC, and a final extension of 5 min at 72 ºC.

For amplification of the *nifH*, PCR was performed in a final volume of 25 µL as described for *nifA* amplification. The primers used were NifHintFW (5'-TCCACCACGTCCCAAAATAC-3') and NifHintRv (5'-CTCTGTAGCCCACCTTGAGC-3'). The amplification program was: 2 min of initial denaturation at 95 °C, 30 cycles of 60 s at 95 ºC, 60 s at 56 °C, 12 s at 72 ºC, and a final extension of 5 min at 72 ºC. The reaction generates a fragment of 200 bp.

The 16S rRNA gene amplification was used to normalize the relative *nifA* and *nifH* transcript abundance. The primers IntF and IntR (Laranjo *et al* 2004), were used to generate a fragment of 199 bp. The PCR reaction was performed in a volume of 25 µL, using 1 µL of cDNA, 1× reaction Green GoTaq® Flexi buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 15 pmol of each primer and 0.125U of GoTaq® G2 Flexi DNA Polymerase (Promega). The amplification program was: 2 min of initial denaturation at 95 °C, 20 cycles of 60 s at 95 ºC, 60 s at 56 °C, 12 s at 72 ºC, and a final extension of 5 min at 72 ºC.

Positive controls using total DNA of V-15b strain as template and negative controls without reverse transcriptase enzyme were performed. Densitometric analysis of ethidium bromide–stained agarose gels was performed using Kodak Digital Science 1D version 2.0.3 (Eastman Kodak Company).

Evaluation of symbiotic performance

In order to evaluate if the presence of the additional *nifA* gene improved the symbiotic performance of the mesorhizobia strains, a plant growth trial under controlled conditions was performed using chickpea plants grown in pots and inoculated with both the wild-type and the transformed strains. Chickpea seeds were surface-sterilized and pre-germinated as previously described. After germination, the seedlings were transferred to plastic pots filled with sterile vermiculite and inoculated (Alexandre et al. 2009).

The rhizobia strains were grown in TY liquid medium at 28° C for 72 h. Cell culture was centrifuged at 10.000 × g, resuspended in fresh TY liquid medium to an OD_{540} of 1.0 and 1 ml of this bacterial suspension was used to inoculate each seedling. Five replicates were used for each treatment. A nitrogen-free nutrient solution (Broughton and Dilworth 1971) was applied three times a week. Uninoculated plants were used as negative control and plants watered with N-supplemented nutrient solution were used as positive control. Plants were grown in a growth chamber under a 16 h-light and 8 h-dark cycle and 24° C-day and 18° C-night temperature at a relative humidity of 65%. After 8 weeks the plants were harvested and several parameters were measured, namely shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN), average weight per nodule (AWN). Symbiotic effectiveness (SE) was calculated using the following formula (Gibson 1987):

SE = (SDW inoculated plants - SDW negative control plants) / (SDW positive control plants - SDW negative control plants).

Statistical analysis included analysis of variance and the data were compared by one-way ANOVA and the Duncan's Multiple Range Test ($P < 0,05$), using SPSS statistics V.21 (SPSS Inc).

Based on the results obtained in the symbiotic performance evaluation, mesorhizobia strain V-15b and its derivates were selected to be used for further analyses.

Growth curves

Triplicates of wild-type strain V-15b as well as its corresponding transformed strains with pRK415 and pRKnifA were cultured in 5 mL of TY medium (supplemented with 15 $\mu g.ml^{-1}$ tetracycline in the case of strains carrying plasmids pRK415 and pRKnifA) during 142 h. The optical densities at 540 nm ($O.D._{540}$) were measured every 24 hours, in order to generate the corresponding growth curves.

Motility assay

To compare the swimming ability between the wild-type (V-15b) and transformed strains (V15bpRK415, V15bpRKnifA), a motility assay was performed in TY plates containing 0.25% agar (Rouws et al. 2008). An aliquot of 10 µl of cell suspension ($O.D._{540}$ of 0.1) was inoculated in the center of the Petri dishes and after 20 days of incubation at 28º C the diameter of the halo formed was measured.

Evaluation of Nodulation Kinetics

The nodulation kinetics were evaluated through a hydroponic assay using chickpea plants inoculated with V15bpRK415 and V15bpRKnifA ($OD_{540\ nm}$ of 0.6). The procedures were conducted as previously described in Brígido et al. (2012b). Eight seeds per treatment were used and the number of nodules was evaluated every three days during 33 days. Plants were kept on a growth chamber under the same conditions describe above for the evaluation of symbiotic performance.

Histological analysis of nodules

Nodules were excised from 7-week-old chickpea plants and processed for sectioning and histological observation by bright field microscopy. Briefly, nodules were fixed in a 4% formaldehyde solution, dehydrated in an increasing ethanol series and embedded in paraffin. Cross-sections of embedded nodules (2 µm) were stained with 0.1% Toluidine Blue solution. Histological sections of nodules were examined under a Nikon SMZ800 stereomicroscope and Nikon Eclipse 80i microscope.

Analysis of rhizobia infection process

Pre-germinated chickpea seeds were inoculated with GFP-tagged (single infection) mesorhizobia strains, as described by Robledo et al. (2011). Chickpea roots and root hairs were stained with 10 µM propidium iodide (Sigma-Aldrich) (Flores-Félix et al. 2015). Projections were made from adjusted individual channels and accumulating stacks using Leica software. The analysis of root hairs 3 to 7 days after inoculation were performed using a Confocal Laser Scanning Microscope (Leica TCS SPE) equipped with solid-state laser, allowing visualization of GFP (488 nm), RFP and propidium iodide (532 nm) fluorescence.

2.4. Results

Confirmation of *nifA* overexpression and evaluation of *nifH* expression

NifA is a positive regulator of genes involved in nitrogen fixation, namely genes encoding the nitrogenase complex, the *nifHDK* operon. In order to confirm that the *nifA* transcript was more abundant in the V15bpRKnifA than in theV15bpRK415 strain as well as to evaluate if the expression of the gene *nifH* was higher in the former strain due to the higher NifA amounts, a semiquantitative analysis of these genes expression was performed by RT-PCR. The results presented in Fig. 1A show that the *nifA* expression levels in free-living V15bpRKnifA cells are approximately four fold higher than in the V15bpRK415 cells. The expression of the same gene was also evaluated in bacteroids (21 days after inoculation) and the transcript levels in V15bpRKnifA were approximately three fold higher than in V15bpRK415 bacteroids (Fig. 1B).

To evaluate if higher levels of *nifA* transcript lead to higher NifA amounts and consequently to higher transcript levels of the NifA regulon genes, the expression levels of the *nifH* gene were evaluated in bacteroids. The results show that in V15bpRKnifA bacteroids the expression of *nifH* is approximately two fold higher than in V15bpRK415 bacteroids (Fig. 1B).

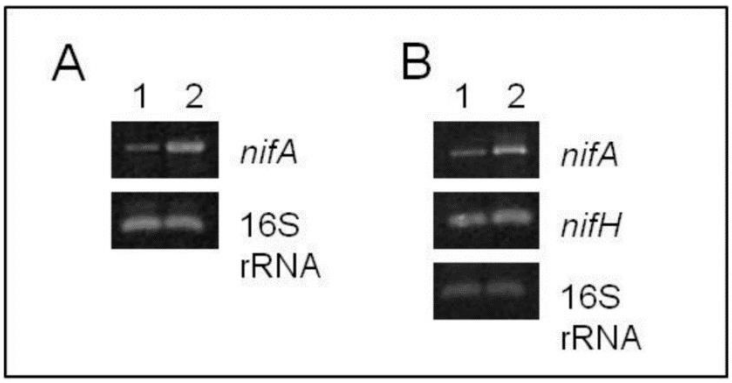

Figure 1. Analysis of *nifA* and *nifH* expression by semiquantitative RT-PCR in V15bpRK415 and V15bpRnifA strains. A - *nifA* gene expression in free-living cells and B - *nifA* and *nifH* genes expression in bacteroids. 1 - V15bpRK415 and 2 - V15bpRKnifA strains.

Evaluation of the symbiotic performance

In order to evaluate the effect of the extra *nifA* copies in the symbiotic performance of the transformed strains, several plant parameters were evaluated in a

plant growth assay. The Number of Nodules (NN), Root Dry Weight (RDW), Shoot Dry Weight (SDW) as well as Symbiotic Effectiveness (SE) were determined using inoculated chickpea plants grown in pots under controlled conditions.

No nodules were found in the roots of plants used as positive control (non-inoculated plants–supplemented with nitrogen) and negative control (non-inoculated plants with no nitrogen source).

Although there were no significant differences in the number of nodules obtained with the three V-15b strains (Table 2), the AWN from plants inoculated with V15bpRKnifA was about three times higher than those inoculated with V15b or V15bpRK415.

In terms of plant growth, the results show that chickpea plants inoculated with V15bpRKnifA have a higher shoot dry weight compared to plants inoculated with V15bpRK415 (Table 2). The SDW data were used to calculate the SE, which was significantly increased by the overexpression of the *nifA* gene (Table 2). Although the increase in SDW, and consequently in SE, of the strain V15bpRKnifA is not statistically significant when compared to the wild type strain, the SE of strain V15bpRKnifA is about 4 times higher than the SE of strain V15bpRK415. No significant differences were obtained between the root dry weight of the plants inoculated with the three strains (Table 2).

With the exception of the number of nodules that was higher in plants inoculated with PMI6pRKnifA, none of the other parameters were improved when plants were inoculated with PMI6pRKnifA, ST2pRKnifA and Ca36pRKnifA compared with their respective strains harboring the pRK415 (Table 2). Therefore, only V-15b and its transformed strains were used for the following assays.

Table 2. Results obtained from pot assays of chickpea plants inoculated with wild type or transformed strains.

Strain	SDW (g)	RDW (g)	NN	AWN (mg)	SE (%)
V-15b	0,507 ± 0,269 ab	0,306 ± 0,169 a	101 ± 56 a	0,659 ± 0,291 b	15,98 ± 18,5 ab
V15bpRK415	0,385 ± 0,151 b	0,274 ± 0,143 a	117 ± 51 a	0,576 ± 0,732 b	7,60 ± 10,3 b
V15bpRKnifA	0,716 ± 0,214 a	0,362 ± 0,134 a	76 ± 22 a	1,684 ± 0,052 a	30,33 ± 14,7 a
PMI-6	1,349 ± 0,389 a	0,636 ± 0,232 a	42 ± 21 b	4,470 ± 1,647 ab	40,87 ± 14,3 a
PMI6pRK415	1,511 ± 0,279 a	0,608 ± 0,080 a	33 ± 5 b	6,459 ± 1,435 a	46,84 ± 10,3 a
PMI6pRKnifA	1,675 ± 0,281 a	0,730 ± 0,260 a	72 ± 15 a	2,912 ± 1,664 b	52,88 ± 10,4 a
UPM-Ca36^T	1,592 ± 0,266 a	0,784 ± 0,113 a	52 ± 16 a	4,335 ± 1,060 a	49,84 ± 9,8 a
Ca36pRK415	1,514 ± 0,200 a	0,766 ± 0,174 a	47 ± 18 a	6,855 ± 2,398 a	46,94 ± 7,4 a
Ca36pRKnifA	1,571 ± 0,391 a	0,703 ± 0,201 a	60 ± 22 a	5,659 ± 3,339 a	49,03 ± 14,4 a
ST-2	1,427 ± 0,442 a	0,833 ± 0,191 a	48 ± 14 a	3,799 ± 1,355 a	43,73 ± 16,2 a
ST2pRK415	1,336 ± 0,211 a	0,850 ± 0,162 a	41 ± 8 a	3,591 ± 1,175 a	40,38 ± 7,8 a
ST2pRKnifA	1,641 ± 0,261 a	0,912 ± 0,243 a	57 ± 13 a	4,253 ± 0,302 a	51,61 ± 9,6 a

Different letters in the same group of strains indicate statistically significant differences (*P<0,05*). SDW - Shoot Dry Weight; RDW - Root Dry Weight; NN - Number of Nodules; AWN - Average Weight per Nodule; SE - Symbiotic Effectiveness

Growth curves

In order to characterize the growth kinetics, V15b, V15bpRK415 and V15bpRKnifA strains were grown in TY medium. A higher growth rate of the wild-type was obtained particularly in the exponential phase, compared to the transformed strains (Fig. 2A). This is probably due to the energy required for the replication of plasmids pRK415 and pRKnifA. The growth curves of the three V-15b strains are different, with the wild-type growing faster and the V15bpRKnifA growing slower.

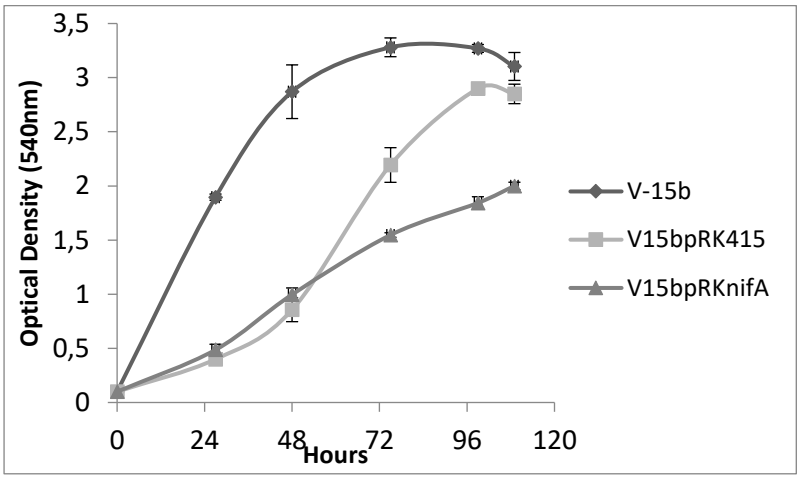

Figure 2. Growth curves in TY of V-15b, V15bpRK415 and V15bpRKnifA strains. Bars represent standard deviation.

Motility assay

The motility can affect the infection ability of rhizobia (Caetano-Anollés *et al* 1988; Gay-Fraret *et al* 2012). In order to test the influence of extra copies of *nifA* in the swimming ability, motility assays were performed using the wild-type and transformed strains of V-15b.

The strain V15bpRKnifA displayed a halo with a radius slightly larger than the wild type and larger than V15bpRK415 at 20 days after inoculation, indicating a faster swimming ability of the strain with extra *nifA* copies. These results suggest that the expression vector alone affects the swimming ability (Fig. 3).

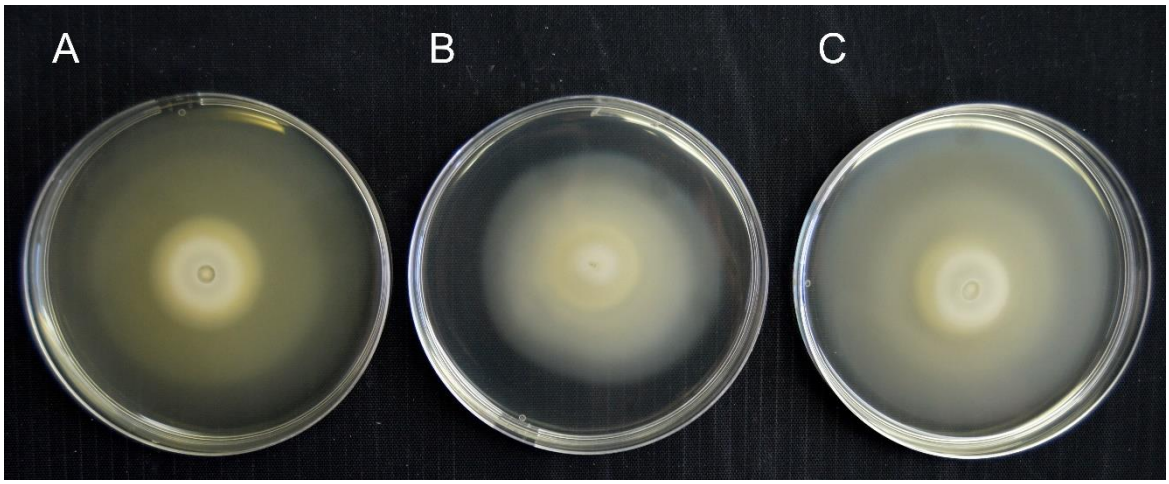

Figure 3. Swimming ability of A - V-15b, B - V15bpRK415 and C - V15bpRKnifA strains in TY culture medium containing 0.25% agar. The strains were grown at 28 ºC for 20 days.

Kinetics of nodulation

In order to evaluate the effect of additional *nifA* copies in the kinetics of nodulation of chickpea plants, a hydroponic assay was conducted and the number of nodules was monitored for 35 days post inoculation (dpi).

An initial delay of, at least, two days was observed in the formation of the first nodules by V15bpRK415 compared to V15bpRKnifA inoculation (Fig. 4). In addition, the plants inoculated with V15bpRKnifA showed a higher number of nodules throughout the

hydroponic trial. At 34 dpi the highest difference was observed in the number of nodules between plants inoculated with the V15bpRKnifA strain and those inoculated with V15bpRK415. These results suggest that the presence of extra copies of the *nifA* gene improve the nodulation efficiency of strain V-15b and that this gene is involved in the chickpea nodulation process.

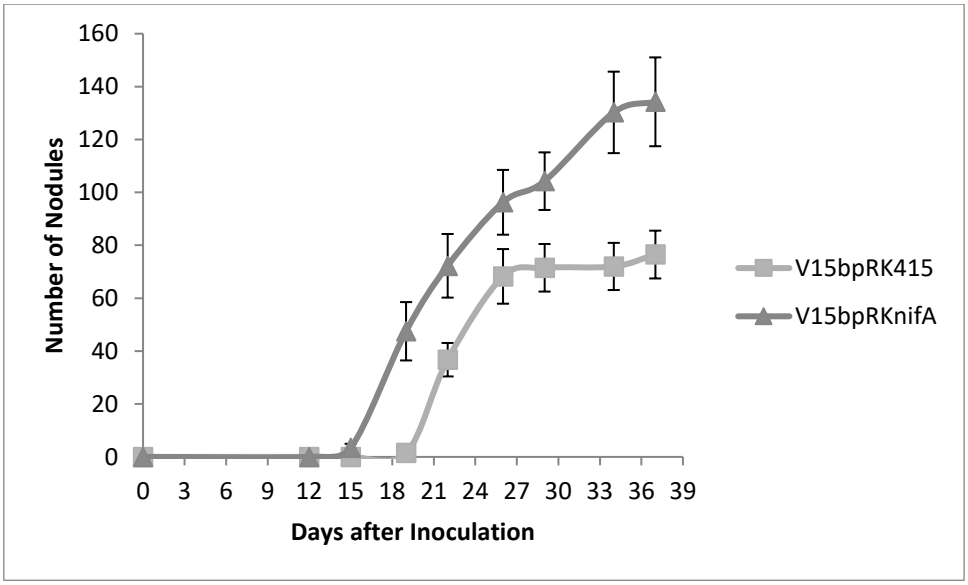

Figure 4. Nodulation kinetics of chickpea plants. Average number of nodules for plants inoculated with V15bpRK415 and V15bpRKnifA strains. Bars represent standard error.

Histological analysis of nodules

Although nodule number was not significantly different among the three treatments in the pot trial performed to evaluate the symbiotic effectiveness, the average weight per nodule from V15bpRKnifA inoculated plants is higher than in the V-15b and V15bpRK415 inoculated plants. In order to determine if there is an alteration of the inner nodule morphology, a histological analysis of the nodules cross-sections was performed.

Histological sections of nodules collected seven weeks after inoculation (Fig. 5) showed that V-15b, V15bpRK415 and V15bpRKnifA formed indeterminate nodules in which nodule zones I, II, III and IV (Vasse et al. 1990) were well-defined (Fig. 5A, E, I). Nodules from V15bpRKnifA treatment showed a more developed fixation zone (Fig. 5I) than V-15b and V15bpRK415 nodules (Fig. 5A, E). Higher magnification of infection (Fig. 5C, G, K) and fixation (Fig. 5D, H, L) zones confirms that there are no structural differences in these zones, showing typical infection threads (IT) ends and cells containing bacteroids, respectively. However, it is noticeable that the fixation zone in V15bpRKnifA nodules occupies a larger area of the nodule than in V-15b and V15bpRK415 nodules. It is remarkable the presence of an extra zone in nodules from

V15bpRK415 and V15bpRKnifA treatments, which harbour some similarities with the infection zones (Fig. 5F, J). This extra zone is not present in the V-15b induced nodules (Fig 5B).

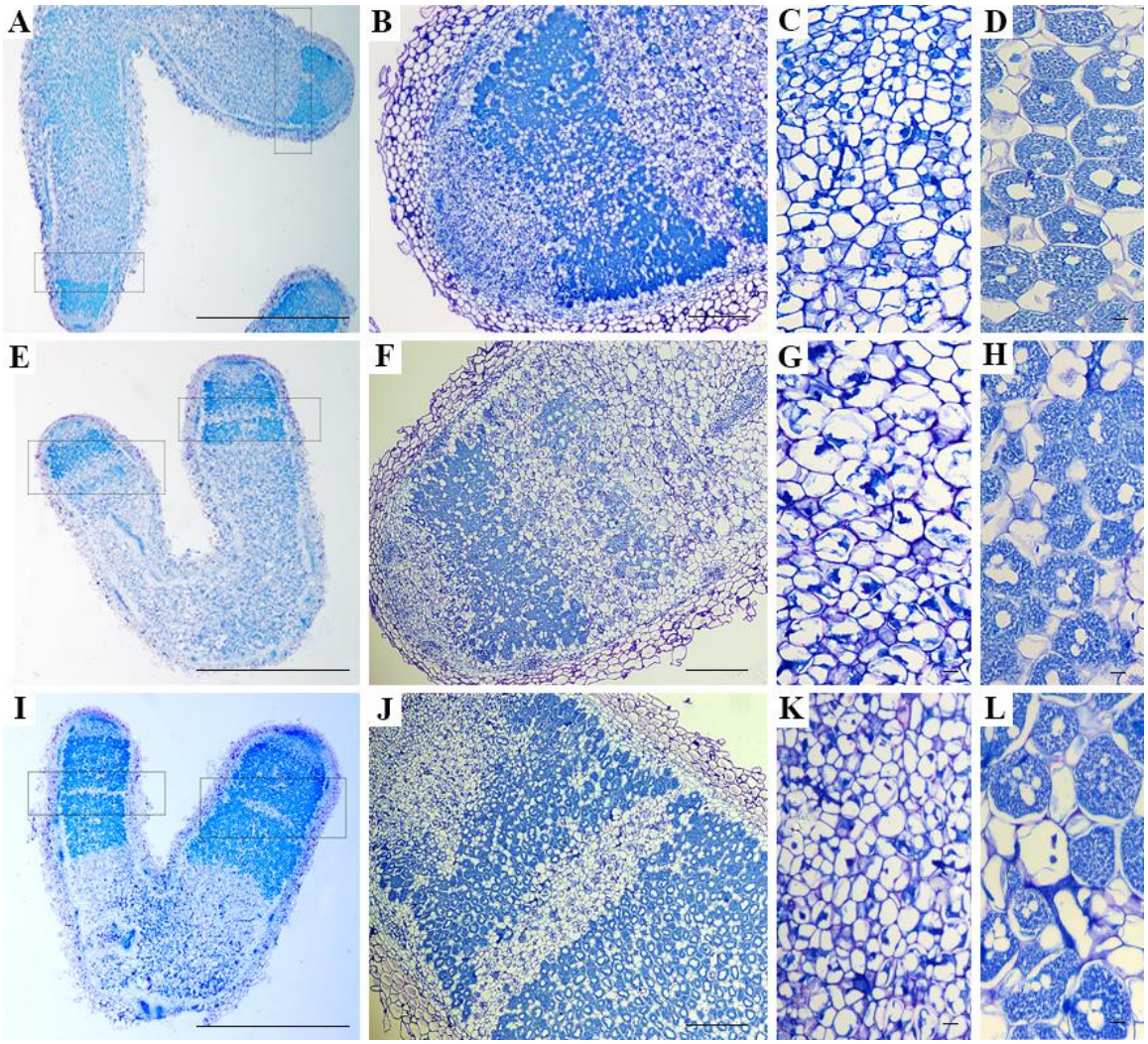

Figure 5. Histological sections of nodules from chickpea plants inoculated with chickpea mesorhizobia V-15b and its derivatives (V15bpRK415 and V15bpRKnifA). Structures of whole nodules induced by A - V-15b, E - V15bpRK415 and I - V15bpRKnifA strains. Panels B, F and J showed the area marked with squares in the panels A, E and I, respectively, showing the fixation zone on a higher magnification. Infection zone are showed in panels C, G, K and the fixation zone in panels D, H, L with a higher magnification. Scale bars are 1mm (A, E and I); 500 µm (B, F and J); 16 µm (C, G, K, D, H and L).

Colonization and infection thread

In order to determine if the overexpression of the *nifA* gene may be inducing changes in the mesorhizobia-chickpea infection process, *in vitro* infection assays were performed.

Mesorhizobial infection process was analysed on roots of chickpea seedlings during the early steps of the interaction (4-7 dpi). V15bGFP, V15bpRK415GFP and V15bpRKnifAGFP were able to effectively colonize and infect chickpea roots and root hairs, showing typical caps, curlings and infection threads.

No clear differences in the structures or levels of infection threads formation were found among V15bGFP, V15bpRK415GFP and V15bpRKnifAGFP inoculated plants. As an example, Fig. 6 shows chickpea root hairs colonized by V15bpRKnifAGFP.

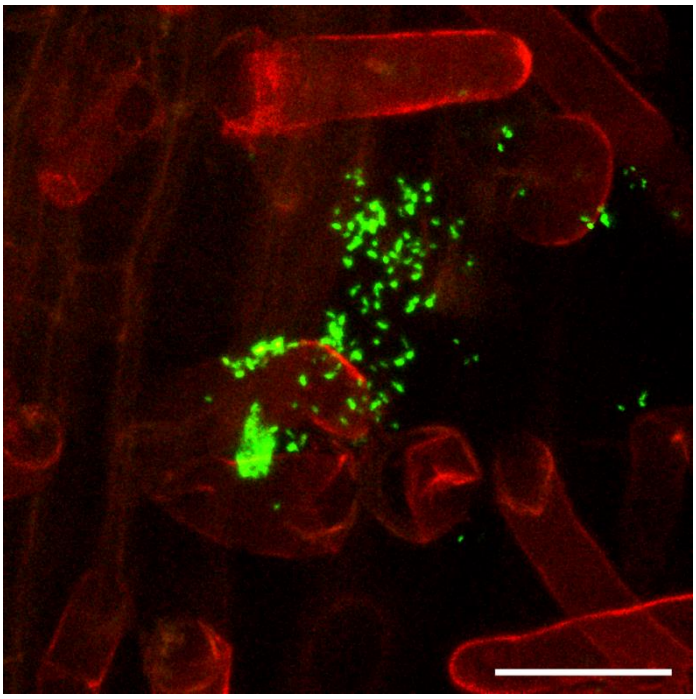

Figure 6. Confocal laser scanning micrograph showing the colonization of chickpea root hairs inoculated with green fluorescent protein–tagged strain V15pRKnifAGFP. Scale bar: 25 µm

2.5. Discussion

In the present work, four *Mesorhizobium* strains able to nodulate chickpea were transformed with the *nifA* gene from *M. mediterraneum* UPM-Ca36^T, cloned in the expression vector pRK415. The aim was to evaluate the effects of extra *nifA* copies in the symbiotic performance of these strains, namely a potential improvement of their SE.

The strains UPM-Ca36^{T}, PMI-6 and ST-2 transformed with extra *nifA* copies did not show any improvement in the SE. On the other hand, V15bpRKnifA showed a significant increase of SE, compared to the V15bpRK415 strain. In addition, the pot assay revealed a higher AWN with V15bpRKnifA inoculation, indicating that the extra *nifA* copies contributed to the development of larger nodules. Thus, the NifA protein appears to influence the size and weight of nodules, probably acting as a regulator of genes involved in nodule development. Larger functional nodules probably contributed to the increase of the symbiotic effectiveness. These results agree with other studies suggesting that NifA influences the number and size of the nodules. For example, nodulation assays with *Bradyrhizobium japonicum* showing that *nifA* mutant strains induce about two times more nodules than the wild-type strain, but the dry weight per nodule was about four times smaller and these nodules were Fix^{-} (Fischer et al. 1986). Similarly, Sanjuan et al. (1989) reported that *S. meliloti nifA* mutants can produce numerous, but small and white root nodules. It was reported that in a *S. meliloti nifA* mutant four nodulation-specific genes (*nodH*, *nodL*, *nolF* and *noeB*) were down-regulated in 30-day nodules (Gong et al. 2006).

Histological analysis of nodules collected seven weeks after inoculation showed that V-15b, V15bpRK415 and V15bpRKnifA formed indeterminate nodules with the expected zones, however the fixation zone observed in nodules from plants inoculated with V15bpRKnifA was larger than that observed in nodules from plants inoculated with V-15b and V15bpRK415. These results confirm that, in addition to the contribution of *nifA* to the AWN increase, it also contributed to increase of the fixation zone of these nodules, which is directly related with the amount of N fixed.

The pot assay showed that the NN was not significantly different between the two transformed strains. Nevertheless, a hydroponic assay was conducted to evaluate the nodulation kinetics. Overall, the results from this assay suggest that the presence of the extra copies of *nifA* in V-15b increases the rate of nodules formation. Particularly at the end of the assay, V15bpRKnifA showed a significantly higher number of nodules compared to both wild-type and V15bpRK415 strains. Similar results were previously obtained with *S. fredii* using extra-copies of *nifA* from *K. pneumoniae* that resulted in an increase in the nodulation activity and nodulation competitiveness on soybean plants (Jieping et al. 2002). Furthermore, Sanjuan and Olivares (1991) reported that multicopy plasmids carrying the *K. pneumoniae nifA* gene enhanced *S. meliloti* nodulation competitiveness on alfalfa. The distinct results obtained in the number of nodules, when the pot trial is compared to the hydroponic assay, is probably due to the different duration of the two trials. The higher number of nodules induced by the inoculation of the

V15bpRKnifA is probably transient and since in the pot trial the number of nodules is only evaluated 7 weeks after inoculation, this difference no longer exists.

The RT-PCR analyses indicate that the introduced *nifA* gene is transcribed. The extra *nifA* copies were confirmed to be expressed in both free-living and in the bacteroids of V15bpRKnifA. Thus, a higher amount of functional NifA protein is expected to be produced and consequently an upregulation of the *nif*, *fix* and other genes from the NifA regulon are likely to occur in this strain (Novichkov et al. 2013). Indeed, two fold higher expression levels of *nifH* were detected by RT-PCR in the bacteroids produced by V15bpRKnifA strain, compared with those found in V15bPRK415 bacteroids. The higher expression of *nif* as well as *fix* genes most probably contributed to the higher SE obtained with strain V15bpRKnifA. On the other hand, the nodulation kinetic assays suggest that additional *nifA* copies can increase the nodulation ability of chickpea rhizobia. It is likely that the higher amount of NifA regulator also induces higher expression of other genes involved in nodule development, bacteroids persistence or competitiveness, such as *nfe* (nodule formation efficiency) and *mos* (rhizopine synthesis) described in *S.meliloti* and *groESL3* reported in *B. japonicum* (Fischer 1994).

Since the extra *nifA* copies are constitutively expressed from the P_{lac} promoter, we could expect that the difference between the expression levels in V15bpRKnifA and V15bpRK415 is higher in the free-living conditions than in the bacteroids. This is due to the endogenous *nifA* gene whose expression is higher in the microaerobic conditions found in the nodules than in free-living cells (Terpolilli et al. 2012). Indeed, in V15bpRKnifA free-living cells, the *nifA* transcript levels were four fold higher than those detected in V15bpRK415 cells, while in V15bpRKnifA bacteroids the expression levels were three fold the levels detected in V15bpRK415 bacteroids.

Growth curves were performed to characterize the growth kinetics of V-15b and its transformed strains. The strain V15bpRKnifA and V15bpRK415 show a slower growth than the wild type strain, probably due to the presence of the large plasmids pRKnifA and pRK415, respectively.

The ability to move in semi-solid culture media can be an indication of the infection efficiency of plant-interacting bacteria (Rouws et al. 2008). Motility assays with *S. meliloti nifA* mutants showed that disruption of the gene affected the swimming ability (Gong et al. 2007). Based on this study, we could expect that extra *nifA* copies could improve the motility of rhizobia. Indeed, V-15b strain transformed with pRKnifA had an improvement in its mobility compared with the wild type and V15bpRK415 strains. This higher motility could have contributed to improve the ability of this strain to colonize the roots and infect the root hair of inoculated plants, contributing to improve the rate of nodules formation.

In order to investigate in more detail the early steps of the infection process, the modified strains, as well as the wild type V-15b were transformed with fluorescent proteins (*gfp*) genes, inoculated in pre-germinated chickpea seeds and observed under confocal microscopy (4-7 dpi). V-15b, V15bpRK415 and V15bpRKnifA were able to effectively colonize and infect chickpea roots and root hairs and show typical caps, curlings and infection threads and no clear differences among these strains were observed in the early infection process.

In spite of the negative effects due to the presence of the plasmid pRK415 *per se* in the mesorhizobia strains (lower growth rate), the overall effects of plasmid pRKnifA in the symbiosis phenotype indicate that the extra *nifA* copies are able to overcome those negative effects, since the strain V15bPRKnifA has a higher symbiotic performance compared to the V15bpRK415 strain.

Future assays in field conditions can provide further information on the benefits of extra *nifA* copies in rhizobia inoculants. Previous field studies with a *S. meliloti* recombinant strain, which had an additional copy of both *nifA* and *dctABD*, showed an increase in alfalfa biomass by 12.9% compared with the yield obtained with the wild-type strain (lBosworth et al. 1994).

Additional studies are required to understand the NifA precise role in the regulation of other genes involved in nitrogen fixation and nodulation. Nevertheless, the present study clearly indicates that the *nifA* overexpression in *Mesorhizobium* is able to benefit the host plant, significantly contributing to improve the symbiotic effectiveness. Therefore, the development of rhizobia strains with increased production of the NifA regulator seems a promising strategy to production of inocula for agronomic purposes. Future studies may involve the integration of an extra *nifA* copy in the genome of a chickpea *Mesorhizobium* strain to avoid the above mentioned negative effects of the expression vector.

2.6. References

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microbial Ecology, 58*, 930-941.

Batut, J., Daveran-Mingot, M. L., David, M., Jacobs, J., Garnerone, A. M., & Kahn, D. (1989). *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *Embo j, 8*(4), 1279-1286.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology, 84*(1), 188-198.

Bosworth, A. H., Williams, M. K., Albrecht, K. A., Kwiatkowski, R., Beynon, J., Hankinson, T. R., . . . Triplett, E. W. (1994). Alfalfa Yield Response to Inoculation with Recombinant Strains of *Rhizobium meliloti* with an Extra Copy of *dct*ABD and/or Modified *nif*A Expression. *Applied and Environmental Microbiology, 60*(10), 3815-3832.

Brígido, C., Alexandre, A., & Oliveira, S. (2012). Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia. *Microbiol Res, 167*(10), 623-629.

Brígido, C., Robledo, M., Menendez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604. doi: 10.1094/mpmi-05-12-0140-r

Broughton, W. J., & Dilworth, M. J. (1971). Control of Leghaemoglobin Synthesis in Snake Beans. *Biochemical Journal, 125*(4), 1075-1080.

Chengtao, Y., Guanqiao, Y., Shanjiong, S. S., & Jiabi, Z. (2004). Functional difference between *Sinorhizobium meliloti* NifA and *Enterobacter cloacae* NifA. *Sci China C Life Sci, 47*(1), 44-51.

Finan, T. M., Kunkel, B., De Vos, G. F., & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol, 167*(1), 66-72.

Fischer, H. M. (1994). Genetic regulation of nitrogen fixation in rhizobia. *Microbiological Reviews, 58*(3), 352-386.

Fischer, H. M., Alvarez-Morales, A., & Hennecke, H. (1986). The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of nif gene expression and formation of determinate symbiosis. *Embo j, 5*(6), 1165-1173.

Flores-Félix, J., Menéndez, E., Marcos-García, M., Celador-Lera, L., & Rivas, R. (2015). Calcofluor white, an Alternative to Propidium Iodide for Plant Tissues Staining in Studies of Root Colonization by Fluorescent-tagged Rhizobia. *Journal of Advances in Biology & Biotechnology, 2*(1), 65-70. doi: 10.9734/jabb/2015/12444

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M. T., . . . Velázquez, E. (2012). *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. *PLoS One, 7*(5), e38122. doi: 10.1371/journal.pone.0038122

Gibson, A. H. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. In G. H. Elkan (Ed.), *Symbiotic Nitrogen Fixation Technology* (pp. 321-363). New York: Marcel Dekker, Inc.

Gilles-Gonzalez, M. A., Ditta, G. S., & Helinski, D. R. (1991). A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature, 350*(6314), 170-172. doi: 10.1038/350170a0

Gong, Z., Zhu, J., Yu, G., & Zou, H. (2007). Disruption of *nifA* gene influences multiple cellular processes in *Sinorhizobium meliloti*. *J Genet Genomics, 34*(9), 783-789. doi: 10.1016/s1673-8527(07)60089-7

Gong, Z. Y., He, Z. S., Zhu, J. B., Yu, G. Q., & Zou, H. S. (2006). *Sinorhizobium meliloti nifA* mutant induces different gene expression profile from wild type in Alfalfa nodules. *Cell Res, 16*(10), 818-829.

Haag, A. F., Arnold, M. F., Myka, K. K., Kerscher, B., Dall'Angelo, S., Zanda, M., . . . Ferguson, G. P. (2013). Molecular insights into bacteroid development during *Rhizobium*-legume symbiosis. *FEMS Microbiol Rev, 37*(3), 364-383. doi: 10.1111/1574-6976.12003

Hoffman, B. M., Lukoyanov, D., Yang, Z. Y., Dean, D. R., & Seefeldt, L. C. (2014). Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem Rev, 114*(8), 4041-4062. doi: 10.1021/cr400641x

Jieping, Z., Xiaomi, D., Ling, X., Jiabi, Z., Shanjiong, S., & Guanqiao, Y. (2002). Extra-copy *nifA* enhances the nodulation efficiency of *Sinorhizobium fredii*. *Chinese Science Bulletin, 47*(7), 565-567. doi: 10.1360/02tb9130

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene, 70*(1), 191-197. doi: 10.1016/0378-1119(88)90117-5

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol Res, 169*(1), 2-17. doi: 10.1016/j.micres.2013.09.012

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W., & Oliveira, S. (2008). Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiology Ecology, 66*(2), 391-400.

Martinez, M., Palacios, J. M., Imperial, J., & Ruiz-Argueso, T. (2004). Symbiotic autoregulation of *nifA* expression in *Rhizobium leguminosarum* bv. *viciae*. *J Bacteriol, 186*(19), 6586-6594.

Moscatiello, R., Alberghini, S., Squartini, A., Mariani, P., & Navazio, L. (2009). Evidence for calcium-mediated perception of plant symbiotic signals in aequorin-expressing *Mesorhizobium loti*. *BMC Microbiol, 9*(1), 206. doi: 10.1186/1471-2180-9-206

Nascimento, F., Brígido, C., Alho, L., Glick, B. R., & Oliveira, S. (2012b). Enhanced chickpea growth-promotion ability of a *Mesorhizobium* strain expressing an exogenous ACC deaminase gene. *Plant and Soil, 353*(1), 221-230. doi: 10.1007/s11104-011-1025-2

Nour, S. M., Cleyet-Marel, J.-C., Normand, P., & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology, 45*(4), 640-648.

Novichkov, P. S., Kazakov, A. E., Ravcheev, D. A., Leyn, S. A., Kovaleva, G. Y., Sutormin, R. A., . . . Rodionov, D. A. (2013). RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. *BMC Genomics, 14*, 745. doi: 10.1186/1471-2164-14-745

Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F., & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis, 22*(6), 1086-1089.

Robledo, M., Jimenez-Zurdo, J. I., Soto, M. J., Velazquez, E., Dazzo, F., Martinez-Molina, E., & Mateos, P. F. (2011). Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial cellulase CelC2. *Mol Plant Microbe Interact, 24*(7), 798-807. doi: 10.1094/mpmi-10-10-0249

Rouws, L. F., Simoes-Araujo, J. L., Hemerly, A. S., & Baldani, J. I. (2008). Validation of a Tn5 transposon mutagenesis system for *Gluconacetobacter diazotrophicus*

through characterization of a flagellar mutant. *Arch Microbiol, 189*(4), 397-405. doi: 10.1007/s00203-007-0330-x

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). New York: Cold Spring Harbor Laboratory.

Sanjuan, J., & Olivares, J. (1989). Implication of *nif*A in regulation of genes located on a *Rhizobium meliloti* cryptic plasmid that affect nodulation efficiency. *J Bacteriol, 171*(8), 4154-4161.

Sanjuan, J., & Olivares, J. (1991). Multicopy plasmids carrying the *Klebsiella pneumoniae nifA* enhance *Rhizobium meliloti* nodulation competitiveness on alfalfa. *Molecular Plant-Microbe Interactions, 4*(4), 365-369. doi: 10.1094/mpmi-4-365

Soupène, E., Foussard, M., Boistard, P., Truchet, G., & Batut, J. (1995). Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. *Proc Natl Acad Sci U S A, 92*(9), 3759-3763.

Sullivan, J. T., Brown, S. D., & Ronson, C. W. (2013). The NifA-RpoN Regulon of *Mesorhizobium loti* Strain R7A and Its Symbiotic Activation by a Novel LacI/GalR-Family Regulator. *PLoS One, 8*(1). doi: e53762

10.1371/journal.pone.0053762

Terpolilli, J. J., Hood, G. A., & Poole, P. S. (2012). What determines the efficiency of N(2)-fixing *Rhizobium*-legume symbioses? *Adv Microb Physiol, 60*, 325-389. doi: 10.1016/B978-0-12-398264-3.00005-X

van Dillewijn, P., Martinez-Abarca, F., & Toro, N. (1998). Multicopy vectors carrying the *Klebsiella pneumoniae nifA* gene do not enhance the nodulation competitiveness of *Sinorhizobium meliloti* on alfalfa. *Mol Plant Microbe Interact, 11*(8), 839-842. doi: 10.1094/mpmi.1998.11.8.839

Vasse, J., de Billy, F., Camut, S., & Truchet, G. (1990). Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J Bacteriol, 172*(8), 4295-4306.

Chapter 3

Additional copies of *nodD* can improve the symbiotic effectiveness and the rate of nodulation of mesorhizobia strains

3.1. Summary

In rhizobia, NodD protein plays an important role in the regulation of genes directly involved in the nodules formation. This protein interacts with flavonoids released by legume plants and this complex triggers the Nod factors synthesis. Nod factors are responsible for many changes of the symbiosis process such as, root hair curl, infection thread formation, induction of cell division and gene expression in the root cortex and pericycle, nodule development and the number of nodules. This study evaluates the effects in the symbiotic performance of three mesorhizobia strains (V-15b, PMI-6 and ST-2) overexpressing extra *nodD* copies. Plants inoculated with V15bpRKnodD showed a higher number of nodules compared with those inoculated with V15bpRK415. Plants inoculated with PMI6pRKnodD and ST2pRKnodD showed an improvement in the shoot dry weight and symbiotic effectiveness. Besides that, extra *nodD* copies seem to have caused an increase in the average weight per nodule in plants inoculated with ST2pRKnodD. A hydroponic assay was performed to evaluate the nodulation kinetics of plants inoculated with PMI6pRKnodD and ST2pRKnodD, compared to the corresponding strains harboring the pRK415. Both strains overexpressing *nodD* were highly efficient in nodules formation showing more nodules during the whole time of the assay. Moreover, PMI6pRKnodD induced the first nodules three days sooner than PMI6pRK415. To evaluate nodule histology, stained sections of several nodules were observed by microscopy. This analysis showed no differences between nodules formed by plants inoculated with ST2pRKnodD and those inoculated with ST2pRK415. Nodules formed by plants inoculated with PMI6pRKnodD, did not show the senescent zone than those inoculated with PMI6pRK415. Colonization and infection assays showed that the number of infection threads and bacteria on the root surface were higher in plants inoculated with strains harboring extra *nodD* copies. These results indicate that overexpressing *nodD* may be a powerful tool to achieve the improvement of mesorhizobia that may be used as inoculant in the future.

3.2. Introduction

Rhizobia can form endosymbiotic associations with legume plants. In this process, rhizobia reduce atmospheric dinitrogen into ammonium that becomes available to host plants. In exchange, legumes release photosynthesis products that are used as a source of energy by the microsymbionts (Oldroyd 2013). Biological nitrogen fixation is a very important process because it allows the reduction of the use of nitrogen fertilizers that have a high financial cost and may pollute the environment (Garg and Geetanjali 2007; Glick 2015; Laranjo et al. 2014).

The symbiosis between rhizobial bacteria and host plants is basically divided into two important steps: nodulation and nitrogen fixation. Before nodulation, a complex molecular “dialog” between legume and rhizobium takes place in the rhizosphere allowing the symbionts to recognize each other (Janczarek et al. 2015; Oldroyd 2013). Many compounds are exudated by legumes during the interaction with the microorganism, among them flavonoids are the most important, acting as chemoattractants to rhizobia (Cooper 2004; Cooper 2007b; Garg and Geetanjali 2007; Janczarek et al. 2015). An important aspect regarding flavonoids is their interactions with the constitutively expressed NodD, a regulator of other rhizobial nodulation genes (e.g. *nodABC*) (Cooper 2004). The combination of these two compounds triggers the synthesis of specific reverse signal molecules by rhizobia called lipochitooligosaccharides (LCOs) or Nod factors. Nod factors are responsible for many advances of the symbiosis process such as, root hair curling, infection thread formation, induction of cell division and gene expression in the root cortex and pericycle and nodule development (Garg and Geetanjali 2007; Laranjo et al. 2014; Oldroyd 2013; van Brussel et al. 2002). In addition, it was reported that an extract containing rhizobial Nod factors increased the number of nodules in plants (Kidaj et al. 2012; Macchiavelli and Brelles-Marino 2004; Maj et al. 2009; Podleśny et al. 2014)

NodD, encoded by the *nodD* gene, is associated to the membrane in several species (Schell 1993). This protein belongs to the LysR family transcriptor regulators and beyond being responsible for the activation of the main nodulation genes, it also may regulate, directly or indirectly, additional important symbiotic features such as polysaccharide production, phytohormone synthesis, motility, quorum-sensing, activation of the type III and IV secretion systems (del Cerro et al. 2015; Hubber et al. 2007; Krause et al. 2002; Lopez-Baena et al. 2008; Pérez-Montaño et al. 2014; Theunis et al. 2004).

It was found through genomic studies that the number of copies of *nodD* in rhizobia may differ among species, varying between one to five copies, and moreover,

the properties of different NodD and their flavonoid preferences in the same strain may also vary (Broughton et al. 2000; del Cerro et al. 2015; Schlaman et al. 1998). *nodD* mutation in strains with only one copy frequently suppresses the nodulation (Nod^-) (e.g. *Rhizobium leguminosarum* bv. *trifolli*) (Broughton et al. 2000). On the other hand, when *nodD* gene is mutated in strains carrying more than one copy, the nodulation suppression may or may not happen. For example, in *Sinorhizobium freddi* NGR234 the inactivation of *nodD1* is sufficient to abolish nodulation, whereas in *Sinorhizobium meliloti* the mutation in three copies is needed to cause Nod^- (Broughton et al. 2000; Honma and Ausubel 1987; Hungria et al. 1992). In *Rhizobium tropici* CIAT 899 the two *nodD* copies are needed to full nodulation of common bean (del Cerro et al. 2015). Machado and Krishnan (2003) showed that a *nodD1* mutant of *Sinorhizobium freddi* USDA 191 was unable to nodulate soybean and the inactivation of *nodD2* or addition of extra copies of *nodD1* or *nodD2* caused delayed nodulation and reduced the number of nodules.

The genus *Mesorhizobium* includes rhizobacteria that can associate with important legumes, such as chickpea (*Cicer arietinum* L.) and biserrula (*Biserrula pelecinus* L.) (Laranjo et al. 2014). Several studies have shown that *Mesorhizobium* strains associated with chickpea plants are diverse in symbiotic effectiveness (Alexandre et al. 2009; Laranjo et al. 2008; Laranjo et al. 2004; Rivas et al. 2007) and stress tolerance (Alexandre and Oliveira 2011; Brígido et al. 2012a; Brígido and Oliveira 2013). As in other rhizobia, the nodulation in mesorhizobia strains is controlled by NodD and some studies show that mutations in *nod* genes regulated by this protein caused diverse phenotypes in the host plant (Rodpothong et al. 2009). For example, *nodZ* and *nolL* mutants in *Mesorhizobium loti* R7A formed uninfected nodule primordia on *Lotus filicaulis* and *Lotus corniculatus* and effective nodules with a delayed nodulation on *Lotus japonicus,* and showed a reduction in infection threads formation, while double mutation of *nodS* and *nolO* did not affect the nodulation. Regarding *nodD* genes, *nodD2* mutants did not affect the nodulation on the host plants tested, however *nodD1* delayed the formation of nodules on *Lotus japonicus* and *Lotus corniculatus* and was unable to nodulate *Leucaena leucocephala* (Hubber et al. 2007; Rodpothong et al. 2009). Double mutant on *nodD1* and *nodD2* completely abolished nodulation, indicating that they are complementary in *M. loti* (Rodpothong et al. 2009).

Previous studies performed with modified *Mesorhizobium* strains overexpressing varied genes could improve the number of nodules or accelerate de nodulation process in chickpea (Brigido et al. 2013; da-Silva 2017; Nascimento et al. 2012b; Nascimento et al. 2012a; Paço et al. 2016). However, the effects of overexpression of the *nodD* gene in mesorhizobia were never analyzed. Therefore, the aim of this study is to evaluate the

symbiotic performance of chickpea *Mesorhizobium* strains transformed with extra *nodD* copies.

3.3. Material and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used are listed in Table 1. Three previously characterized chickpea mesorhizobia strains isolated form Portuguese soils were used: V-15b-Viseu, ST-2-Setúbal, PMI-6-Portimão (Alexandre et al. 2009; Brígido et al. 2012a; Laranjo et al. 2008). These strains have only one copy of the gene *nodD* (Eliziário 2015).

The mesorhizobia strains were grown in Tryptone-yeast (TY) medium at 28°C (Beringer 1974b) and tetracycline (15 $\mu g.ml^{-1}$) was added into the medium for pRK415-transformed strains. Kanamycin (50 $\mu g.ml^{-1}$) and gentamycin (15 $\mu g.ml^{-1}$) were added to TY medium for bacteria transformed with pMRGFP and pMP4661, respectively. *Escherichia coli* DH5α harboring pRK415 and MT616 (harboring pRK600) strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001) supplemented with 15 $\mu g.ml^{-1}$ of tetracycline and 25 $\mu g.ml^{-1}$ of chloramphenicol, respectively.

Table 1 Bacterial strains and plasmids used in this work.

Plasmids/Strains	Characteristics	Reference
pRK600	pRK2013 *npt*::Tn9. Cm^r	(Finan et al. 1986)
pRK415	Broad host-range vector; Tc^r	(Keen et al. 1988)
pRKnodD	Plasmid pRK415 containing the *nodD* gene from *M. mediterraneum* UPM-$Ca36^T$; Tc^r	(Eliziário 2015)
pMRGFP	Plasmid containing the *gfp* gene; Km^r	(García-Fraile et al. 2012)
pMP4661	Plasmid containing the *rfp* gene; Gm^r	(Bloemberg et al. 2000)
E. coli		
MT616	Strain harboring the helper plasmid pRK600	(Finan et al. 1986)
DH5α	Competent cells	NZYTech
Mesorhizobium		
V-15b	*Mesorhizobium* sp. V-15b-Viseu isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
ST-2	*Mesorhizobium* sp. ST-2-Setubal isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
PMI-6	*Mesorhizobium* sp. PMI-6-Portimão isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
UPM-$Ca36^T$	*Mesorhizobium mediterraneum* UPM-$Ca36^T$ isolated from chickpea root nodules from Spain	(Nour et al. 1995)
V15bpRKnodD	V-15b strain harboring pRKnodD	(Eliziário 2015)
ST2 pRKnodD	ST-2 strain harboring pRKnodD	(Eliziário 2015)
PMI6pRKnodD	PMI-6 strain harboring pRKnodD	(Eliziário 2015)
PMI6pRKnodDGFP	PMI-6 strain harboring pRKnodD and pMRGFP	This work
ST2pRKnodDGFP	ST-2 strain harboring pRKnodD and pMRGFP	This work
V15bpRK415	V-15b strain harboring pRK415	This work
ST2pRK415	ST-2 strain harboring pRK415	This work
PMI6pRK415	PMI-6 strain harboring pRK415	This work
PMI6pRK415GFP	PMI-6 strain harboring pRK415 and pMRGFP	This work
PMI6pRK415RFP	PMI-6 strain harboring pRK415 and pMP4661	This work
ST2pRK415GFP	ST-2 strain harboring pRK415 and pMRGFP	This work
ST2pRK415RFP	ST-2 strain harboring pRK415 and pMP4661	This work

Transforming the strains with the *nodD* gene

As mentioned in Table 1, strains V15bpRKnodD, ST2pRKnodD and PMI6pRKnodD strains were constructed in a previous work (Eliziário, 2015), according to the following procedures. The *nodD* gene sequence used in this study was amplified from *Mesorhizobium mediterraneum* UPM-$Ca36^T$ strain. The PCR reaction was prepared in 50 µl, with 1 µl of template DNA, 1 U of Taq polymerase (Fermentas), 1X buffer, 5 mM MgCl2, 0.2 mM dNTP's and 30 pmol of each primer: nodD-CMG6-F-PstI (5' - CTGCAGTAT GCGTTTCAAAGGACTTG - 3') and nod-CMG6-R (5' - GCATGCTCACAGCGG GGCAGCCAT CC - 3'). The amplification program used was as follows: 2 minutes at 95°C in the initial denaturation stage, followed by 30 cycles of 20

sec denaturation at 95°C, 10 sec hybridization at 58°C and 1 min extension at 70 ° C, ending with a 5 min final extension at 70 ° C.

V15bpRKnodD, ST2pRKnodD and PMI6pRKnodD strains (Eliziário 2015) were generated by triparental mating with their respective wild type, *E. coli* DH5α cells containing the pRKnodD plasmid and *E. coli* MT616 cells with pRK600 acting as helper, as described by Nascimento et al. (2012a). The exogenous *nodD* gene is under the control of the *lac* promoter. The wild type mesorhizobia strains were also transformed with pRK415.

Previous work shows that the expression vector pRK415 allows the expression of the cloned gene under both free-living and symbiosis conditions (chapter 2).

In order to evaluate the infection process, transformed strains harboring pRKnodD plasmid were transformed with pMRGFP (containing the *gfp* gene) and strains harboring pRK415 were transformed with pMRGFP or pMP4661 (containing the *rfp* gene) by triparental mating, as described above. These transformations were confirmed by observing the cells on a fluorescence microscope.

Evaluation of the symbiotic performance

The three mesorhizobia wild types as well as the corresponding transformed strains were inoculated on chickpea plants in order to evaluate whether additional *nodD* copies improved the symbiotic effectiveness of these mesorhizobia strains. Chickpea seeds were first surface-sterilized and germinated in plates containing water agar medium (Alexandre et al. 2009). Once transferred to plastic pots filled with sterile vermiculite, 1 ml of bacterial suspension in TY liquid medium (OD_{540} of 1.0) was inoculated in each seedling.

Plants were watered with a nitrogen-free nutrient solution (Broughton and Dilworth 1971) three times a week. Uninoculated plants and plants watered with N-supplemented nutrient solution were used as negative and positive control, respectively. Five replicates were performed per treatment. The conditions in the plant growth chamber were set to 16 h-light and 8 h-dark cycle and 24°C-day and 18°C-night temperature at a relative humidity of 65%. Plants were harvested after 7 weeks and several parameters were measured, such as shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN) and average weight per nodule (AWN). Symbiotic effectiveness (SE) was obtained using the shoot dry weight values, including those from the positive and negative controls (Gibson 1987). Statistical analysis was performed using SPSS statistics V.21 (SPSS Inc; IBM New York, USA) and included analysis of variance, namely one-way ANOVA, and the Tukey's Multiple Range Test ($P < 0,05$).

Only PMI-6 and ST-2 transformed strains were selected to be used for the following analyses based on the results obtained in the symbiotic performance evaluation.

Bacterial growth

To evaluate the growth of ST-2, PMI-6 and their respective transformed strains, bacterial cultures were grown in TY medium at 28° C with orbital shaking. In the case of the transformed strains, 15 $\mu g.ml^{-1}$ of tetracycline was added to the medium. The initial OD_{540nm} was 0,1 for each strain. Triplicates were used for each strain and the growth was monitored by measuring the optical density at 540 nm every 24 hours.

Evaluation of nodulation kinetics

In order to evaluate the nodulation kinetics, a hydroponic assay, as described by Brígido et al. (2012b), was performed using chickpea plants inoculated with suspensions of PMI6pRK415, PMI6pRKnodD, ST2pRK415 and ST2pRKnodD strains ($OD_{540\ nm}$ of 0.6). Eight seeds per treatment were used and the number of nodules was evaluated every three days during 33 days. The same controlled conditions describe above for the evaluation of symbiotic performance were used in this experiment.

Histological analysis of nodules

After 35 days of inoculation in hydroponic assay, nodules were excised and processed for light microscopy. Nodules were fixed in 4% formaldehyde, dehydrated in an increasing ethanol series and finally embedded in paraffin (Brígido et al. 2012b). Toluidine blue-stained sections (6 µm) of embedded nodules were observed by bright-field microscopy.

Analysis of rhizobia infection process

GFP-tagged (single infection) or RFP+GFP-tagged (co-infection) mesorhizobia strains were inoculated in pre-germinated chickpea seeds, as described by Robledo et al. (2011) in order to evaluate if extra *nodD* copies increased the ability of those strains to colonize and infect root hairs. The analysis of root hairs after 4 or 6 days of inoculation were performed using a Confocal Laser Scanning Microscope (Leica TCS SPE) equipped with solid-state laser, allowing visualization of GFP (488 nm), RFP and

propidium iodide (532 nm) fluorescence. Propidium iodide at 10 µM (Sigma-Aldrich) was used to stain chickpea roots and root hairs. Projections were made from adjusted individual channels and accumulating stacks using Leica software.

3.4. Results

Most *Mesorhizobium* species seem to encode only one copy of *nodD* and phylogenetic analysis of this gene showed that *Mesorhizobium* species group according to the host plant and not to their species affiliation (Eliziário 2015). In addition, only one copy of *nodD* was found in the genome of UPM-Ca36T, the strain used to amplify the cloned gene (data not shown), however this genome is still in the draft stage. Although the *nodD* sequence similarity is very high among chickpea mesorhizobia, the three isolates used in this study have different *nodD* sequences and also different from the one clone in the expression vector, which was obtained from *M. mediterraneum* UPM-Ca36^T (Eliziário 2015).

Symbiotic effectiveness assays

Several mesorhizobia strains were transformed with extra *nodD* copies, in order to analyze their effect in the symbiotic effectiveness (SE) and nodulation process in chickpea plants. Although plants inoculated with V15bpRKnodD strain showed a number of nodules higher than those inoculated with the strain harboring the empty vector, there were no significant differences in other parameters, such as the SDW, RDW, AWN and SE (Table 2). On the other hand, plants inoculated with PMI-6 strain harboring extra *nodD* copies did not show any significant difference in the NN, AWN and RDW when compared with plants inoculated wild type and PMI6pRK415, however the SDW and SE were significantly higher (Table 2).

Chickpea plants were also inoculated with ST-2 strain harboring pRKnodD plasmid and the results show that extra *nodD* copies in this strain seem to contribute to the improvement of AWN, SDW and, consequently the SE when compared with ST-2 wild type. As observed in plants inoculated with PMI6pRKnodD, those inoculated with ST2pRKnodD also did not show any improvement in the NN and RDW. None of the transformed strains improved the root dry weight of inoculated plants (Table 2). Based on the results obtained in the symbiotic performance evaluation, mesorhizobia strains PMI-6 and ST-2 and their derivates were selected to be used for further analyses, even ST2pRKnodD did not showing a significant improvement in the SE compared with ST2pRK415.

Table 2 Results obtained from pots assays of chickpea plants inoculated with wild type or transformed strains.

Strain	SDW (g)	RDW (g)	NN	AWN (mg)	SE (%)
V-15b	1.267 ± 0.183 a	0.641 ± 0.213 a	92 ± 15.7 ab	1.971 ± 0.241 a	40.95 ± 8.63 a
V15bpRK415	1.406 ± 0.232 a	0.472 ± 0.115 a	71 ± 16.3 b	1.650 ± 0.224 a	47.44 ± 10.94 a
V15bpRKnodD	1.529 ± 0.069 a	0.604 ± 0.084 a	138 ± 46.9 a	2.042 ± 1.717 a	53.25 ± 3.27 a
ST-2	1.104 ± 0.330 b	0.524 ± 0.138 a	61 ± 18.2 a	1.749 ± 0.314 b	33.28 ± 15.15 b
ST2pRK415	1.227 ± 0.225 ab	0.539 ± 0,077 a	51 ± 21.5 a	2.114 ± 0.737 ab	39.06 ± 10.59 ab
ST2pRKnodD	1.556 ± 0.311 a	0.633 ± 0.089 a	62 ± 15.5 a	2.544 ± 0.579 a	54.51 ± 14.61 a
PMI-6	1.139 ± 0.138 ab	0.467 ± 0.116 a	38 ± 14.3 a	4.534 ± 2.023 a	31.37 ± 9.08 ab
PMI6pRK415	1.008 ± 0.227 b	0.317 ± 0.104 a	28 ± 12.6 a	4.737 ± 3.111 a	22.83 ± 14.86 b
PMI6pRKnodD	1.337 ± 0.060 a	0.461 ± 0.157 a	39 ± 20.7 a	4.235 ± 1.288 a	44.29 ± 3.98 a

Different letters in the same group of strains indicate statistically significant differences (*P<0,05*). SDW - Shoot Dry Weight; RDW - Root Dry Weight; NN - Number of Nodules; AWN - Average Weight per Nodule; SE - Symbiotic Effectiveness

Growth curves

Rhizobial growth rate may be related with their ability to colonize and infect root hairs. The strains that showed an improvement in the symbiotic effectiveness at pot assay were used to evaluate the growth kinetics. PMI-6 wild type strain showed faster growth at exponential phase than the modified strains, most likely due the energetic cost of carrying the introduced expression vector (Fig 1A). The ST-2 wild type strain also grew faster during exponential phase, however at the stationary phase the strain harboring pRK415 plasmid reached very similarly OD values to those of the wild type. Both strains showed OD values close to 3.5 at stationary phase, whereas ST2pRKnodD grew at a slower rate and reached the stationary phases with an OD of approximately 2 (Fig 1B).

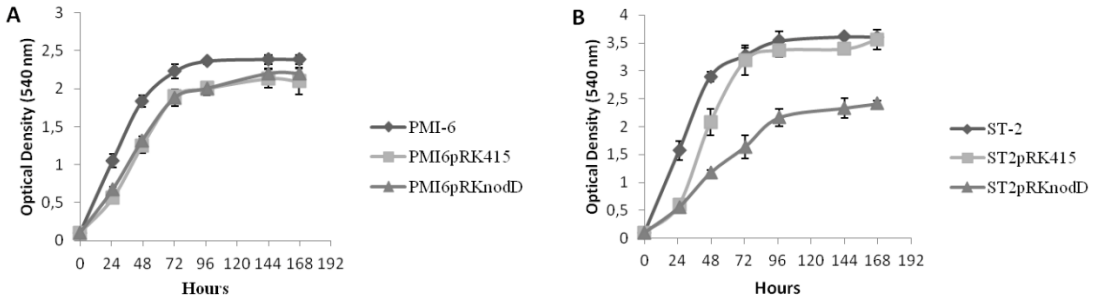

Figure 1. Growth curves of A - PMI-6, PMI6pRK415 and PMI6pRKnodD. B - ST-2, ST2pRK415 and ST2pRKnodD during about 170 hours of growth in TY medium. Bars represent standard deviation.

Nodulation Kinetics

A hydroponic assay was conducted in order to evaluate the nodulation kinetics of plants inoculated with PMI6pRKnodD and ST2pRKnodD, compared to plants inoculated with the corresponding strains harboring the empty vector pRK415. At 11 days after inoculation the first nodules were observed in plants inoculated with PMI6pRKnodD, whereas plants inoculated with PMI6pRK415 showed a delay of three days, showing the first nodules only at 14 days after inoculation (Fig 2A). Plants inoculated with PMI6pRKnodD continuously showed a significantly higher number of nodules until the end of the experiment with about 4-fold more nodules than plants inoculated with PMI6pRK415 (Fig 2A). All the nodules had a reddish interior, which indicates accumulation of active leghemoglobin (Fig 3E2 and F2). Although the hydroponic conditions are clearly not favorable to the shoot development of chickpea plants, the shoot of plants inoculated with PMI6pRKnodD seemed to be more developed (Fig 3 A and B).

ST2pRKnodD strain also improved the NN of chickpea plants, compared to ST2pRK415. Even though the difference between NN of plants inoculated with both ST-2 modified strains is smaller than the one observed for PMI-6 modified strains, ST2pRKnodD also induced a significant higher number of nodules throughout the assay (Fig 2B). The largest difference was detected at 21 days after inoculation, when the NN of plants inoculated with ST2pRKnodD were about 3-fold that of plants inoculated with ST2pRK415. The development of the shoots and root nodules showed in fig 4A to D indicates that extra *nodD* copies contributed to the improvement of these parameters.

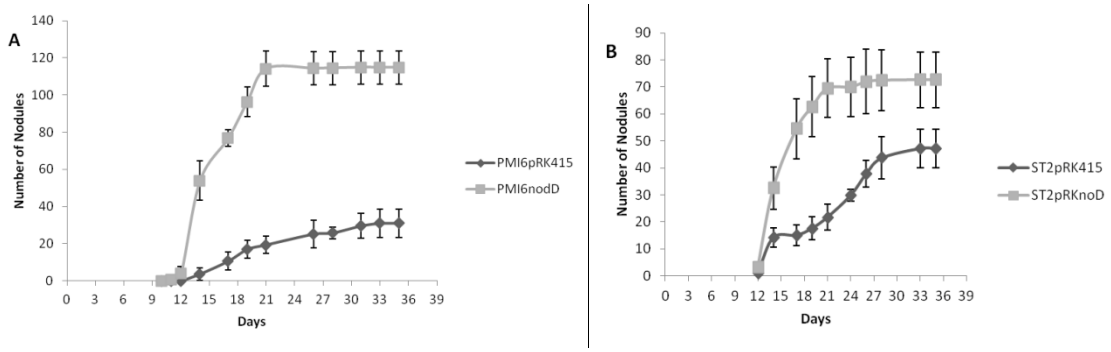

Figure 2. Nodulation kinetics of chickpea plants inoculated with PMI-6 and ST-2 modified strains. Average Number of nodules for plants inoculated with A-PMI6pRK415 and PMI6pRKnodD. B - ST2pRK415 and ST2pRKnodD. Bars represent standard error.

Histological analysis of nodules

In order to perform a histological analysis of nodules from plants inoculated with PMI-6 and ST-2 transformed strains, nodule sections were stained and observed under bright-field microscopy.

The large senescent zone observed in nodules from plants inoculated with PMI6pRK415 was absent in those from plants inoculated with PMI6pRKnodD (fig 3G and H). The other zones showed the typical organization of indeterminate nodules, although the fixation zone from nodules inoculated with PMI6pRK415 seems to show a higher number of non-invaded plant cells (Fig 3I-L).

There was no difference at the histological level between nodules from plants inoculated with ST2pRK415 and ST2pRKnodD (Fig 4E-J).

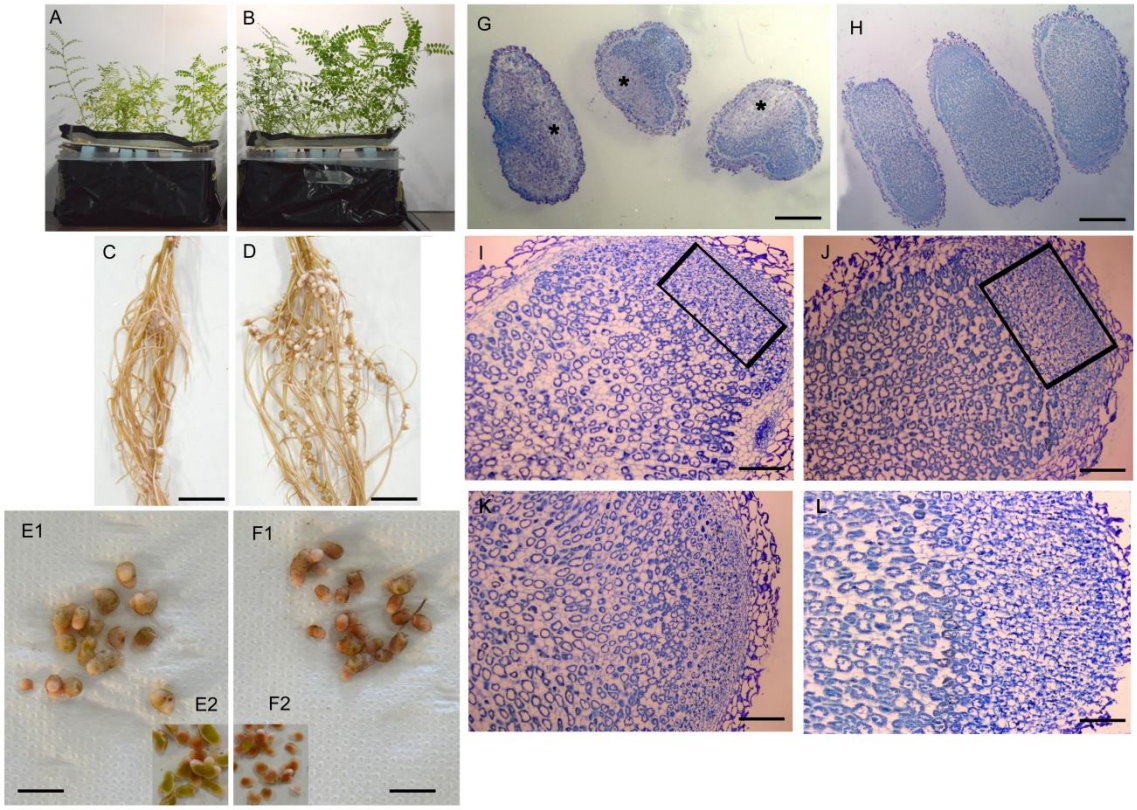

Figure 3. Chickpea Plants grown in hydroponic conditions and the corresponding nodules. Plants at 35 days after inoculation inoculated with A – PMI6pRK415 and B - PMI6pRKnodD. Roots showing nodules formed at 35 days after inoculation of C – PMI6pRK415 and D - PMI6pRKnodD. E1 - Nodules from plants inoculated with PMI6pRK415; E2 - Nodule section from plants inoculated with PMI6pRK415; F1 - Nodules from plants inoculated with PMI6pRKnodD; F2 - Nodule section from plants inoculated with PMI6pRKnodD; G, I and K - Nodules sectioned and stained with toluidine blue from plants inoculated with PMI6pRK415; H, J and L - Nodules sectioned and stained with toluidine blue form plants inoculated with PMI6pRKnodD; G - Asterisks indicate senescent zones; I and J - Square indicates meristematic zones. Scale bars: 5 cm (C and D); 1 cm (E1 and F1); 1 mm (G and H); 400µm (I and J); 200 µm (K and L).

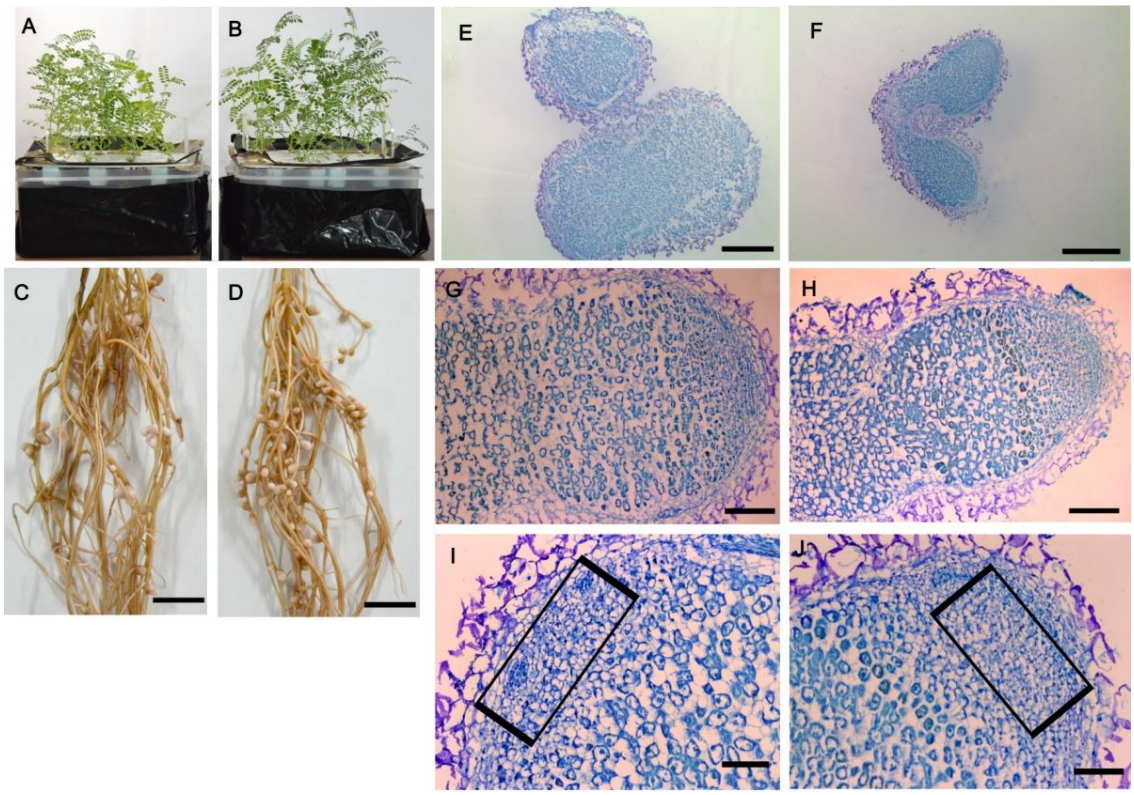

Figure 4. Chickpea Plants grown in hydroponic conditions and the corresponding nodules. Plants at 35 days after inoculation inoculated with A – ST2pRK415 and B - ST2pRKnodD. Roots showing nodules formed at 35 days after inoculation of C – ST2pRK415 and D ST2pRKnodD. E, G and I - Nodules sectioned and stained with toluidine blue from plants inoculated with ST2pRK415; F, H and J - Nodules sectioned and stained with toluidine blue form plants inoculated with ST2pRKnodD; I and J - Square indicates meristematic zones. Scale bars: 5 cm (C and D); 1 mm (E and F); 400µm (G and H); 200 µm (I and J).

Colonization and infection thread

The high number of nodules observed in hydroponic assay may be related with a more efficient colonization and infection processes; therefore, these first stages of symbiosis were observed by confocal microscopy in plants inoculated with the transformed strains harboring a fluorescent protein.

Both ST2pRKnodDGFP and PMI6pRKnodDGFP strains are more efficient in colonizing the surface of the roots than those harboring the empty pRK415 plasmid (Fig 5A, B, D and E). In addition, the number of infection threads was higher in plants inoculated with ST2pRKnodDGFP than in plants inoculated with ST2pRK415GFP (Fig

5A and B). The root hairs from plants inoculated with PMI6pRKnodDGFP displayed more curling than those inoculated with the empty vector strain (Fig 5D and E).

The strains with extra *nodD* copies and tagged with GFP protein were co-inoculated with the strains harboring the empty vector tagged with RFP (red fluorescent protein) and then observed in confocal microscopy. These co-inoculation experiments confirmed the previous observations made by single inoculation, i. e., both ST2pRKnodDGFP and PMI6pRKnodDGFP showed a higher colonization of the root surface and induced the formation of a higher number of infection threads than ST2pRK415RFP and PMI6pRK415RFP, respectively (Fig 5C and F).

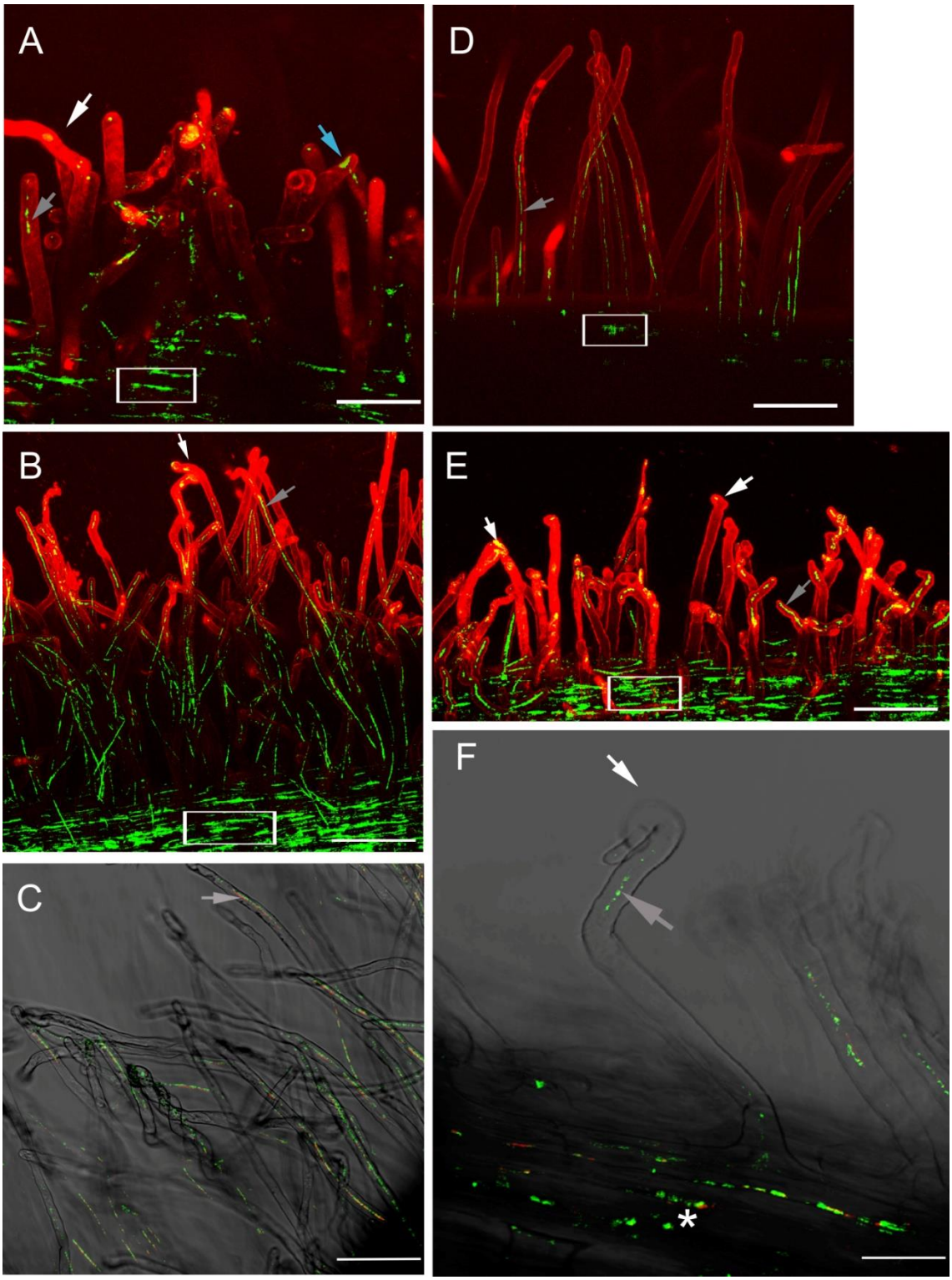

Figure 5. Confocal laser scanning micrographs of chickpea roots inoculated with green and red fluorescent protein–tagged rhizobia. A – ST2pRK415, B – ST2pRKnodD, D - PMI6pRK415 and E - PMI6pRKnodD strains tagged with green fluorescent protein, showing the initial infection process in chickpea roots (stained with propidium iodide), at 5 (ST-2 transformed strains) and 4 (PMI-6 transformed strains) days after inoculation; C – ST2pRK415RFP (red) and ST2pRKnodDGFP (green) strains co-inoculated in

chickpea roots at 6 days after inoculation; F - PMI6pRK415RFP (red) and PMI6pRKnodDGFP (green) co-inoculated in chickpea roots at 3 days after inoculation. Root hair curling (white arrows); infection threads on root hairs (gray arrows); cap on root hair tip (blue arrow), rhizobial attachment on roots (square) and empty vector strains as well as those harboring extra *nodD* copies sharing the same intercellular space (asterisk) are shown. Scale bars: 50 µm (A); 75 µm (B, C and D); 100 µm (E); 25 µm (F).

3.5. Discussion

NodD is a regulator of other rhizobial nodulation genes (e.g. *nodABC*) (Oldroyd 2013). When this protein interacts with flavonoids released by legume plants, it forms a protein-phenolic complex that triggers the Nod factors (Cooper 2004). Among the several functions in the symbiosis process, the Nod factors act mainly at root hair curling, infection thread formation, induction of cell division and gene expression in the root cortex and pericycle, nodule development and the number of nodules (Garg and Geetanjali 2007; Laranjo et al. 2014; Oldroyd 2013; van Brussel et al. 2002).

To evaluate whether extra *nodD* copies improve the symbiotic parameters in mesorhizobia strains, a chickpea pot assay with sterile vermiculite as substrate was performed under controlled conditions. Although the number of nodules from plants inoculated with V-15b harboring the pRKnodD plasmid was approximately two-fold more than those inoculated with V15bpRK415, it did not improve the symbiotic effectiveness, probably due the high demand of energy required to form the large number of nodules. Terpolilli et al. (2008) reported the inoculation of *Sinorhizobium meliloti* 1021 in *Medicago truncatula* formed several nodules, however they were small, pale, more widely distributed on the root system and with fewer infected cells. Regarding the number of nodules and its relationship with NodD, some studies have been shown distinct effects when different copies of *nodD* gene are deleted or overexpressed. For example, a mutation in *nodD1* of *Sinorhizobium fredii* USDA191 blocked nodulation in soybean cultivars Peking and McCall, whereas the *nodD2* mutant delayed nodulation on Peking and reduced the number of nodules on McCall. The overexpression of the two copies caused the same effects observed when the *nodD2* was deleted (Machado and Krishnan 2003). Bacteria harboring only one copy of NodD often are Nod^- when *nodD* is deleted (Broughton et al. 2000). It has been shown that Nod factors, regulated by NodD, control the number of nodules formed on a root system by inducing an autoregulation response in the host plant (van Brussel et al. 2002). Contrary to what was observed in plants inoculated with V-15b, the ST-2 and PMI-6 strains harboring extra *nodD* copies did not show any increase in the number of nodules on inoculated chickpea plants, however the

shoot dry weight as well as the symbiotic effectiveness was statistically higher than those harboring the empty pRK415.

PMI-6 and ST-2 strains transformed with extra *nodD* copies were characterized in terms of their growth kinetics. Both PMI6pRKnodD and ST2pRKnodD strains showed a slower growth in the exponential phase compared to the wild type strain. This was expected because the size and copy number of the plasmid could slow down the replication process. Nevertheless, PMI6pRKnodD showed a very similar growth to that of the strain harboring the empty vector (PMI6pRK415), while ST2pRKnodD showed a much slower growth rate in the exponential phase than the strain harboring the empty vector (ST2pRK415) and reached stationary phase at a much lower OD.

To evaluate the kinetics of nodules formation, a hydroponic assay was performed. Both PMI-6 and ST-2 transformed strains with extra *nodD* copies showed a remarkable increase in the number of nodules. In addition, the PMI6pRKnodD strain developed the first nodules earlier. This apparent discrepancy between the results of two different plant assays is most likely due the shorter duration of the hydroponic assay (5 weeks), compared with the pot assay (8 weeks). These results suggest that there might be a transient higher number of nodules in plants inoculated with PMI6pRKnodD and ST2pRKnodD, which may benefit the plant in terms of N-fixation, yet in a later stage of the symbiosis that difference is not detected any longer. Specific Nod factors application induces the nodule primordial formation and in most rhizobia Nod factors are required to induce nodules in host plants (Broughton et al. 2000; D'Haeze and Holsters 2002; Oldroyd 2013; Perret et al. 2000; Spaink 2000; Spaink et al. 1991), therefore the extra *nodD* copies expressed constitutively may increase the Nod factor production that, in turn may account for the higher number of nodules observed.

Unlike previously described situations where a high number of nodules was actually seen to be harmful to the host plant (Terpolilli et al. 2008), in this case the presence of more nodules seems to contribute to development of the plants, as shown in fig. 3B and 4B, since the higher number of active nodules probably contributed to the shoot dry weight improvement observed in pots assay. To further analyses the structures of these nodules, nodules sections were stained and analyzed by microscopy. Nodules from plants inoculated with ST2pRKnodD showed no difference when compared with those from plants inoculated with ST2pRK415. On the other hand, extra *nodD* copies in PMI-6 seems to have contributed to maintain the infection zone without the premature senescence zone observed in nodules from plants inoculated with PMI6pRK415. Nod factors are responsible for the induction the early nodulin genes (Limpens and Bisseling 2009; Vijn et al. 1995). Nodulins are proteins specific to nodule development and nitrogen fixation and the best known among these proteins is leghemoglobin, which is

found in all legumes and regulates oxygen tension in nodules (Manen et al. 1991; Wittenberg 1974). In plants inoculated with PMI6pRKnodD the nodules were pink with a reddish interior, a typical coloration of active leghemoglobin. This finding suggests that extra *nodD* copies may affect the regulation of nodulin genes and consequently improve the concentration of leghemoglobins.

Among the several roles of Nod factors in the symbiosis process, deformation of root hair and pre-infection threads are probably the most important (Lerouge et al. 1990; Oldroyd 2013; van Brussel et al. 1992). Mutants defective in Nod factor production can no longer associate with the host legume (Dénarié et al. 1996; Oldroyd and Downie 2004; Oldroyd 2013). In order to investigate whether the improvement of the symbiotic effectiveness of plants inoculated with ST2pRKnodD and PMI6pRKnodD could be related with colonization and infection threads formation, these strains and their respective controls (ST2pRK415 and PMI6pRK415) were tagged with the *gfp* or *rfp* protein and analyzed by confocal microscopy. Extra *nodD* copies may indeed have contributed to enhance the colonization and infection of the transformed bacteria, since the number of infection threads and bacteria on the root surface was higher in root plants inoculated with these strains. Moreover, the number of curling on root hairs observed in plants inoculated with PMI6pRKnodD were higher than those observed in PMI6pRK415. Co-infection assays were performed with the strains harboring extra *nodD* copies transformed with *gfp* with those harboring pRK415 transformed with *rfp* and the results were similar, with a higher density of PMI6pRKnodDGFP and ST2pRKnodDGFP than PMI6pRK415RFP and ST2pRK415GFP.

Despite the importance of mesorhizobia and their contribution to the amount of fixed nitrogen in legumes, little is still known about regulation of nodulation and fixation genes in this genus. Therefore, this study aimed to investigate the first levels of the regulation of nodules formation in three mesorhizobia strains overexpressing the *nodD* gene. The study demonstrated that indeed the NodD regulator plays an important role in the strains tested increasing the shoot dry weight and improving the symbiotic effectiveness, as well as contributing to nodules formation.

3.6. References

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microbial Ecology, 58*, 930-941.

Alexandre, A., & Oliveira, S. (2011). Most heat-tolerant rhizobia show high induction of major chaperone genes upon stress. *FEMS Microbiology Ecology, 75*, 28-36.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology, 84*(1), 188-198.

Bloemberg, G. V., Wijfjes, A. H., Lamers, G. E., Stuurman, N., & Lugtenberg, B. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microbe Interact, 13*(11), 1170-1176. doi: 10.1094/mpmi.2000.13.11.1170

Brígido, C., Alexandre, A., & Oliveira, S. (2012). Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia. *Microbiol Res, 167*(10), 623-629.

Brigido, C., Nascimento, F. X., Duan, J., Glick, B. R., & Oliveira, S. (2013). Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Mesorhizobium* spp. reduces the negative effects of salt stress in chickpea. *FEMS Microbiol Lett, 349*(1), 46-53. doi: 10.1111/1574-6968.12294

Brígido, C., & Oliveira, S. (2013). Most acid-tolerant chickpea mesorhizobia show induction of major chaperone genes upon acid shock. *Microbial Ecology, 65*(1), 145-153.

Brígido, C., Robledo, M., Menendez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604. doi: 10.1094/mpmi-05-12-0140-r

Broughton, W. J., & Dilworth, M. J. (1971). Control of Leghaemoglobin Synthesis in Snake Beans. *Biochemical Journal, 125*(4), 1075-1080.

Broughton, W. J., Jabbouri, S., & Perret, X. (2000). Keys to symbiotic harmony. *J Bacteriol, 182*(20), 5641-5652.

Cooper, J. E. (2004). Multiple Responses of Rhizobia to Flavonoids During Legume Root Infection. *Advances in Botanical Research, 41*, 1-62. doi: http://dx.doi.org/10.1016/S0065-2296(04)41001-5

Cooper, J. E. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *Journal of Applied Microbiology, 103*(5), 1355-1365. doi: doi:10.1111/j.1365-2672.2007.03366.x

D'Haeze, W., & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology, 12*(6), 79R-105R.

da-Silva, J. R., Alexandre, A, Brígido, C, Oliveira, S. (2017). Can stress response genes be used to improve the symbiotic performance of rhizobia? *AIMS Microbiology, 3*(3), 365-382.

del Cerro, P., Rolla-Santos, A. A. P., Gomes, D. F., Marks, B. B., Pérez-Montaño, F., Rodríguez-Carvajal, M. Á., . . . Hungria, M. (2015). Regulatory *nodD1* and *nodD2* genes of *Rhizobium tropici* strain CIAT 899 and their roles in the early stages of molecular signaling and host-legume nodulation. *BMC Genomics, 16*(1), 251. doi: 10.1186/s12864-015-1458-8

Dénarié, J., Debellé, F., & Promé, J. C. (1996). *Rhizobium* Lipo-Chitooligosaccharide Nodulation Factors: Signaling Molecules Mediating Recognition and Morphogenesis. *Annual Review of Biochemistry, 65*(1), 503-535.

Eliziário, F. (2015). *Análise e Sobre-expressão de Genes de Simbiose de Rizóbios de Grão-de-bico.* (Master), Évora University, Évora.

Finan, T. M., Kunkel, B., De Vos, G. F., & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol, 167*(1), 66-72.

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M. T., . . . Velázquez, E. (2012). *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. *PLoS One, 7*(5), e38122. doi: 10.1371/journal.pone.0038122

Garg, N., & Geetanjali. (2007). Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. *Agron Sustain Dev, 27*. doi: 10.1051/agro:2006030

Gibson, A. H. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. In G. H. Elkan (Ed.), *Symbiotic Nitrogen Fixation Technology* (pp. 321-363). New York: Marcel Dekker, Inc.

Glick, B. R. (2015). *Beneficial Plant-Bacterial Interactions*: Springer.

Honma, M. A., & Ausubel, F. M. (1987). *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. *Proceedings of the National Academy of Sciences, 84*(23), 8558-8562.

Hubber, A. M., Sullivan, J. T., & Ronson, C. W. (2007). Symbiosis-induced cascade regulation of the *Mesorhizobium loti* R7A VirB/D4 type IV secretion system. *Mol Plant Microbe Interact, 20*(3), 255-261. doi: 10.1094/mpmi-20-3-0255

Hungria, M., Johnston, A. W., & Phillips, D. A. (1992). Effects of flavonoids released naturally from bean (*Phaseolus vulgaris*) on *nodD*-regulated gene transcription in *Rhizobium leguminosarum* bv. *phaseoli*. *Mol Plant Microbe Interact, 5*(3), 199-203.

Janczarek, M., Rachwał, K., Marzec, A., Grządziel, J., & Palusińska-Szysz, M. (2015). Signal molecules and cell-surface components involved in early stages of the legume-*rhizobium* interactions. *Applied Soil Ecology, 85*, 94-113. doi: 10.1016/j.apsoil.2014.08.010

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene, 70*(1), 191-197. doi: 10.1016/0378-1119(88)90117-5

Kidaj, D., Wielbo, J., & Skorupska, A. (2012). Nod factors stimulate seed germination and promote growth and nodulation of pea and vetch under competitive conditions. *Microbiol Res, 167*(3), 144-150. doi: 10.1016/j.micres.2011.06.001

Krause, A., Doerfel, A., & Gottfert, M. (2002). Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. *Mol Plant Microbe Interact, 15*(12), 1228-1235. doi: 10.1094/mpmi.2002.15.12.1228

Laranjo, M., Alexandre, A., & Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol Res, 169*(1), 2-17. doi: 10.1016/j.micres.2013.09.012

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W., & Oliveira, S. (2008). Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiology Ecology, 66*(2), 391-400.

Laranjo, M., Machado, J., Young, J. P. W., & Oliveira, S. (2004). High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiology Ecology, 48*(1), 101-107.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., & Denarie, J. (1990). Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature, 344*(6268), 781-784. doi: 10.1038/344781a0

Limpens, E., & Bisseling, T. (2009). Nod Factor Signal Transduction in the Rhizobium–Legume Symbiosis. In A. M. C. Emons & T. Ketelaar (Eds.), *Root Hairs* (pp. 249-276). Berlin, Heidelberg: Springer Berlin Heidelberg.

Lopez-Baena, F. J., Vinardell, J. M., Perez-Montano, F., Crespo-Rivas, J. C., Bellogin, R. A., Espuny Mdel, R., & Ollero, F. J. (2008). Regulation and symbiotic significance of nodulation outer proteins secretion in *Sinorhizobium fredii* HH103. *Microbiology, 154*(Pt 6), 1825-1836. doi: 10.1099/mic.0.2007/016337-0

Macchiavelli, R. E., & Brelles-Marino, G. (2004). Nod factor-treated *Medicago truncatula* roots and seeds show an increased number of nodules when inoculated with a limiting population of *Sinorhizobium meliloti*. *J Exp Bot, 55*(408), 2635-2640. doi: 10.1093/jxb/erh261

Machado, D., & Krishnan, H. B. (2003). *nodD* alleles of *Sinorhizobium fredii* USDA191 differentially influence soybean nodulation, *nodC* expression, and production of exopolysaccharides. *Current Microbiology, 47*(2), 134-137. doi: 10.1007/s00284-002-3972-6

Maj, D., Wielbo, J., Marek-Kozaczuk, M., Martyniuk, S., & Skorupska, A. (2009). Pretreatment of clover seeds with nod factors improves growth and nodulation of Trifolium pratense. *J Chem Ecol, 35*(4), 479-487. doi: 10.1007/s10886-009-9620-x

Manen, J. F., Simon, P., Van Slooten, J. C., Osterås, M., Frutiger, S., & Hughes, G. J. (1991). A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. *The Plant Cell, 3*(3), 259-270. doi: 10.1105/tpc.3.3.259

Nascimento, F., Brígido, C., Alho, L., Glick, B. R., & Oliveira, S. (2012b). Enhanced chickpea growth-promotion ability of a *Mesorhizobium* strain expressing an exogenous ACC deaminase gene. *Plant and Soil, 353*(1), 221-230. doi: 10.1007/s11104-011-1025-2

Nascimento, F. X., Brigido, C., Glick, B. R., Oliveira, S., & Alho, L. (2012a). *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. *Lett Appl Microbiol, 55*(1), 15-21. doi: 10.1111/j.1472-765X.2012.03251.x

Nour, S. M., Cleyet-Marel, J.-C., Normand, P., & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology, 45*(4), 640-648.

Oldroyd, G. E., & Downie, J. A. (2004). Calcium, kinases and nodulation signalling in legumes. *Nat Rev Mol Cell Biol, 5*(7), 566-576. doi: 10.1038/nrm1424

Oldroyd, G. E. D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology, 11*(4), 252-263. doi: 10.1038/nrmicro2990

Paço, A., Brígido, C., Alexandre, A., Mateos, P. F., & Oliveira, S. (2016). The Symbiotic Performance of Chickpea Rhizobia Can Be Improved by Additional Copies of the *clpB* Chaperone Gene. *PLoS One, 11*(2), e0148221. doi: 10.1371/journal.pone.0148221

Pérez-Montaño, F., Jiménez-Guerrero, I., Del Cerro, P., Baena-Ropero, I., López-Baena, F. J., Ollero, F. J., . . . Espuny, R. (2014). The Symbiotic Biofilm of *Sinorhizobium fredii* SMH12, Necessary for Successful Colonization and Symbiosis of *Glycine max* cv Osumi, Is Regulated by Quorum Sensing Systems and Inducing Flavonoids via NodD1. *PLoS One, 9*(8), e105901. doi: 10.1371/journal.pone.0105901

Perret, X., Staehelin, C., & Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews, 64*(1), 180-201.

Podleśny, J., Wielbo, J., Podleśna, A., & Kidaj, D. (2014). The pleiotropic effects of extract containing rhizobial Nod factors on pea growth and yield. *Central European Journal of Biology, 9*(4), 396-409. doi: 10.2478/s11535-013-0277-7

Rivas, R., Laranjo, M., Mateos, P. F., Oliveira, S., Martinez-Molina, E., & Velázquez, E. (2007). Strains of *Mesorhizobium amorphae* and *Mesorhizobium tianshanense*, carrying symbiotic genes of common chickpea endosymbiotic species, constitute

a novel biovar (*ciceri*) capable of nodulating *Cicer arietinum*. *Letters in Applied Microbiology, 44*(4), 412-418. doi: doi:10.1111/j.1472-765X.2006.02086.x

Robledo, M., Jimenez-Zurdo, J. I., Soto, M. J., Velazquez, E., Dazzo, F., Martinez-Molina, E., & Mateos, P. F. (2011). Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial cellulase CelC2. *Mol Plant Microbe Interact, 24*(7), 798-807. doi: 10.1094/mpmi-10-10-0249

Rodpothong, P., Sullivan, J. T., Songsrirote, K., Sumpton, D., Cheung, K. W., Thomas-Oates, J., . . . Ronson, C. W. (2009). Nodulation gene mutants of *Mesorhizobium loti* R7A-*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. *Mol Plant Microbe Interact, 22*(12), 1546-1554. doi: 10.1094/mpmi-22-12-1546

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). New York: Cold Spring Harbor Laboratory.

Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol, 47*, 597-626. doi: 10.1146/annurev.mi.47.100193.003121

Schlaman, H. R. M., Phillips, D. A., & Kondorosi, E. (1998). Genetic Organization and Transcriptional Regulation of Rhizobial Nodulation Genes. In H. P. Spaink, A. Kondorosi & P. J. J. Hooykaas (Eds.), *The Rhizobiaceae: Molecular Biology of Model Plant-Associated Bacteria* (pp. 361-386). Dordrecht: Springer Netherlands.

Spaink, H. P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol, 54*. doi: 10.1146/annurev.micro.54.1.257

Spaink, H. P., Sheeley, D. M., van Brussel, A. A., Glushka, J., York, W. S., Tak, T., . . . Lugtenberg, B. J. (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. *Nature, 354*(6349), 125-130. doi: 10.1038/354125a0

Terpolilli, J. J., O'Hara, G. W., Tiwari, R. P., Dilworth, M. J., & Howieson, J. G. (2008). The model legume *Medicago truncatula* A17 is poorly matched for N2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. *New Phytol, 179*(1), 62-66. doi: 10.1111/j.1469-8137.2008.02464.x

Theunis, M., Kobayashi, H., Broughton, W. J., & Prinsen, E. (2004). Flavonoids, NodD1, NodD2, and *nod*-box NB15 modulate expression of the *y4wEFG* locus that is required for indole-3-acetic acid synthesis in *Rhizobium* sp. strain NGR234. *Mol Plant Microbe Interact, 17*(10), 1153-1161. doi: 10.1094/mpmi.2004.17.10.1153

van Brussel, A. A., Bakhuizen, R., van Spronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J., & Kijne, J. W. (1992). Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. *Science, 257*(5066), 70-72. doi: 10.1126/science.257.5066.70

van Brussel, A. A., Tak, T., Boot, K. J., & Kijne, J. W. (2002). Autoregulation of root nodule formation: signals of both symbiotic partners studied in a split-root system of *Vicia sativa* subsp. nigra. *Mol Plant Microbe Interact, 15*(4), 341-349. doi: 10.1094/mpmi.2002.15.4.341

Vijn, I., Martinez-Abarca, F., Yang, W. C., das Neves, L., van Brussel, A., van Kammen, A., & Bisseling, T. (1995). Early nodulin gene expression during Nod factor-induced processes in Vicia sativa. *Plant J, 8*(1), 111-119.

Wittenberg, J. B. (1974). Facilitated oxygen diffusion. The role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. *J Biol Chem, 249*(13), 4057-4066.

Chapter 4

The chaperone GroEL can improve the symbiotic effectiveness of mesorhizobia strains

This chapter is based on the manuscript:

da-Silva JR, Alexandre, A, Brígido, C, Oliveira, S (2017) Can stress response genes be used to improve the symbiotic performance of rhizobia? AIMS Microbiology 3: 365-382

4.1. Summary

Successful colonization of the host root by free-living rhizobia requires that these bacteria are able to deal with adverse conditions in the soil. In addition, rhizobia have to be able to endure stresses that may occur during their endosymbiotic life inside the root nodules. Stress response genes, such as *otsAB*, *groEL*, *clpB*, *rpoH* play an important role in tolerance of free-living rhizobia to different environmental conditions and some of these genes have been shown to be involved in the symbiosis. The aim of this study was to evaluate whether extra *groEL* copies could improve the symbiotic effectiveness as well as other parameter related with the chickpea-mesorhizobia symbiosis. Chickpea plants inoculated with ST2pRKgroEL showed a symbiotic effectiveness approximately 1.5 fold higher than plants inoculated with the wild-type. On the other hand, Ca36pRKgroEL, V15bpRKgroEL and PMI6pRKgroEL did not show improvement of the symbiotic effectiveness. Nodulation kinetics was also evaluated and showed that after 35 days plants inoculated with ST2pRKgroEL had 78 % more nodules than those inoculated with ST2pRK415. Section of nodules analyzed by microscopy revealed no histological difference between nodules from plants inoculated with ST2pRKgroEL and ST2pRK415. Colonization and infection tests were performed and showed that ST2pRKgroEL and ST2pRK415 seem to colonize and infect the root hairs similarly. The growth of the transformed ST-2 strains was evaluated under different stress conditions and after a heat shock at 48º C for 15 min, ST2pRKgroEL showed slightly higher growth rate, when compared with the strain harboring the empty vector (ST2pRK415). Despite the promising increase in the SE obtained by transforming ST-2 with additional copies of *groEL*, three other mesorhizobia strains also overexpressing *groEL* did not show a significant improvement of their symbiotic effectiveness. This indicates that the effects of higher levels of GroEL on the symbiosis might be strain specific.

4.2. Introduction

Bacteria are not only able of colonizing extreme environments, but also of living inside a wide diversity of hosts. Regardless the particular natural environment where each species can be found, bacteria are often subjected to adverse conditions. The main factors studied as bacterial stressors are temperature, salt, pH and nutrient starvation. Many of the genes involved in stress response are conserved across bacterial species, which is remarkable, taking into account the range of different environmental niches where bacteria can live.

The symbiotic process between rhizobia and the host legume is mainly divided into two major events: bacterial infection and nodule organogenesis (Oldroyd and Downie 2008). For a successful symbiotic association, it is essential that these two phenomena are coordinated in both spatial and temporal manner, to ensure nodule formation at the site of bacterial infection (for review see (Oldroyd and Downie 2008)). When compatible molecular signals are recognized by the host legumes, a series of events, such as growth of polarized root hair tip and invagination associated with bacterial infection, are initiated in the host plant leading to the development of specialized structures, called nodules (Fournier et al. 2008; Gage 2004).

Since early events in the symbiosis process such as molecular signalling and rhizobial attachment, are particularly sensitive to high temperatures, salinity, acidity and other environmental stresses (Hungria and Stacey 1997; Hungria and Vargas 2000; Zhang and Smith 1996), rhizobia have to be able to physiologically adapt to environmental conditions, in order to ensure a successful symbiosis with its legume partner. These stresses that negatively affect the microsymbiont in free-living conditions as well as during the symbiotic relationship can lead to a delay in infection and nodule formation, development of non-fixing nodules or even to failure of the nodulation process (Zahran 1999). Therefore, rhizobia must be able to overcome stress conditions both outside and within the nodule, to achieve a complete and effective nitrogen-fixing symbiosis. Therefore, the role of stress response genes must be an important or even fundamental part of the symbiotic process. In fact, it was suggested that among the genes required for bacteroid formation, some are specific for symbiosis and others are involved in physiological adaptation to the environmental conditions within and outside the nodule (Oke and Long 1999).

Transcriptomic and proteomic analyses of rhizobia in symbiosis with their host legumes also suggest the involvement of stress response genes, mainly heat shock proteins such as ClpB and GroESL, in the symbiotic process. For example, overexpression of the ClpB and GroEL/ES proteins was detected in nodules formed by *Bradyrhizobium*

japonicum and *Sinorhizobium meliloti* strains (Djordjevic 2004; Djordjevic et al. 2003a; Nomura et al. 2010a; Sarma and Emerich 2005; Sarma and Emerich 2006). These findings are reinforced through transcriptomic analyses where up-regulation of these genes was observed in root nodules (Karunakaran et al. 2009; Pessi et al. 2007b; Uchiumi et al. 2004).

The most studied molecular chaperone in terms of its involvement in the symbiosis is GroEL. Particular copies of this chaperone gene, usually upregulated in the bacteroids, seem to play a fundamental role in the formation of functional NodD and nitrogenase complex (Fischer et al. 1999; Ogawa and Long 1995). For example, among the five *groESL* operons in the *S. meliloti* genome only one operon ($groEL_1$) was found to be involved in symbiosis (Ogawa and Long 1995). Fischer et al. (1993) found a co-regulation between $groESL_3$ and nitrogen fixation genes in *B. japonicum*, yet none of the *B. japonicum* mutants that individually lack one *groEL* gene were depleted in their symbiotic phenotype (Bittner et al. 2007; Fischer et al. 1999). However, double mutation on $groEL_3$ and $groEL_4$ genes in *B. japonicum* affects the symbiotic performance, since these copies are required for the formation of a functional nitrogenase (Fischer et al. 1999). These two copies are the most abundant in the GroEL pool in bacteroids (Fischer et al. 1993). Studies on the symbiotic performance of strains mutated in the *dnaJ* gene, encoding the co-chaperone DnaJ, also revealed distinct results using different rhizobia species. For example, a *B. japonicum dnaJ* mutant strain was able to establish fully effective symbiosis with soybeans (Minder et al. 1997). More recently, the involvement of the ClpB chaperone in the symbiotic process was evaluated. Although a *Mesorhizobium clpB* mutant strain was able to establish symbiosis with chickpea plants, the ClpB absence caused a delay in nodule formation and development (Brígido et al. 2012b), indicating its involvement in the symbiotic process.

Several genes involved in stress response have been overexpressed in rhizobia as an attempt to improve their symbiotic performance, particularly under stress conditions such as salt, oxidative, drought, heat or biotic stress. Overexpression of genes related to protection of bacteria from salt stress has contributed to the improvement of rhizobia strains under stressful conditions. A *S. meliloti* strain overexpressing the *betS* gene, involved in the rapid acquisition of betaines by cells subjected to osmotic shock, showed a better maintenance of nitrogen fixation activity in salinised alfalfa plants than the wild-type strain (Boscari et al. 2006). The *otsA* gene encodes the enzyme trehalose-6-phosphate synthase involved in the biosynthesis of trehalose (Elbein et al. 2003). Moussaid et al. (2015) overexpressed *otsA* from *S. meliloti* in *Mesorhizobium ciceri* and found an increase of the *otsA*-overexpressing strain growth in saline media. Chickpea plants inoculated with *M. ciceri* carrying extra *otsA* copies formed more nodules and

accumulated more shoot biomass than the wild-type inoculated plants, when grown in the presence of NaCl. Also, *P. vulgaris* inoculated with *R. etli* overexpressing *otsA* showed more nodules with increased nitrogenase activity and higher biomass compared with plants inoculated with the wild-type strain. Only plants inoculated with the *otsA*-overexpressing strain fully recovered from drought stress (Suarez et al. 2008).

The first successful improvement of a rhizobium with a chaperone gene was achieved using the chickpea nodulating strain *M. mediterraneum* UPM-Ca36^T modified with extra-copies of the *clpB* gene (Paço et al. 2016). The nodulation kinetics analysis showed a higher rate of nodule development as well as a higher number of nodules in plants inoculated with the *clpB*-transformed strain. More interestingly the symbiotic effectiveness of the *clpB*-overproducing strain increased ~60% at pH 5 and ~83% at pH 7, compared to the wild-type strain. This improved symbiotic phenotype may be related to an increased expression of symbiosis genes, as detected for the nodulation genes *nodA* and *nodC* (Paço et al. 2016).

In this study the potential of the chaperone gene *groEL* in the improvement of chickpea *Mesorhizobium* symbiotic performance was investigated as well as its effect on the tolerance to several stresses.

4.3. Material and Methods

Bacterial strains and Growth Conditions

Bacterial strains and plasmids are listed in Table 1. Three chickpea mesorhizobia isolates from Portuguese soils were selected (Alexandre et al. 2009; Brígido et al. 2012a; Laranjo et al. 2008), in addition to the type strain *Mesorhizobium mediterraneum* UPM-Ca36^T (Nour et al. 1995)

The mesorhizobia strains were routinely grown at 28ºC in tryptone-yeast (TY) medium (Beringer 1974a). The growth medium for pRK415-transformed mesorhizobia strains was supplemented with tetracycline (15 μg.ml^{-1}). For bacteria transformed with *gfp* and *rfp* genes, kanamycin (50 μg.ml^{-1}) and gentamycin (15 μg.ml^{-1}) were added to TY medium, respectively. *Escherichia coli* DH5α and MT616 strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001) at 37ºC. For *E. coli* strains containing pRK415, 15 μg.ml^{-1} of tetracycline was used, while for MT616 with pRK600, the medium was supplemented with 25 μg.ml^{-1} of chloramphenicol.

Table 1 Bacterial strains and plasmids used in this work.

Plasmids/Strains	Characteristics	Reference
pRK600	pRK2013 *npt*::Tn9. Cm^r	(Finan et al. 1986)
pRK415	Broad host-range vector; Tc^r	(Keen et al. 1988)
pRKgroEL	Plasmid pRK415 containing the *groEL* gene from *M. mediterraneum* UPM-$Ca36^T$; Tc^r	This work
pMRGFP	Plasmid containing the *gfp* gene; Km^r	(García-Fraile et al. 2012)
pMP4661	Plasmid containing the *rfp* gene; Gm^r	(Bloemberg et al. 2000)
E. coli		
MT616	Strain harboring the helper plasmid pRK600	(Finan et al. 1986)
DH5α	Competent cells	NZYTech
Mesorhizobium		
V-15b	*Mesorhizobium* sp. V-15b-Viseu isolated from chickpea root nodules	(Alexandre et al. 2009)
ST-2	*Mesorhizobium* sp. ST-2-Setubal isolated from chickpea root nodules	(Alexandre et al. 2009)
PMI-6	*Mesorhizobium* sp. PMI-6-Portimão isolated from chickpea root nodules	(Alexandre et al. 2009)
UPM-$Ca36^T$	*Mesorhizobium mediterraneum* UPM-$Ca36^T$ isolated from chickpea root nodules	(Nour et al. 1995)
V15bpRKgroEL	V-15b strain harboring pRKgroEL	This work
ST2pRKgroEL	ST-2 strain harboring pRKgroEL	This work
PMI6pRKgroEL	PMI-6 strain harboring pRKgroEL	This work
Ca36pRKgroEL	UPM-$Ca36^T$ strain harboring pRKgroEL	This work
ST2pRKgroELGFP	ST-2 strain harboring pRKgroEL and pMRGFP	This work
ST2pRK415GFP	ST-2 strain harboring pRK415 and pMRGFP	This work
V15bpRK415	V-15b strain harboring pRK415	This work
ST2pRK415	ST-2 strain harboring pRK415	This work
PMI6pRK415	PMI-6 strain harboring pRK415	This work
Ca36pRK415	UPM-$Ca36^T$ strain harboring pRK415	This work
ST2pRK415RFP	ST-2 strain harboring pRK415 and pMP4661	This work

Transforming the strains with *groEL* gene

A PCR amplification was performed in order to obtain the full sequence of a particular copy of the *groEL* gene from *Mesorhizobium mediterraneum* UPM-$Ca36^T$, a type strain that nodulates chickpea (Nour et al. 1995). The amplified *groEL* copy is the one that shares the highest similarity with the copy overexpressed in *M. japonicum* MAFF303099 bacteroids (Uchiumi et al. 2004). Performed in 50 µl, the PCR was made using 0.5 µL of DNA, 1× Buffer for Phusion GC Buffer, 0.2 mM of each dNTP, 15 pmol of each primer and 0.4 U of Phusion DNA Polymerase (Thermo Fisher Scientific). The primers used were groEL-F (5'- GAATTCAATGGCTGCCAAAGACGTAAA -3') and groEL-R (5'- GAATTCTTAGAAATCCATACCGCCCA -3'). The amplification program was: 30 s at 98°C, 30 cycles of 10 s at 98ºC, 20 s at 53°C, 33 s at 72 ºC and a final extension of 5 min at 72 ºC. The obtained *groEL* fragment of 1659 bp was cloned in pCR-

Blunt™ vector (ThermoFisher Scientific), sequenced and subcloned in the expression vector pRK415, previously digested with *Eco*RI.

The exogenous *groEL* gene is under the control of the *lac* promoter. The chickpea mesorhizobia strains were modified by triparental mating with the pRKgroEL plasmid. *E. coli* DH5α cells containing the plasmid pRKgroEL were used as the donor, mesorhizobia isolates were the recipient and *E. coli* MT616 cells, with pRK600 acted as helper, as described by Nascimento et al. (2012a). The pRKgroEL-modified strains were named V15bpRKgroEL, ST2pRKgroEL, PMI6pRKgroEL and Ca36pRKgroEL. The empty plasmid pRK415 also was used to transform the same mesorhizobia strains.

In order to confirm the transformation of mesorhizobia cells with pRKgroEL and pRK415, total DNA was extracted according to (Rivas et al. 2001) and used to amplify the region of the expression vector that includes the *groEL* gene. A DNA fragment of 1781 bp was expected using the universal primers M13F and M13R-pUC to the fragment with the insert and and 122 pb to the region without it. The PCR reaction was performed in a final volume of 50 µL, using 5 µL of DNA, 1× reaction Green GoTaq® Flexi buffer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 15 pmol of each primer and 0.625U of GoTaq® G2 Flexi DNA Polymerase (Promega). The amplification program was: 2 min of initial denaturation at 95 °C, 30 cycles of 60 s at 95 ºC, 45 s at 56 °C, 85 s at 72 ºC, and a final extension of 5 min at 72 ºC.

In addition, strain ST2pRKgroEL was transformed with pMRGFP (containing the *gfp* gene) and strain ST2pRK415 was transformed with pMRGFP or pMP4661 (containing the *gfp* or *rfp* gene) by triparental mating, as described above.

Evaluation of the symbiotic performance

A plant growth trial under controlled conditions was performed using both the wild type and the transformed strains to evaluate whether additional *groEL* copies improved the symbiotic effectiveness of chickpea mesorhizobia strains. After surface-sterilization and germination (Alexandre et al. 2009), chickpea seeds were transferred to plastic pots filled with sterile vermiculite and inoculated.

Mesorhizobia strains were grown in TY liquid medium at 28°C for 72 h. Cell culture was centrifuged at 10000 × g, resuspended in fresh TY liquid medium to an OD_{540nm} of 1.0 and 1 ml of this bacterial suspension was used to inoculate each seedling. Five replicates were performed per treatment. A nitrogen-free nutrient solution (Broughton and Dilworth 1971) was applied three times a week. Uninoculated plants were used as negative control and plants watered with N-supplemented nutrient solution were used as positive control. Plants were grown in a growth chamber under a 16 h-light

and 8 h-dark cycle and 24°C-day and 18°C-night temperature, at a relative humidity of 65%. After 7 weeks, plants were harvested and several parameters were measured, such as shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN) and average weight per nodule (AWN). Symbiotic effectiveness (SE) was calculated using the shoot dry weight values, including those from the positive and negative controls (Gibson 1987). Statistical analysis was performed using SPSS statistics V.21 (SPSS Inc; IBM New York, USA) and included analysis of variance, one-way ANOVA and the Tukey's Multiple Range Test ($P < 0,05$).

Mesorhizobia strain ST-2, ST2pRK415 and ST2pRKgroEL were selected to be used for further analyses, based on the results obtained in the symbiotic performance evaluation.

Evaluation of nodulation kinetics

Chickpea plants inoculated with cell suspensions of ST2pRK415 and ST2pRKgroEL strains at an $OD_{540\,nm}$ of 0.6 were used to evaluate the nodulation kinetics through a hydroponic assay. The procedures were conducted as described in Brígido et al. (2012b). Eight seeds per treatment were used and the number of nodules was evaluated every three days during 33 days.

Histological analysis of nodules

Nodules were excised from hydroponic plants 35 days after inoculation and processed for light microscopy. Nodules were fixed in 4% formaldehyde, dehydrated in an increasing ethanol series and embedded in paraffin. Toluidine blue-stained sections (6 µm) of embedded nodules were examined by bright-field microscopy.

Analysis of rhizobia infection process

Pre-germinated chickpea seeds were inoculated with the mesorhizobia strains ST2pRKgroELGFP (GFP-tagged) or ST2pRK415RFP (RFP -tagged), as described by Robledo et al. (2011). Inoculation of both strains simultaneously (1:1 ratio) was done in order to analyze competition in co-infection conditions. Analysis of roots and root hairs stained with 10 µM propidium iodide (Sigma-Aldrich) or with 50mg/l calcofluor white (Sigma- Aldrich) and 10% potassium hydroxide solution (Flores-Félix et al. 2015) 5 or 6 days after of inoculation was performed using a Confocal Laser Scanning Microscope

(Leica TCS SPE) equipped with solid-state laser, allowing visualization of GFP (488 nm), RFP and propidium iodide (532 nm) and calcofluor white (405 nm) fluorescence. Projections were made from adjusted individual channels and accumulation in the image stacks using Leica software.

Bacterial growth curves under controlled and abiotic stress conditions

In order to evaluate free-living growth of wild type ST-2, as well as its derivates harboring pRK415 and pRKgroEL, strains were grown under control conditions at 28°C in 5 mL of TY medium (supplemented with 15 $\mu g.ml^{-1}$ tetracycline in the case of transformed strains) during 144 h. The same strains were submitted to the following stress conditions: acidity (TY adjusted to pH 5); high temperature (34°C), and a heat shock (48°C during 15 min). The optical densities at 540 nm were measured every 24 hours and triplicates were used for each strain.

4.4. Results

The *groEL* gene is found in multiple copies in many genomes of *Alphaproteobacteria* (Lund 2009). This is also the case in rhizobia, where different copies are known to have distinct regulation mechanisms (Bittner et al. 2007; Bittner and Oke 2006; Rodriguez-Quinones et al. 2005). Previous studies indicated the involvement of particular *groEL* copies in the symbiosis (Fischer et al. 1993; Ogawa and Long 1995). To investigate the potential of the chaperone gene *groEL* in the improvement of rhizobia symbiotic performance, four chickpea mesorhizobia strains were modified by the addition of extra *groEL* copies (Table 2). The *M. mediterraneum* UPM-Ca36^T *groEL* copy that shares the highest similarity with the copy overexpressed in *M. japonicum* MAFF303099 bacteroids (Uchiumi et al. 2004), was cloned in the expression vector pRK415, allowing constitutive expression of these gene in the transformed strains.

Evaluation of the symbiotic performance

To evaluate the effects of extra *groEL* copies in the symbiotic performance, a plant growth trial using the transformed strains was carried out for seven weeks under controlled conditions and the symbiotic effectiveness as well as other parameters were evaluated.

From the four strains overexpressing *groEL*, only plants inoculated with ST2pRKgroEL showed a significant increase in the shoot dry weight, and consequently in the symbiotic effectiveness, compared with those inoculated with the corresponding ST2pRK415 and wild type. The SDW and SE of plants inoculated with ST2pRKgroEL were 43 % and 64 % higher than those showed in plants inoculated with ST2pRK415, respectively (Fig 2). This improvement of symbiotic effectiveness was not associated to significant differences in the number of nodules or in the average weight per nodule.

Although there was no significant improvement in the SE of strains V-15b and PMI-6 transformed with pRKgroEL, these strains induced the development of a higher number of nodules than the corresponding strains harboring the pRK415 plasmid. In the case of the homologous overexpression, the plant assay showed no significant improvement in the symbiotic performance of Ca36pRKgroEL. Nevertheless, a lower average weight per nodule was detected for both Ca36pRKgroEL and Ca36pRK415 when compared with the wild type strain. This was the only difference detected in the symbiotic parameters between plants inoculated with the wild type and those inoculated with the strain carrying the expression vector pRK415. The presence of this vector alone does not seem to affect the symbiotic performance of the tested strains (chapter 2).

Considering the results from the evaluation of the symbiotic performance of mesorhizobia strains overexpressing *groEL*, strain ST-2 and its transformed derivatives were selected for further studies, since ST2pRKgroEL was the only strain leading to an improvement in the shoot dry weight of chickpea plants, and consequently an increase of approx. 64% in the symbiotic effectiveness.

Table 2. Results obtained from pots assays of chickpea plants inoculated with wild type or transformed strains.

Strain	SDW (g)	RDW (g)	NN	AWN (mg)	SE (%)
V-15b	1.267 ± 0.183 a	0.641 ± 0.213 a	92 ± 16 ab	1.971 ± 0.241 a	40.95 ± 8.6 a
V15bpRK415	1.406 ± 0.232 a	0.472 ± 0.115 a	71 ± 16 b	1.650 ± 0.224 a	47.44 ± 10.9 a
V15bpRKgroEL	1.509 ± 0.369 a	0.591 ± 0.089 a	120 ± 24 a	1.431 ± 0.493 a	52.30 ± 17.3 a
PMI6pRK415	1.602 ± 0.460 a	0.698 ± 0.059 a	67 ± 17.3 b	2.334 ± 0.650 a	56.69 ± 21.6 a
PMI6pRKgroEL	1.683 ± 0.219 a	0.677 ± 0.279 a	99 ± 16.5 a	2.318 ± 0.650 a	60.50 ± 10.3 a
UPM-Ca36^T	1.120± 0.234 a	0.471 ± 0.066 a	61 ± 27 a	4.659 ± 1.027 a	34.00 ± 10.53 a
Ca36pRK415	1.289± 0.294 a	0.562 ± 0.115 a	66 ± 7.2 a	2.536 ± 0.775 b	41.98 ± 13.82 a
Ca36pRKgroEL	1.489± 0.224 a	0.554 ± 0.139 a	77 ± 9.9 a	2.108 ± 0.613 b	51.35 ± 11.84 a
ST-2	1.104 ± 0.330 b	0.524 ± 0.138 a	61 ± 18.2 a	1.749 ± 0.314 a	36.10 ± 15.55 b
ST2pRK415	1.227 ± 0.225 b	0.539 ± 0.077 a	54 ± 14.1 a	2.222 ± 0.609 a	39.06 ± 10.59 b
ST2pRKgroEL	1.765 ± 0.318 a	0.638 ± 0.173a	77 ± 44.2 a	2.019 ± 0.785 a	64.35 ± 14.98 a

Data correspond to the mean and standard deviation of five replicates per treatment. Different letters in the same group of strains are statistically significant (*P<0.05*). SDW - Shoot Dry Weight; RDW - Root Dry Weight; NN - Number of Nodules; AWN - Average Weight per Nodule; SE - Symbiotic Effectiveness

Bacterial growth curves under different stresses

Considering the well-established role of GroEL in stress tolerance, the growth of ST-2 and its respective transformed strains was evaluated under different stress conditions. For all the conditions tested, which included heat, salinity and acidic stresses, the wild type strains showed a higher growth rate (Fig 1). This is probably due to the energy required to replicate the expression vector and to express the corresponding antibiotic resistance genes, which hampers the growth of the transformed strains (Fig 1A-D). This effect is particularly striking in the case of salinity stress (1,5 % NaCl), since the wild type grows slightly slower that in control conditions, while both transformed strains are unable to grow (data not shown). Fig 1B shows the growth curve after a heat shock at 48°C during 15 min followed by control condition (28°C). Only in this condition, the ST-2 strain with extra *groEL* copies shows a slight acceleration in the growth from 133 hours onwards, when compared with ST2pRK415. Extra *groEL* copies did not increase the growth of ST-2 under other stresses, namely at 34°C and pH 5 (Fig 1C-D).

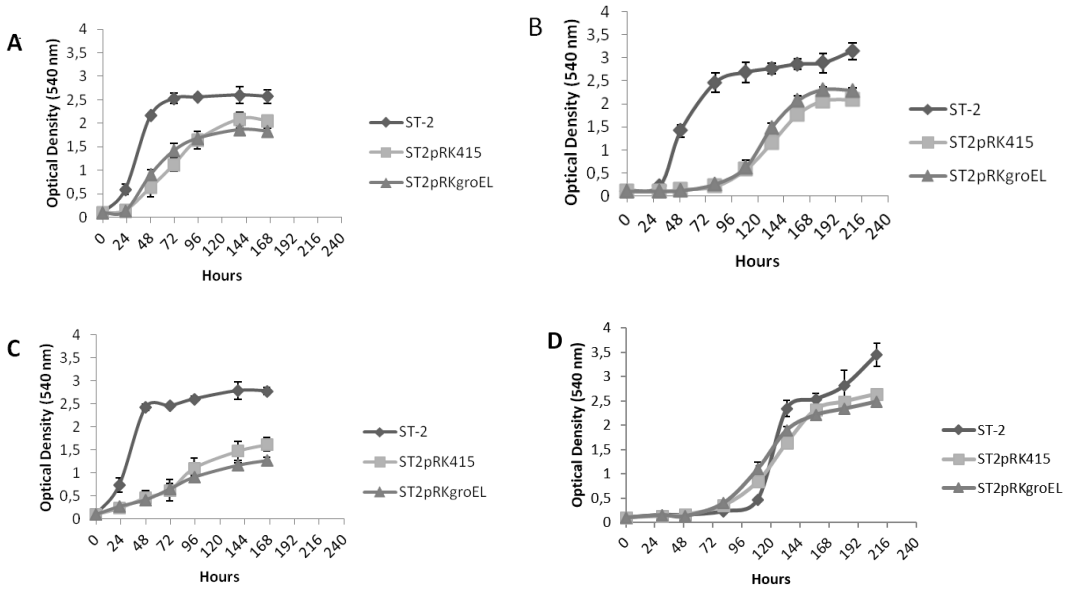

Figure 1. Growth curves of ST-2, ST2RK415 and ST2pRKgroEL in TY medium. A - Control condition (28°C); B - Heat shock at 48°C during 15 min followed by control condition; C – Continuous heat stress at 34°C; D - Continuous acidity stress (pH 5). Wild type strain statistically differs to transformed strains in all conditions. ST2RK415 and ST2pRKgroEL statistically differ in B (heat shock) from 133 hours onwards ($P < 0.05$), detected using T-test, implemented in SPSS V.21 software (SPP Inc., Chicago, U.S.A). Bars represent standard deviation.

Nodulation kinetics

In order to evaluate the dynamics of nodule development, nodulation on chickpea plants inoculated with ST-2 transformed strains were analyzed under hydroponic conditions during 35 days (Fig 2). Both strains induced the formation of the first nodules at 12 days after inoculation. Although in an early time point, plants inoculated with ST2pRK415 show a higher number of nodules, at 17 days after inoculation a similar number of nodules was detected in plants inoculated with ST2pRK415 and with ST2pRKgroEL. From this point onwards, the strain with extra *groEL* copies shows a higher number of nodules in the final time points of the nodulation kinetics evaluation, plants inoculated with ST2pRKgroEL showed approx. 78% more nodules than ST2pRK415 (Fig 2).

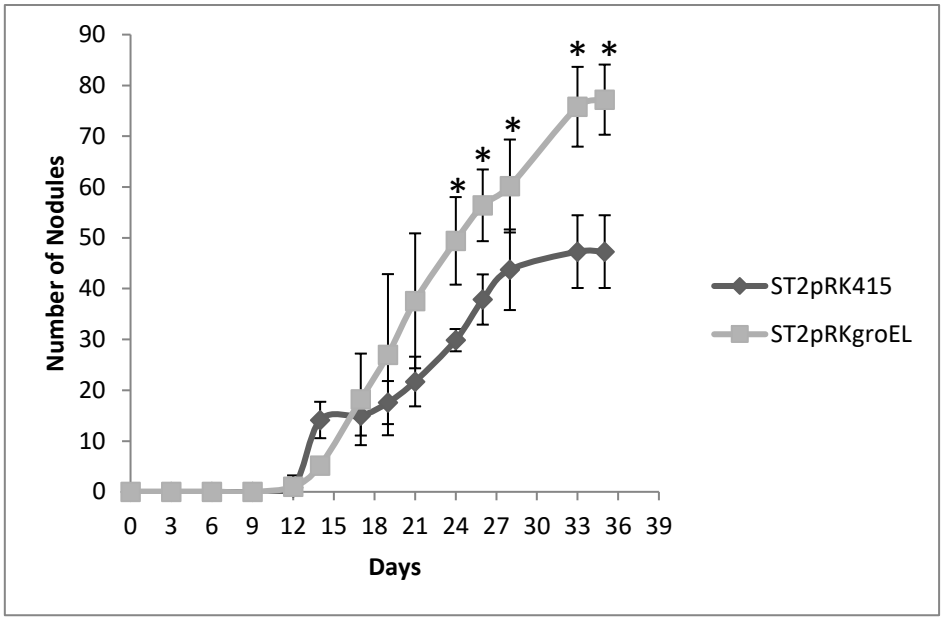

Figure 2. Nodulation kinetics of chickpea plants inoculated with ST2pRK415 and ST2pRKgroEL. Asterisks indicate statistical differences between the two strains ($P < 0.05$), using T-test, implemented in SPSS V.21 software (SPP Inc., Chicago, U.S.A). Bars represent standard deviation.

Histological analysis of nodules

Nodules from the hydroponic assay were embedded in paraffin and sectioned for analysis using light microscopy, in order to verify possible differences in their histology. Nodules from plants inoculated with both ST2pRK415 and ST2pRKgroEL showed the typical zones of an indeterminate nodule with meristematic, infection and fixation zones (Fig 3A and B). These nodules did not show a senescent zone probably due the short duration of the hydroponic trial. Overall, no differences were detected between nodules from plants inoculated with each of the transformed strains.

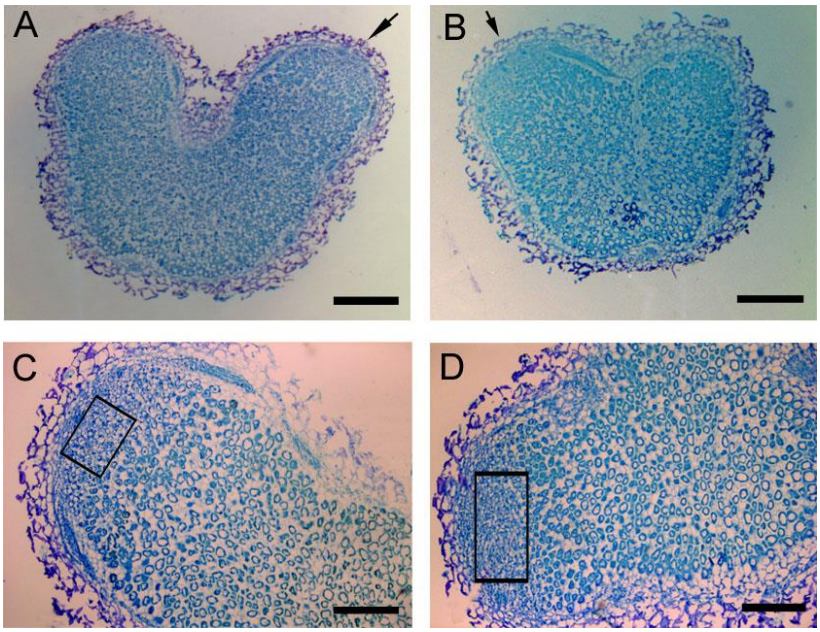

Figure 3. Nodule sections from chickpea plants grown in hydroponic conditions. Sections were stained with Toluidine Blue. A and C - ST2pRK415; B and D - ST2pRKgroEL; C and D - squares indicate infection zones. Scale bars: 1 mm (A and B); 400 µm (C and D).

Colonization and infection thread development

In order to investigate whether extra *groEL* copies could affect the first steps of the symbiosis process, both colonization and infection of root hairs by ST2pRK415 and ST2pRKgroEL were analysed by confocal microscopy. For this purpose, strains were transformed with an additional plasmid carrying *gfp* or *rfp* genes (Table 1). At seven days after inoculation both strains showed curling and infection threads on chickpea root hairs (Fig 4A and B). No difference in the infection process was detected between the two strains. Co-infection analysis at six days after inoculation confirmed that both strains seem to colonize equally the roots surface and form infection threads with a similar rate (Fig 4C).

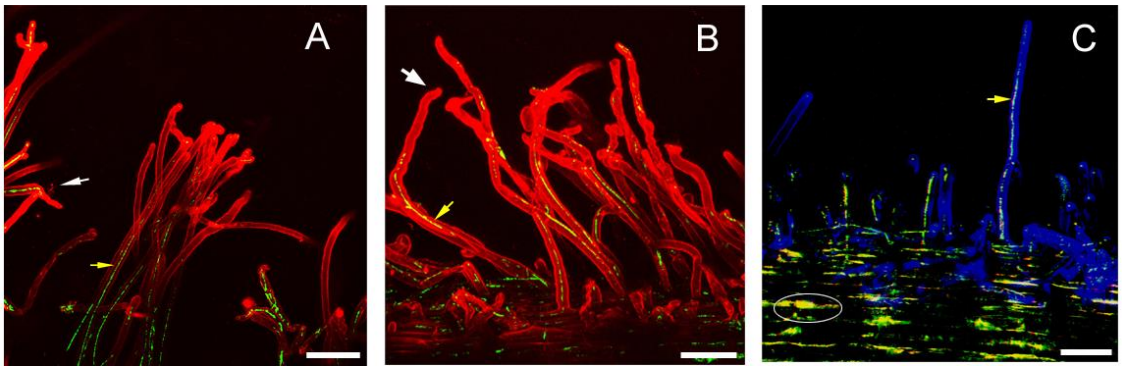

Figure 4. Confocal laser scanning micrographs showing the initial infection process of chickpea roots inoculated with green and red fluorescent protein–tagged rhizobia. Single inoculation: A - ST2pRK415 and B - ST2pRKgroEL strains tagged with green fluorescent protein and chickpea roots stained with propidium iodide, at 7 days after inoculation; Co-inoculation: C - ST2pRK415 (red) and ST2pRKgroEL (green) strains co-inoculated in chickpea roots stained with calcofluor white, at 6 days after co-inoculation. Root hair curling (white arrows); infection threads inside root hairs (yellow arrows); both strains sharing the same intercellular space (ellipse). Scale bars: 75 µm (A and B) and 50 µm (C).

4.5. Discussion

The role of chaperone system GroESL in protein folding and its importance for stress tolerance has been widely studied (Guisbert et al. 2004; Weiss et al. 2016). Alexandre and Oliveira (2013) review the role of different number of GroEL copies with different regulation in rhizobia. For example, in *B. japonicum* five *groESL* were annotated, however only three of them seemed to be heat induced (Babst et al. 1996). *S. meliloti* also has five *groEL* copies, while *R. leguminosarum* has three (Bittner et al. 2007; Rodriguez-Quinones et al. 2005). In *Mesorhizobium* sp. MAFF303099 only one of the five copies of *groEL* is highly expressed after a heat shock (Alexandre et al. 2014). Some studies have shown that GroEL is involved in the regulation of genes related with the nodulation process. It was reported that this chaperone is required for early regulation of *nod* genes in *S. meliloti* and that its mutation delayed nodules formation and caused a Fix^- phenotype (Ogawa and Long 1995). In *Bradyrhizobium japonicum* GroEL is required for the formation of a functional nitrogenase (Fischer et al. 1999) and the levels of GroEL in soybean nodules were seen to be seven times higher than in free-living conditions (Choi et al. 1991). In *S. meliloti* was found that NodD proteins are *in vitro* substrates of the GroESL system and that these chaperones also modulate the NodD binding to *nod* box sequence (Peck et al. 2013; Yeh et al. 2002). Farkas et al (2014)

reported that in *S. meliloti* GroEL1 is required for efficient infection, differentiation of bacteroids and nitrogen fixation in alfalfa. In the present study, four *Mesorhizobium* strains were transformed with extra *groEL* copies and its effect on the symbiotic performance was evaluated using different assays. From the five *groEL* copies found in the UPM-Ca36^T strain the copy selected for overexpression was that one which shares the highest similarity with the copy previously reported to be overexpressed in *M. japonicum* MAFF303099 bacteroids (Uchiumi et al. 2004). Only the ST-2 strain was able to improve the SDW of plants as well as its SE when modified with extra *groEL* copies. This suggests the involvement of the *groEL* gene in the symbiosis process in *Mesorhizobium* which was already suggested by its up regulation in bacteroids (Uchiumi et al. 2004). Others stress response genes related to saline, oxidative, drought, heat or biotic conditions were associated with symbiotic performance improvement when overexpressed (Boscari et al. 2006; Moussaid et al. 2015; Paço et al. 2016; Shvaleva et al. 2010; Talbi et al. 2012). For example, the *Mesorhizobium* strain UPM-Ca36^T modified with extra copies of the chaperone gene *clpB* (Paço et al. 2016) showed a higher rate of nodule development as well as a higher number of nodules. More interestingly, the symbiotic effectiveness of the *clpB*-overproducing strain increased ~60% at pH 5 and ~83% at pH 7, compared to the wild-type strain (Paço et al. 2016). Previously, Brígido et al (2012b) also showed that the chaperone ClpB was involved in chickpea root nodulation by *Mesorhizobium*. *clpB* mutants showed a 6- to 8-day delay in nodule appearance and analysis of *nodC* expression showed lower levels of this transcript on the mutant strain.

In the present study, the UPM-Ca36^T as well as PMI-6 and V-15b strains were also transformed with extra *groEL* copies, however there was no improvement in any of the plant parameters evaluated in chickpea plants inoculated with these strains. Nevertheless, the NN resulting from PMI6pRKgroEL and V15bpRKgroEL inoculation was higher than that of the corresponding strains transformed with the pRK415 plasmid. Nelson and Sadowsky (2015) reported that a large amount of nodules without any improvement or with negative effects in the host plants may indicate that bacteria are causing a pathogenic result. Nevertheless, no significant decrease in the plant parameters analyzed was observed for chickpea plants inoculated with the transformed strains that induced a higher number of nodules.

Although the SE was higher in plants inoculated with ST2pRKgroEL, the NN was not statistically different. As the role of GroEL in the symbiosis process seems to be related with the early *nod* genes regulation (Ogawa and Long 1995), it was expected that extra *groEL* copies would improve the NN, since one of the *nod* genes function is to regulate the NN on root hair (Limpens and Bisseling 2009). In order to observe the

continuous formation of nodules, a hydroponic assay was performed and plants inoculated with the strain with extra *groEL* copies indeed showed a higher number of nodules during 35 days, suggesting that *groEL* may influence indirectly the nodulation process. This difference in the number of nodules was not detected in the pot assay probably due to the larger duration of this experiment (seven weeks). It is likely that the higher number of nodules induced by the inoculation of ST2pRKgroEL, compared to ST2pRK415, is transient and that later on the number of nodules on plants inoculated with both strains reaches similar values as reported for the pot assay.

Analysis of histological sections of nodules from plants inoculated with ST2pRKgroEL showed similar zones compared to nodules resulting from ST2pRK415 inoculation. In addition, no differences were detected in root attachment or the infection. These results suggest that probably the GroEL relationship with the symbiosis is not related with the colonization or infection, besides it does not seem to have any evolvement with the infection of the nodules. Therefore, the higher SE observed in the ST2pRKgroEL is justified by the increase of the NN.

Since GroEL is known to be involved in temperature stress tolerance in several bacteria including in rhizobia (Alexandre and Oliveira 2011; Ogawa and Long 1995), the growth of ST2pRKgroEL was evaluated under different stress conditions. ST2pRKgroEL showed faster growth than ST2pRK415 after a heat shock at 48 °C for 15 minutes, however the modified strains were indistinguible under the other stress conditions evaluated. This suggests that the extra copies of *groEL* most likely helped bacteria to recover after the heat shock. In chickpea rhizobia GroEL was found to be consistently overproduced when isolates were submitted to heat stress (Laranjo and Oliveira 2011; Rodrigues et al. 2006). Moreover, higher levels of *groEL* transcript were previously suggested to be associated to higher tolerance to heat shock in mesorhizobia (Alexandre and Oliveira 2011).

Despite the promising results obtained in the improvement of rhizobial symbiotic performance with the overexpression of stress response genes, further studies with more bacterial species are required to validate this approach as a strategy to engineer rhizobial strains that can be useful as crop inoculants, particularly under challenging soil and climatic conditions.

In addition to its main role as heat shock protein, GroEL is known to be involved in the nitrogen fixation process (Ogawa and Long 1995), thus this study showed that increasing the *groEL* expression may be enough to improve the symbiotic effectiveness.

4.6. References

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microbial Ecology, 58*, 930-941.

Alexandre, A., Laranjo, M., & Oliveira, S. (2014). Global transcriptional response to heat shock of the legume symbiont *Mesorhizobium loti* MAFF303099 comprises extensive gene downregulation. *DNA Res, 21*(2), 195-206. doi: 10.1093/dnares/dst050

Alexandre, A., & Oliveira, S. (2011). Most heat-tolerant rhizobia show high induction of major chaperone genes upon stress. *FEMS Microbiology Ecology, 75*, 28-36.

Alexandre, A., & Oliveira, S. (2013). Response to temperature stress in rhizobia. *Critical Reviews in Microbiology, 39*(3), 219-228.

Babst, M., Hennecke, H., & Fischer, H. M. (1996). Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. *Molecular Microbiology, 19*(4), 827-839.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol, 84*.

Bittner, A. N., Foltz, A., & Oke, V. (2007). Only one of five *groEL* genes is required for viability and successful symbiosis in *Sinorhizobium meliloti*. *J. Bacteriol., 189*(5), 1884-1889. doi: 10.1128/jb.01542-06

Bittner, A. N., & Oke, V. (2006). Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. *J. Bacteriol., 188*(10), 3507-3515.

Bloemberg, G. V., Wijfjes, A. H., Lamers, G. E., Stuurman, N., & Lugtenberg, B. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microbe Interact, 13*(11), 1170-1176. doi: 10.1094/mpmi.2000.13.11.1170

Boscari, A., Van de Sype, G., Le Rudulier, D., & Mandon, K. (2006). Overexpression of BetS, a *Sinorhizobium meliloti* high-affinity betaine transporter, in bacteroids from *Medicago sativa* nodules sustains nitrogen fixation during early salt stress adaptation. *Molecular Plant-Microbe Interactions, 19*(8), 896-903.

Brígido, C., Alexandre, A., & Oliveira, S. (2012). Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia. *Microbiological Research, 167*(10), 623-629.

Brígido, C., Robledo, M., Menendez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604. doi: 10.1094/mpmi-05-12-0140-r

Broughton, W. J., & Dilworth, M. J. (1971). Control of Leghaemoglobin Synthesis in Snake Beans. *Biochemical Journal, 125*(4), 1075-1080.

Choi, E. Y., Ahn, G. S., & Jeon, K. W. (1991). Elevated levels of stress proteins associated with bacterial symbiosis in amaeba-proteus and soybean root nodules cells. *Biosystems, 25*(3), 205-212. doi: 10.1016/0303-2647(91)90006-7

Djordjevic, M. A. (2004). *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. *PROTEOMICS, 4*(7), 1859-1872.

Djordjevic, M. A., Chen, H. C., Natera, S., Van Noorden, G., Menzel, C., & Taylor, S. (2003). A global analysis of protein expression profiles in Sinorhizobium meliloti: discovery of new genes for nodule occupancy and stress adaptation. *Mol Plant Microbe Interact, 16*. doi: 10.1094/mpmi.2003.16.6.508

Elbein, A. D., Pan, Y. T., Pastuszak, I., & Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology, 13*(4), 17r-27r. doi: 10.1093/glycob/cwg047

Farkas, A., Maroti, G., Durgo, H., Gyorgypal, Z., Lima, R. M., Medzihradszky, K. F., . . . Kondorosi, E. (2014). Medicago truncatula symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. *Proc Natl Acad Sci U S A, 111*(14), 5183-5188. doi: 10.1073/pnas.1404169111

Finan, T. M., Kunkel, B., De Vos, G. F., & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol, 167*(1), 66-72.

Fischer, H. M., Babst, M., Kaspar, T., Acuña, G., Arigoni, F., & Hennecke, H. (1993). One member of a *gro*ESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *The Embo Journal, 12*(7), 2901-2912.

Fischer, H. M., Schneider, K., Babst, M., & Hennecke, H. (1999). GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum*. *Archives of Microbiology, 171*(4), 279-289.

Flores-Félix, J., Menéndez, E., Marcos-García, M., Celador-Lera, L., & Rivas, R. (2015). Calcofluor white, an Alternative to Propidium Iodide for Plant Tissues Staining in Studies of Root Colonization by Fluorescent-tagged Rhizobia. *Journal of Advances in Biology & Biotechnology, 2*(1), 65-70. doi: 10.9734/jabb/2015/12444

Fournier, J., Timmers, A. C. J., Sieberer, B. J., Jauneau, A., Chabaud, M., & Barker, D. G. (2008). Mechanism of Infection Thread Elongation in Root Hairs of *Medicago truncatula* and Dynamic Interplay with Associated Rhizobial Colonization. *Plant Physiol, 148*(4), 1985-1995. doi: 10.1104/pp.108.125674

Gage, D. J. (2004). Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. *Microbiol. Mol. Biol. Rev., 68*(2), 280-300.

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M. T., . . . Velázquez, E. (2012). *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. *PLOS ONE, 7*(5), e38122. doi: 10.1371/journal.pone.0038122

Gibson, A. H. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. In G. H. Elkan (Ed.), *Symbiotic Nitrogen Fixation Technology* (pp. 321-363). New York: Marcel Dekker, Inc.

Guisbert, E., Herman, C., Lu, C. Z., & Gross, C. A. (2004). A chaperone network controls the heat shock response in *E. coli*. *Genes Dev, 18*(22), 2812-2821. doi: 10.1101/gad.1219204

Hungria, M., & Stacey, G. (1997). Molecular signals exchanged between host plants and rhizobia: Basic aspects and potential application in agriculture. *Soil Biology and Biochemistry, 29*(5), 819-830. doi: http://dx.doi.org/10.1016/S0038-0717(96)00239-8

Hungria, M., & Vargas, M. A. T. (2000). Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research, 65*(2-3), 151-164.

Karunakaran, R., Ramachandran, V. K., Seaman, J. C., East, A. K., Mouhsine, B., Mauchline, T. H., . . . Poole, P. S. (2009). Transcriptomic Analysis of *Rhizobium leguminosarum* Biovar *viciae* in Symbiosis with Host Plants *Pisum sativum* and *Vicia cracca*. *J Bacteriol, 191*(12), 4002-4014. doi: 10.1128/JB.00165-09

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene, 70*(1), 191-197. doi: 10.1016/0378-1119(88)90117-5

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W., & Oliveira, S. (2008). Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiology Ecology, 66*(2), 391-400.

Laranjo, M., & Oliveira, S. (2011). Tolerance of *Mesorhizobium* type strains to different environmental stresses. *Antonie van Leeuwenhoek, 99*(3), 651-662.

Limpens, E., & Bisseling, T. (2009). Nod Factor Signal Transduction in the Rhizobium–Legume Symbiosis. In A. M. C. Emons & T. Ketelaar (Eds.), *Root Hairs* (pp. 249-276). Berlin, Heidelberg: Springer Berlin Heidelberg.

Lund, P. A. (2009). Multiple chaperonins in bacteria - why so many? *Fems Microbiology Reviews, 33*(4), 785-800. doi: 10.1111/j.1574-6976.2009.00178.x

Minder, A. C., Narberhaus, F., Babst, M., Hennecke, H., & Fischer, H. M. (1997). The *dnaKJ* operon belongs to the sigma(32)-dependent class of heat shock genes in *Bradyrhizobium japonicum*. *Molecular & General Genetics, 254*(2), 195-206.

Moussaid, S., Domínguez-Ferreras, A., Muñoz, S., Aurag, J., Berraho, E. B., & Sanjuán, J. (2015). Increased trehalose biosynthesis improves *Mesorhizobium ciceri* growth and symbiosis establishment in saline conditions. *Symbiosis, 67*(1), 103-111. doi: 10.1007/s13199-015-0338-y

Nascimento, F. X., Brigido, C., Glick, B. R., Oliveira, S., & Alho, L. (2012a). *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. *Lett Appl Microbiol, 55*(1), 15-21. doi: 10.1111/j.1472-765X.2012.03251.x

Nelson, M. S., & Sadowsky, M. J. (2015). Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. *Frontiers in Plant Science, 6*, 491. doi: 10.3389/fpls.2015.00491

Nomura, M., Arunothayanan, H., Dao, T. V., Le, H. T. P., Kaneko, T., Sato, S., . . . Tajima, S. (2010). Differential protein profiles of Bradyrhizobium japonicum USDA110 bacteroid during soybean nodule development. *Soil Science and Plant Nutrition, 56*(4), 579-590. doi: 10.1111/j.1747-0765.2010.00500.x

Nour, S. M., Cleyet-Marel, J.-C., Normand, P., & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology, 45*(4), 640-648.

Ogawa, J., & Long, S. R. (1995). The *Rhizobium meliloti groELc* locus is required for regulation of early nod genes by the transcription activator *nodD*. *Genes & Development, 9*(6), 714-729.

Oke, V., & Long, S. R. (1999). Bacteroid formation in the Rhizobium-legume symbiosis. *Current Opinion in Microbiology, 2*(6), 641-646.

Oldroyd, G. E. D., & Downie, J. M. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes *Annual Review of Plant Biology* (Vol. 59, pp. 519-546).

Paço, A., Brígido, C., Alexandre, A., Mateos, P. F., & Oliveira, S. (2016). The Symbiotic Performance of Chickpea Rhizobia Can Be Improved by Additional Copies of the *clpB* Chaperone Gene. *PLOS ONE, 11*(2), e0148221. doi: 10.1371/journal.pone.0148221

Peck, M. C., Fisher, R. F., Bliss, R., & Long, S. R. (2013). Isolation and characterization of mutant *Sinorhizobium meliloti* NodD1 proteins with altered responses to luteolin. *J Bacteriol, 195*(16), 3714-3723. doi: 10.1128/jb.00309-13

Pessi, G., Ahrens, C. H., Rehrauer, H., Lindemann, A., Hauser, F., Fischer, H. M., & Hennecke, H. (2007). Genome-wide transcript analysis of Bradyrhizobium japonicum bacteroids in soybean root nodules. *Molecular Plant-Microbe Interactions, 20*(11), 1353-1363. doi: 10.1094/mpmi-20-11-1353

Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F., & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis, 22*(6), 1086-1089.

Robledo, M., Jimenez-Zurdo, J. I., Soto, M. J., Velazquez, E., Dazzo, F., Martinez-Molina, E., & Mateos, P. F. (2011). Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial

cellulase CelC2. *Mol Plant Microbe Interact, 24*(7), 798-807. doi: 10.1094/mpmi-10-10-0249

Rodrigues, C., Laranjo, M., & Oliveira, S. (2006). Effect of heat and pH stress in the growth of chickpea mesorhizobia. *Current Microbiology, 53*(1), 1-7.

Rodriguez-Quinones, F., Maguire, M., Wallington, E. J., Gould, P. S., Yerko, V., Downie, J. A., & Lund, P. A. (2005). Two of the three *groEL* homologues in *Rhizobium leguminosarum* are dispensable for normal growth. *Archives of Microbiology, 183*(4), 253-265.

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). New York: Cold Spring Harbor Laboratory.

Sarma, A. D., & Emerich, D. W. (2005). Global protein expression pattern of *Bradyrhizobium japonicum* bacteroids: A prelude to functional proteomics. *PROTEOMICS, 5*(16), 4170-4184. doi: 10.1002/pmic.200401296

Sarma, A. D., & Emerich, D. W. (2006). A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. *Proteomics, 6*(10), 3008-3028. doi: 10.1002/pmic.200500783

Shvaleva, A., Coba de la Peña, T., Rincón, A., Morcillo, C. N., García de la Torre, V. S., Lucas, M. M., & Pueyo, J. J. (2010). Flavodoxin overexpression reduces cadmium-induced damage in alfalfa root nodules. *Plant and Soil, 326*(1), 109-121. doi: 10.1007/s11104-009-9985-1

Suarez, R., Wong, A., Ramirez, M., Barraza, A., Orozco, M. d. C., Cevallos, M. A., . . . Iturriaga, G. (2008). Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. *Molecular Plant-Microbe Interactions, 21*(7), 958-966. doi: 10.1094/mpmi-21-7-0958

Talbi, C., Sanchez, C., Hidalgo-Garcia, A., Gonzalez, E. M., Arrese-Igor, C., Girard, L., . . . Delgado, M. J. (2012). Enhanced expression of *Rhizobium etli cbb*(3) oxidase improves drought tolerance of common bean symbiotic nitrogen fixation. *J Exp Bot, 63*(14), 5035-5043. doi: 10.1093/jxb/ers101

Uchiumi, T., Ohwada, T., Itakura, M., Mitsui, H., Nukui, N., Dawadi, P., . . . Minamisawa, K. (2004). Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J Bacteriol, 186*(8), 2439-2448.

Weiss, C., Jebara, F., Nisemblat, S., & Azem, A. (2016). Dynamic Complexes in the Chaperonin-Mediated Protein Folding Cycle. *Frontiers in Molecular Biosciences, 3*, 80. doi: 10.3389/fmolb.2016.00080

Yeh, K. C., Peck, M. C., & Long, S. R. (2002). Luteolin and GroESL modulate in vitro activity of NodD. *J Bacteriol, 184*(2), 525-530.

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews, 63*(4), 968-989.

Zhang, F., & Smith, D. L. (1996). Genistein accumulation in soybean (Glycine max L Merr) root systems under suboptimal root zone temperatures. *Journal of Experimental Botany, 47*(299), 785-792. doi: 10.1093/jxb/47.6.785

Chapter 5

The osmolarity sensor protein EnvZ improves the symbiotic effectiveness of mesorhizobia strains

5.1. Summary

Bacteria are able to sense oscillation on the environment and adapt themselves to survive under different stress conditions. The two-component signal transduction system EnvZ/OmpR present in *Escherichia coli* mediates responses when bacteria are under osmotic stress. In addition, EnvZ/OmpR has been shown to also regulate genes involved in virulence, fatty acid uptake, exopolysaccharide (EPS) production, peptide transportation and flagella production. Some of these mechanisms are known to be important for a successful symbiosis. Therefore, the aim of this study is to evaluate the effects of extra *envZ* copies in chickpea *Mesorhizobium* strains. The *Mesorhizobium* sp. V-15b, PMI-6, ST-2, EE-7 and UPM-Ca36^T were transformed with pRKenvZ (pRK415 harboring *envZ* gene from *M. mediterraneum* UPM-Ca36^T). The symbiotic performance was evaluated in a pot trial, which showed that the symbiotic effectiveness (SE) of plants inoculated with PMI6pRKenvZ and EE7pRKenvZ was 64 % and 96% higher than that of those inoculated with PMI6pRK415 and EE7pRK415, respectively. Overexpressing *envZ* on UPM-Ca36^T, ST-2 and V-15b strains did not improved the SE, and for that reason further analyses were performed using only EE-7 and PMI-6 strains. Under hydroponic conditions, plants inoculated with the PMI6pRKenvZ strain showed the first nodules 4 days before those inoculated with the empty vector strain (PMI6pRK415). Furthermore, PMI6pRKenvZ started to show a significantly higher nodule number at 28 days after inoculation. Despite the fact that both strains were Fix+, the interior of the nodules resulting from PMI6pRKenvZ inoculation showed a more intense red coloration than those from PMI6pRK415, which presented large senescence zones and a lower number of bacteroid-occupied cells, as revealed by histological analyzes. Moreover, chickpea seedlings inoculated with PMI6pRKenvZ showed a higher number of infection threads than those inoculated with PMI6pRK415. In addition, plants inoculated with PMI6pRKenvZ showed a higher number of secondary roots and an increase in the density of root hairs. On the other hand, plants inoculated with EE7pRKenvZ did not show statistical differences in the number of nodules under hydroponic conditions when compared to EE7pRK415. No differences were also observed in root hairs infection, nodule histology, number of root hairs, secondary roots or changes in the production of exopolysaccharides. The present study suggests EnvZ/OmpR involvement in the symbiosis process and represents an important starting point to evaluate the role of this system in the rhizobia-legume symbiosis.

5.2. Introduction

Interaction with the external environment is vital to bacterial survival (Hubber et al. 2004; Sugawara et al. 2013; Tseng et al. 2009). However, changes in the surroundings can lead these microorganisms to stress conditions, limiting their survival, development and interactions with other prokaryotic or eukaryotic organisms. To handle these fluctuations, bacteria have developed versatile response systems that allow them to detect information about their environment (Boor 2006), as temperature, osmotic pressure, pH and nutrient availability.

Bacteria can mediate responses to environmental stress through two-component regulatory systems (TCRS), namely a histidine kinase (HK) and a cognate response regulator (RR) (Foo et al. 2015; Wang et al. 2012). In a general way, the sense of an environmental stress by the HK allows the autophosphorylation of the conserved histidine residue (Wang et al. 2012), and this phosphoryl group is then transferred by the conserved aspartic acid residue of the RR. Once phosphorylated, the RR usually binds to DNA target sequences stimulating the transcription of appropriate gene targets (Yoshida et al. 2002).

The EnvZ/OmpR system, one of the best-characterized TCRS, is activated in response to osmotic stress and is responsible for the regulation of the expression of outer membrane porins OmpF and OmpC (Alphen and Lugtenberg 1977; Wang et al. 2012). At high osmolarity EnvZ is autophosphorylated and transfers the phosphoryl group to OmpR, which in turn binds to the promoter regions of outer membrane porin genes *ompF* and *ompC* (Yoshida et al. 2002; Yuan et al. 2011). Both porins act in the nutrient exchange, but at high osmolarity OmpC has a slower flow rate and becomes the major porin in the outer membrane, while at low osmolity *ompC* is repressed and OmpF becomes the major porin (Nikaido 2003; Wang et al. 2012). EnvZ also acts as a phosphatase, which dephosphorylates the OmpR-P when the osmotic stress disappears (Mattison and Kenney 2002).

Mutation in *envZ*/*ompR* of *E. coli* affects directly or indirectly the expression of more than 100 genes, including genes related to amino acid biosynthesis, such as isoleucine and cysteine, iron and maltose transport, and flagellar synthesis (Oshima et al. 2002). *ompR* mutation in *Yersinia pestis* affected the expression of 224 genes, indicating a global regulatory role (Gao et al. 2011). Besides its relation to osmotolerance, EnvZ/OmpR system has also been associated with other functions, such as virulence in pathogens, fatty acid uptake, exopolysaccharide (EPS) production, peptide transport and flagella production (Bernardini et al. 1990; Feng et al. 2003; Li et al. 2014; Mills et al. 1998; Pickard et al. 1994; Shin and Park 1995; Vidal et al. 1998;

Yuan et al. 2011). In addition, EnvZ/OmpR regulates the type III secretion system genes in pathogenic bacteria, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Erwinia amylovora* and *Pseudomonas syringae* (Brzostek et al. 2007; Feng et al. 2003; Feng et al. 2004; Li and Zhao 2011).

As shown above, several studies have been aiming to understand the function of EnvZ/OmpR in numerous pathogenic bacteria. Nevertheless, there are no reports on the function of this two-component system in symbiotic bacteria. The aim of this study was to evaluate the effects of overexpressing *envZ* in the rhizobia-host interaction, namely to evaluate whether a higher expression of this gene could lead to an improvement of the symbiotic performance in chickpea mesorhizobia strains.

5.3. Material and Methods

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. A group of four chickpea mesorhizobia strains isolated form Portuguese soils and previously characterized were used: V-15b-Viseu, ST-2-Setúbal, EE-7-Elvas, PMI-6-Portimão (Alexandre et al. 2009; Brígido et al. 2012a; Laranjo et al. 2008). In addition, the strain *M. mediterraneum* UPM-Ca36^T was also used (Nour et al. 1995).

Tryptone-yeast (TY) medium at 28°C was used to grow mesorhizobia strains (Beringer 1974b) and tetracycline (15 μg.ml^{-1}) was added into the medium for pRK415-transformed mesorhizobia strains. For bacteria transformed with *gfp* and *rfp* genes, kanamycin (50 μg.ml^{-1}) and gentamycin (15 μg.ml^{-1}) were added to TY medium, respectively. *Escherichia coli* DH5α and MT616 strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell 2001) at 37º C. For *E. coli* strains containing pRK415, 15 μg.ml^{-1} of tetracycline was used, while for MT616 with pRK600, the medium was supplemented with 25 μg.ml^{-1} of chloramphenicol.

Table 1 Bacterial strains and plasmids used in this work.

Plasmids/Strains	Characteristics	Reference
pRK600	pRK2013 *npt*::Tn9. Cm^r	(Finan et al. 1986)
pRK415	Broad host-range vector; Tc^r	(Keen et al. 1988)
pRKenvZ	Plasmid pRK415 containing the *envZ* gene from *M. mediterraneum* UPM-$Ca36^T$; Tc^r	This work
pMRGFP	Plasmid containing the *gfp* gene; Km^r	(García-Fraile et al. 2012)
pMP4661	Plasmid containing the *rfp* gene; Gm^r	(Bloemberg et al. 2000)
E. coli		
MT616	Strain harboring the helper plasmid pRK600	(Finan et al. 1986)
DH5α	Competent cells	NZYTech
Mesorhizobium		
V-15b	*Mesorhizobium* sp. V-15b-Viseu isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
ST-2	*Mesorhizobium* sp. ST-2-Setubal isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
PMI-6	*Mesorhizobium* sp. PMI-6-Portimão isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
EE-7	*Mesorhizobium* sp. EE-7-Elvas isolated from chickpea root nodules (Portugal)	(Alexandre et al. 2009)
UPM-$Ca36^T$	*Mesorhizobium mediterraneum* UPM-$Ca36^T$ isolated from chickpea root nodules from Spain	(Nour et al. 1995)
V15bpRKenvZ	V-15b strain harboring pRKenvZ	This work
ST2pRKenvZ	ST-2 strain harboring pRKenvZ	This work
PMI6pRKenvZ	PMI-6 strain harboring pRKenvZ	This work
PMI6pRKenvZGFP	PMI-6 strain harboring pRKenvZ and pMRGFP	This work
EE7pRKenvZ	EE-7 strain harboring pRKenvZ	This work
EE7pRKenvZGFP	EE-7 strain harboring pRKenvZ and pMRGFP	This work
Ca36pRKenvZ	UPM-$Ca36^T$ strain harboring pRKenvZ	This work
V15bpRK415	V-15b strain harboring pRK415	This work
ST2pRK415	ST-2 strain harboring pRK415	This work
PMI6pRK415	PMI-6 strain harboring pRK415	This work
PMI6pRK415GFP	PMI-6 strain harboring pRK415 and pMRGFP	This work
PMI6pRK415RFP	PMI-6 strain harboring pRK415 and pMP4661	This work
EE7pRK415	EE-7 strain harboring pRK415	This work
EE7pRK415GFP	EE-7 strain harboring pRK415 and pMRGFP	This work
EE7pRK415RFP	EE-7 strain harboring pRK415 and pMP4661	This work
Ca36pRK415	UPM-$Ca36^T$ strain harboring pRK415	This work

Transforming the strains with the *envZ* gene

The full sequence of the *envZ* gene from *Mesorhizobium mediterraneum* UPM-$Ca36^T$ was amplified by PCR. The PCR was performed in 25 µl using 0,5 µL of DNA, 1× Buffer for Phusion GC Buffer, 0,2 mM of each dNTP, 7.5 pmol of each primer and 0,4 U of Phusion DNA Polymerase (Thermo Fisher Scientific). The primers used were envZ-HindIII-F (5'-AAGCTTAATGAGACGTTTCCTGCCGCA-3') and envZ-BamHI-R (5'-GGATCCCTACGTTGCCAGCGGCAAGC-3'). The amplification program was: 30 s at 98°C, 30 cycles of 10 s at 98ºC, 20 s at 56°C, 28 s at 72 ºC and a final extension of 5

min at 72 ºC. The obtained *envZ* fragment of 1407 bp was cloned in pCR-Blunt™ vector (Thermo Fisher Scientific), sequenced and subcloned in the expression vector pRK415, previously digested with *Hind*III and *Bam*HI.

Five chickpea mesorhizobia strains were transformed by triparental mating with the pRKenvZ plasmid, containing the exogenous *envZ* gene under the control of the *lac* promoter. Based on previous work, it is already known that pRK415 allows the expression of the cloned gene both under free-living and symbiotic conditions (chapter 2). *E. coli* DH5α cells harboring the plasmid pRKenvZ or pRK415 were used as donor and *E. coli* MT616 cells with pRK600 acted as helper, as described by Nascimento et al. (2012a). The pRKenvZ-transformed strains were named V15bpRKenvZ, ST2pRKenvZ, PMI6pRKenvZ, EE7pRKenvZ and Ca36pRKenvZ. The same mesorhizobia strains were transformed with pRK415, and named V15bpRK415, ST2pRK415, PMI6pRK415, EE7pRK415 and Ca36pRK415.

To confirm the transformation of mesorhizobia cells with pRKenvZ and pRK415, total DNA was extracted according to Rivas et al (2001) and used to amplify the region of the expression vector that includes the *envZ* gene. Using the universal primers M13F and M13R-pUC, a DNA fragment of 1529 bp will be amplified from pRKenvZ and 122 pb will be amplified from pRK415. The PCR reaction was performed in a final volume of 50 µL, using 5 µL of DNA, 1× reaction Green GoTaq® Flexi buffer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 15 pmol of each primer and 0.625U of GoTaq® G2 Flexi DNA Polymerase (Promega). The amplification program was: 2 min of initial denaturation at 95 °C, 30 cycles of 60 s at 95 ºC, 45 s at 56 °C, 85 s at 72 ºC, and a final extension of 5 min at 72 ºC.

Once the transformations were confirmed, strains harboring pRKenvZ were transformed with pMRGFP (containing the *gfp* gene) and strains harboring pRK415 were transformed with pMRGFP or pMP4661 (containing the *rfp* gene) by triparental mating, as described above. These transformations were confirmed by fluorescence microscopy observations.

Evaluation of the symbiotic performance

In order to evaluate whether additional *envZ* copies improved the symbiotic effectiveness of chickpea mesorhizobia strains, a plant growth trial under controlled conditions was performed using both the wild-type and the transformed strains. After surface-sterilization and germination, chickpea seeds were transferred to plastic pots filled with sterile vermiculite and inoculated (Alexandre et al. 2009).

For inocula preparation, rhizobia strains were grown in TY liquid medium at 28°C for 72 h. Cell culture was centrifuged at 10.000 × g, resuspended in fresh TY liquid medium to an OD_{540nm} of 1.0 and 1 ml of this bacterial suspension was used to inoculate each seedling. Five replicates per treatment were performed. A nitrogen-free nutrient solution (Broughton and Dilworth 1971) was applied three times a week. Uninoculated plants were used as negative control and plants watered with N-supplemented nutrient solution were used as positive control. Plants were grown in a growth chamber under a 16 h-light and 8 h-dark cycle with 24°C-day and 18°C-night temperature at a relative humidity of 65%. After 7 weeks, plants were harvested and several parameters were measured, such as shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN) and average weight per nodule (AWN). Symbiotic effectiveness (SE) was calculated using the shoot dry weight values, including those from the positive and negative controls (Gibson 1987). Statistical analysis was performed using SPSS statistics V.21 (SPSS Inc; IBM New York, USA) and included analysis of variance, namely one-way ANOVA, and the Tukey's Multiple Range Test ($P < 0,05$).

Based on the results obtained in the symbiotic performance evaluation, mesorhizobia strains PMI-6 and EE-7 and their derivates were selected to be used for further analyses.

Bacterial growth

In order to evaluate free-living growth of wild type PMI-6 and EE-7 as well as their derivatives harboring pRK415 and pRKenvZ, strains were grow in 5 mL of TY medium (supplemented with 15 $\mu g.ml^{-1}$ tetracycline in the case of the transformed strains) during 144 h. The optical densities at 540 nm were measured every 24 hours and triplicates were used for each strain.

In order to evaluate differences in the mucoid phenotype between the strains harboring pRK415 or pRKenvZ plasmids, bacterial growth was observed in yeast manitol agar (YMA).

Evaluation of nodulation kinetics

The nodulation kinetics were evaluated through a hydroponic assay using chickpea plants inoculated with PMI6pRK415, PMI6pRKenvZ, EE7pRK415 and EE7pRKenvZ strains ($OD_{540\ nm}$ of 0.6). The procedures were conducted as previously described in Brígido et al. (2012b). Eight seeds per treatment were used and the number of nodules was evaluated every three days during 33 days. Plants were kept on a growth

chamber under the same conditions describe above for the evaluation of symbiotic performance.

Histological analysis of nodules

Nodules were excised from hydroponic plants 35 days after inoculation and processed for light microscopy. Nodules were fixed in 4% formaldehyde, dehydrated in an increasing ethanol series, and embedded in paraffin (Brígido et al. 2012b). Toluidine blue-stained sections (6 µm) of embedded nodules were examined by bright-field microscopy.

Induction of root hairs formation

To evaluate the effects of extra *envZ* copies on the initial root development of chickpea plants, pre-germinated seedlings were inoculated with strains harboring pRK415 or pRKenvZ plasmids in water-agar plates and the number of secondary roots and the density of root hairs were evaluated seven days after inoculation.

Analysis of rhizobia infection process

Pre-germinated chickpea seeds were inoculated with GFP-tagged (single infection) or RFP+GFP-tagged (co-infection) mesorhizobia strains, as described by Robledo et al. (2011). Chickpea roots and root hairs were stained with 10 µM propidium iodide (Sigma-Aldrich) or with 50mg/l calcofluor white (Sigma- Aldrich) and 10% potassium hydroxide solution (Flores-Félix et al. 2015). Projections were made from adjusted individual channels and accumulating stacks using Leica software. The analysis of root hairs 4 or 6 days after of inoculation were performed using a Confocal Laser Scanning Microscope (Leica TCS SPE) equipped with solid-state laser, allowing visualization of GFP (488 nm), RFP and propidium iodide (532 nm) and calcofluor white (405 nm) fluorescence.

5.4. Results

The mesorhizobia strains V-15b, ST-2, EE-7, PMI-6 and UMP-Ca36^T were transformed with the pRK415 plasmid containing the gene *envZ* (pRKenvZ) or with the empty vector (prK415) as control. The cloned *envZ* gene was amplified from *Mesorhizobium mediterraneum* UPM-Ca36^T. Despite the presence of genes encoding

the EnvZ/OmpR two-component system in the genome of UPM-Ca36^T, the two genes usually regulated by this system sensor (*omprF* and *ompC*) were not identified (data not shown). This genome is still in the draft stage, so the presence of *ompF* and *ompR* cannot be completely ruled out. In addition, the identity of the cloned sequence was 99 % with a osmolarity sensor protein EnvZ in *Mesorhizobium mediterraneum* (accession number (WP_095484204) and 94 % with *Mesorhizobium ciceri* (accession number WP_029349880).

Evaluation of the symbiotic performance

To evaluate whether extra *envZ* copies could increase the symbiotic effectiveness of mesorhizobia strains, a chickpea pot assay was performed (Table 2). Despite the fact that *envZ* overexpression did not change the number of nodules develop on chickpea plants for any of the five strains tested, the symbiotic effectiveness (SE) was improved for PMI6pRKenvZ and EE7pRKenvZ with an increase of 64 % and 96 %, respectvely. SDW, which is used to calculate the SE, was significantly higher in plants inoculated with PMI6pRKenvZ and EE7pRKenvZ, than in those inoculated with PMI6pRK415 and EE7pRK415 or the corresponding wild type strains. From these two strains, only EE7pRKenvZ showed statistical differences in the average weight per nodule (AWN), which was higher in plants inoculated with EE7pRKenvZ, when compared to plants inoculated with the corresponding strain harboring pRK415. Nodules from plants inoculated with ST2pRKenvZ also showed a significant increase on AWN, yet in this case, no differences on SE or shoot dry weight (SDW) were detected. The results obtained for ST-2 and its transformed derivatives showed that an increase on AWN is not directly associated to an improvement of SE, since plants inoculated with ST2pRKenvZ showed the highest increase in the AWN and yet its SE was not significantly higher. In terms of root dry weight, significantly higher values were obtained in plant inoculated with EE-7, PMI-6 and ST-2 strains overexpressing *envZ*, when compared to the corresponding strains harboring pRK415.

In addition, overexpressing *envZ* on UPM-Ca36^T and V-15b strains did not improved any of the parameters analyzed on chickpea pot assay. Taking these results in account, further analyses were performed using only EE-7 and PMI-6 strains and their respective transformed strains.

Table 2 Results obtained from pot assays of chickpea plants inoculated with wild type or transformed strains.

Strain	SDW (g)	RDW (g)	NN	AWN (mg)	SE (%)
V-15b	0.675 ± 0.057 a	0.369 ± 0.037 a	94 ± 17 a	0.949 ± 0.232 a	29.78 ± 5.57 a
V15bpRK415	0.699 ± 0.126 a	0.335 ± 0.078 a	67 ± 35 a	1.567 ± 0.493 a	32.22 ± 12.4 a
V15bpRKenvZ	0.756 ± 0.099 a	0.432 ± 0.062 a	73 ± 25 a	1.332 ± 0.277 a	37.78 ± 9.71 a
UPM-Ca36^T	0.801 ± 0.0960 a	0.356 ± 0.023 a	55 ± 21 a	2.036± 0.797 a	42.19 ± 9.5 a
Ca36pRK415	0.789 ± 0.191 a	0.342 ± 0.080 a	57 ± 17 a	1.994± 0.574 a	41.07 ± 18.8 a
Ca36pRKenvZ	0.848 ± 0.081 a	0.296 ± 0.048 a	77 ± 8 a	1.333± 0.122 a	46.78 ± 8 a
ST-2	1.537 ± 0.207 a	0.512 ± 0.058 ab	57± 6 a	2.495 ± 0.583 b	42.06 ± 10.1 a
ST2pRK415	1.469 ± 0.215 a	0.478 ± 0.075 b	52 ± 13 a	2.546 ± 0.704 b	38.76 ± 10.5 a
ST2pRKenvZ	1.724 ± 0.265 a	0.603 ± 0.047 a	47 ± 9 a	3.754 ± 0.514 a	51.15 ± 12.9 a
PMI-6	1.174 ± 0.080 b	0.490 ± 0.057 ab	69 ± 10 a	2.305 ± 0.262 a	24.29 ± 3.9 b
PMI6pRK415	1.325 ± 0.254 b	0.436 ± 0.071 b	66 ± 29 a	2.411 ± 0.983 a	31.78 ± 12.3 b
PMI6pRKenvZ	1.728 ± 0.178 a	0.527 ± 0.044 a	55 ± 19 a	3.405 ± 1.643 a	51.40 ± 8.6 a
EE-7	0.629 ± 0.131 b	0.321 ± 0.036 b	53 ± 9 a	1.293 ± 0.340 ab	25.3 ± 12.9 b
EE7pRK415	0.680 ± 0.083 b	0.319 ± 0.022 b	65 ± 17 a	1.099 ± 0.324 b	30.3 ± 8.2 b
EE7pRKenvZ	0.973 ± 0.055 a	0.556 ± 0.069 a	63 ± 12 a	1.707 ± 0.294 a	59.1 ± 5.4 a

Different letters in the same group of strains indicate statistically significant differences (*P<0,05*). SDW - Shoot Dry Weight; RDW - Root Dry Weight; NN - Number of Nodules; AWN - Average Weight per Nodule; SE - Symbiotic Effectiveness

Bacterial growth curves

To characterize the growth kinetics of the transformed strains, bacterial growth was evaluated on liquid media (Fig. 1). On the first 48 hours, wild type and PMI6pRKenvZ strains growth is indistinguible, whereas the strain harboring the pRK415 plasmid already shows a slower growth (Fig 1A). From this point onwards, the strain with extra *envZ* copies begins to outgrow the growth of the other two strains, reaching the stationary phase at approx. the same time as the other strains (72 hours), yet with a much higher OD value.

In the case of EE-7 and its derivatives, the three growth curves were very similar, with the stationary phase also starting at approx. 72 h of growth (Fig 1B).

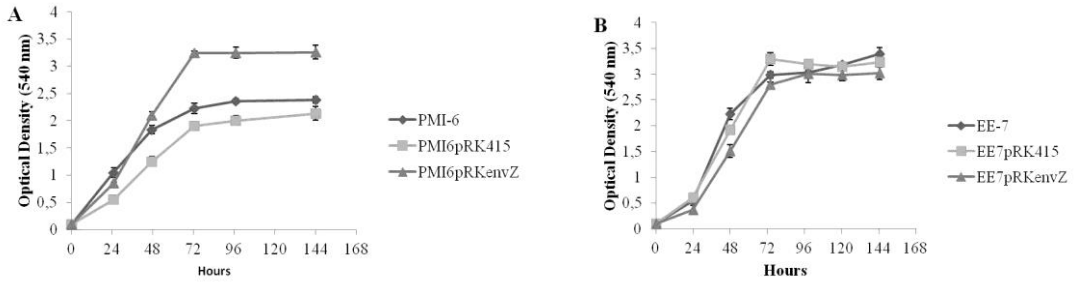

Figure 1. Growth curves in TY medium of A – PMI-6, PMI6pRK415 and PMI6pRKenvZ and B – EE-7, EE7pRK415 and EE7pRKenvZ. Significant differences were found between the growth of PMI6pRK415 and PMI6pRKenvZ strains from 24 hours onwards (*P <0.05*), using T-test. Bars represent standard deviation.

Evaluation of nodulation kinetics

In order to evaluate the effects of extra-*envZ* copies in the rate of nodules formation, the *Mesorhizobium* strains for which a SE improvement was detected previously were used in an assay with chickpea plants conducted in hydroponic conditions (Fig 2). Seedlings were inoculated with PMI6pRK415 or PMI6pRKenvZ and EE7pRK415 or EE7pRKenvZ. Plants inoculated with PMI6pRKenvZ showed the first nodules at 10 days after inoculation (DAI), while chickpea plants inoculated with PMI6pRK415 showed the first nodules only at 14 DAI. Although there is a significantly higher number of nodules in plants inoculated with PMI6pRK415 in an early phase (namely 17 and 19 DAI), at 28 DAI the strain harboring extra *envZ* copies began to show a significantly higher number of nodules and the values are kept higher until the end of the experiment (Fig 2A). Through visual inspection of nodules formed by both strains, it can be observed that many nodules from plants inoculated with PMI6pRK451 were greenish, which is a typical coloration of senescence nodules, inactive in terms of nitrogen fixation (Fig 3A1 and A2). On the other hand, most of the nodules in plants inoculated with PMI6pRKenvZ showed a reddish interior, which results from the accumulation of active leghemoglobin, and many of them were larger than those from plants inoculated with empty vector strain (Fig 3E1 and E2).

The time-course of nodule formation was also evaluated in plants inoculated with EE7pRK415 and EE7pRKenvZ, in order to verify whether the heterologous *envZ* gene could improve nodule number or nodule development rate. Although as a trend, plants inoculated with EE7pRKenvZ strain showed more nodules than plants inoculated with EE7pRK451, this difference is not statistically significant (Fig 2 B). Based on visual inspection, nodules resulting from both strains inoculation seemed very similar (data not shown).

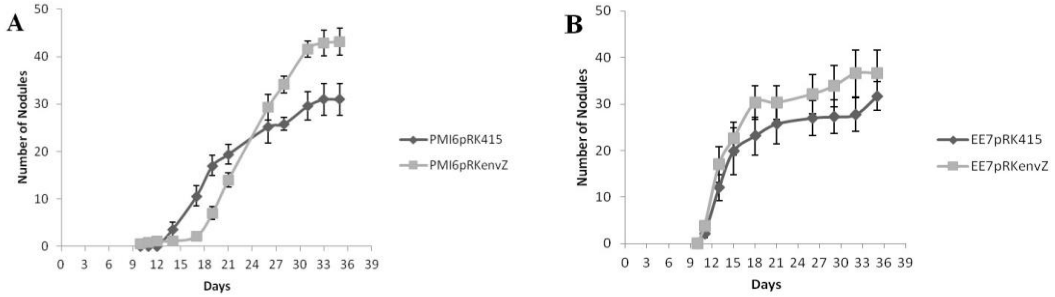

Figure 2. Nodulation kinetics of chickpea plants. Average Number of nodules for plants inoculated with A - PMI6pRK415 and PMI6pRKenvZ strains. B - EE7pRK415 and EE7pRKenvZ strains. Bars represent standard error.

Histological analysis of nodules

In order to verify possible differences in the histology of nodules resulting from the inoculation of chickpea plants with PMI6pRK415 and PMI6pRKenvZ, sections of nodules embedded in paraffin were stained and observed under bright-field microscopy. All nodules analyzed presented the expected zones of effective indeterminate nodule with meristematic, infection and fixation zones (Fig 3B and F). However, PMI6pRK415-induced nodules had a larger senescence zone (black arrow in Fig 3B and D) than those induced by PMI6pRKenvZ (Fig. 3F). In addition, nodules harboring the strain with extra *envZ* copies showed a higher number of bacteroid-occupied cells and each invaded plant cell shows a larger area occupied by differentiated bacteria than in nodules induced by the PMI6pRK415 strain (Fig 3 C and G).

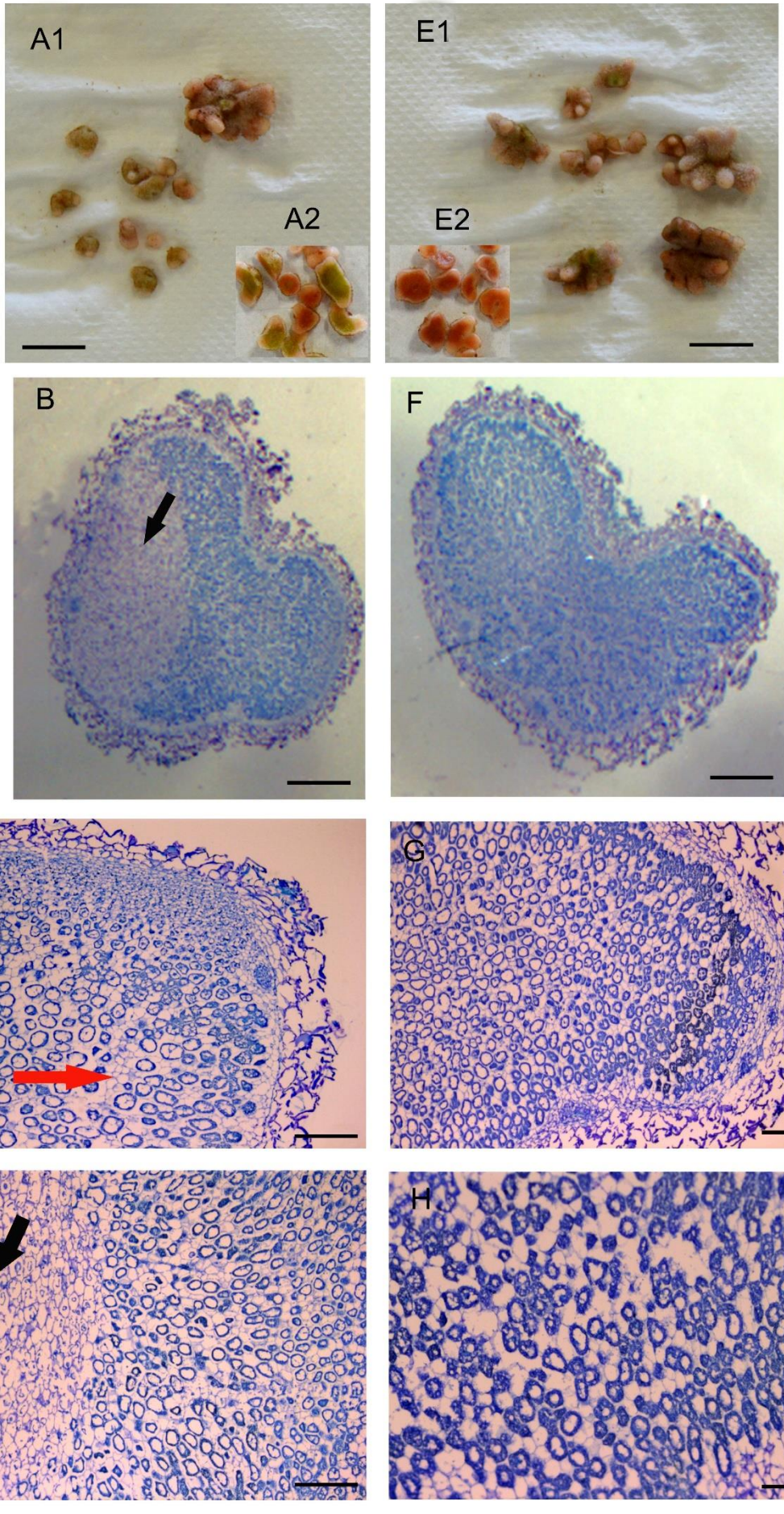
A1
A2
E1
E2
B
F
C
G
D
H

Figure 3. Nodules from chickpea plants 35 DAI. A1 - Nodules from plants inoculated with PMI6pRK415; A2 - Nodule section from plants inoculated with PMI6pRK415; B, C and D - Nodules sectioned and stained with toluidine blue from plants inoculated with PMI6pRK415; E1 - Nodules from plants inoculated with PMI6pRKenvZ; E2 - Nodule section from plants inoculated with PMI6pRKenvZ; F, G and H - Nodules sectioned and stained with toluidine blue form plants inoculated with PMI6pRKenvZ; B and D - Black arrows indicate senescent zone; C - Red arrow indicates uninfected cells. Scale bars: 1 cm (A1 and E1); 800 µm (B and F); 400 µm (C and G); 200 µm (D and H).

Colonization and infection thread

In order to verify whether the higher number of nodules observed in the hydroponic trial in plants inoculated with PMI6pRKenvZ and EE7pRKenvZ strains relied on a higher efficiency of these strains in the first stages of the interaction with plants roots, their infection processes were compared with those of the respective strains harboring the empty vector (PMI6pRK415 and EE7pRK415). All strains were transformed with an additional plasmid encoding the green fluorescent protein gene (*gfp)* and analyzed using confocal microscopy. At four days after inoculation, no differences were detected between chickpea seedlings inoculated with EE7pRK415GFP and EE7pRKenvZGFP strains, regarding important infection parameters such as curling, formation of infection threads and formation of caps in the presence of curling and infection threads (Fig 4A and B). On the other hand, also at four days after inoculation, chickpea seedlings inoculated with the PMI6pRKenvZGFP strain showed a higher number of infection threads than roots inoculated with PMI6pRK415GFP (Fig 4D and E). In addition, colonization of the surface of the roots seemed more efficient for the strain with extra *envZ* copies than for PMI6pRK415GFP.

In order to performed confocal microscopy analysis of chickpea roots simultaneously inoculated with the strain overexpressing *envZ* and the corresponding strain harboring the empty vector, strains PMI6pRK415 and EE7pRK415 were transformed with an additional plasmid carrying the *rfp* gene (red fluorescent protein). Although no differences were detected in the infection process when EE-7 derivative strains were compared separately (Fig 4A and B), upon co-inoculation, a higher amount of green-tagged bacteria (EE7pRKenvZGFP) was observed in intracellular zones and in infection threads, suggesting a higher efficiency on these early symbiosis processes of the strain with extra *envZ* copies (Fig 4C). Similar observations resulted from the analysis of co-inoculation of PMI6pRKenvZGFP and PMI6pRKK415RFP strains (Fig 4F), in agreement with the results obtained by the single inoculation analysis. Since strains were

inoculated in a 1:1 mixture, this analysis seems to indicate that competitiveness at root-hair infection stage is higher in the strains overexpressing *envZ*.

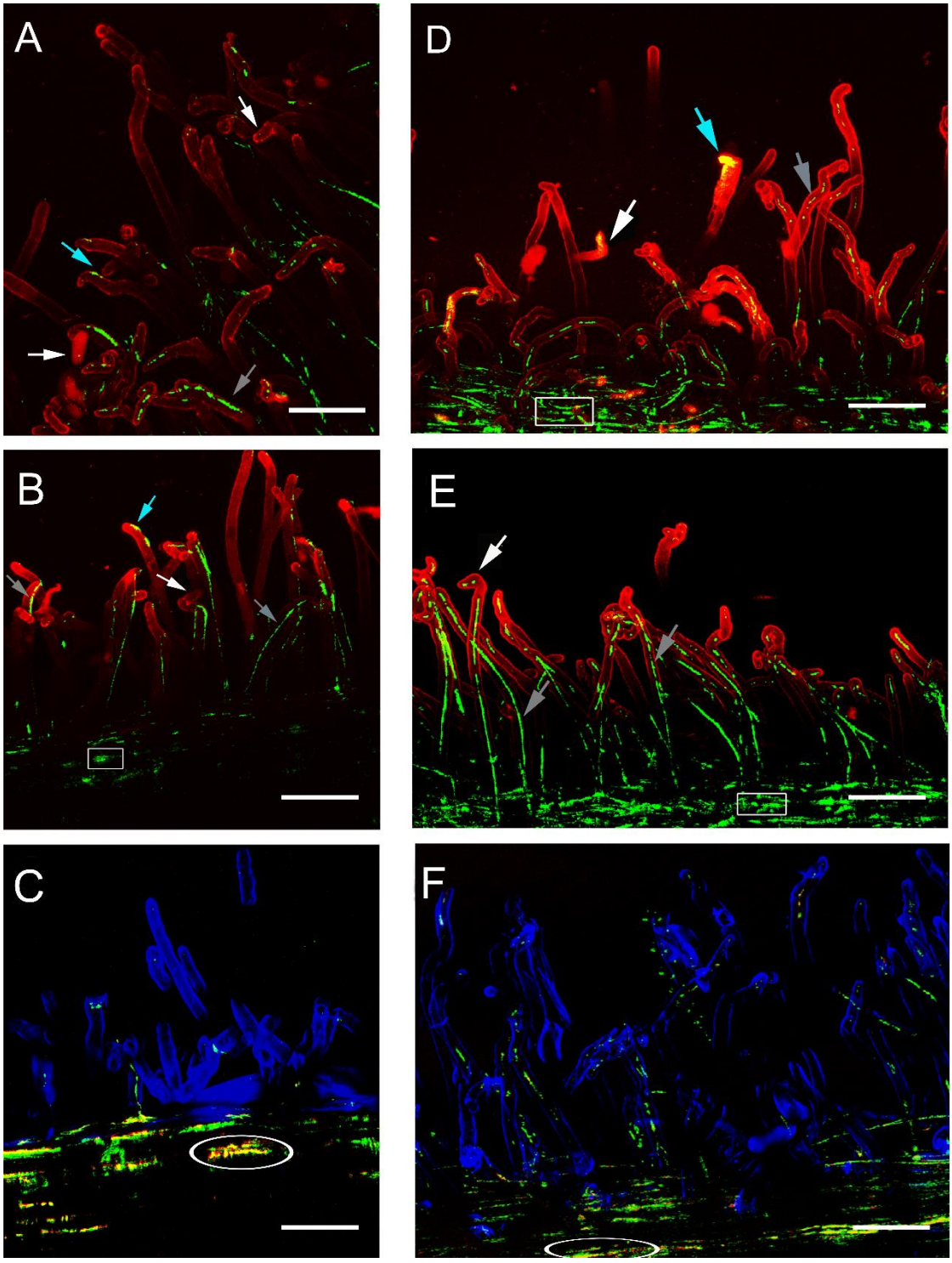

Figure 4. Confocal laser scanning micrographs showing the initial infection process of chickpea roots inoculated with green and red fluorescent protein–tagged rhizobia. Single inoculation: A - EE7pRK415, B - EE7pRKenvZ, D - PMI6pRK415 and E - PMI6pRKenvZ

strains tagged with green fluorescent protein and chickpea roots stained with propidium iodide, at 4 days after inoculation. Co-inoculation: C - EE7pRK415 (red) and EE7pRKenvZ (green) and F - PMI6pRK415 (red) and PMI6pRKenvZ (green) strains co-inoculated in chickpea roots stained with calcofluor white, at 6 days after inoculation. Root hair curling (white arrows); infection threads (gray arrows); caps on root hairs tips (blue arrows), rhizobial attachment on roots (square) and empty vector strains sharing the same intercellular space with those harboring pRKenvZ (ellipses) are shown. Scale bars: 75 μm (A, B, D and E); 50 μm (C and F).

Exopolysaccharides and induction of root hairs formation

A plate assay was performed in order to verify whether *envZ* overexpression influences the mucoid phenotype, which is typical of most rhizobia. When growing in yeast mannitol agar (YMA), strain PMI6pRKenvZ showed a stronger mucoid phenotype than strain PMI6pRK415 (Fig 5A1 and A2) or the wild type strain (data not shown). Despite the fact that the EE-7 strain is naturally very mucoid, the strain transformed with extra copies of *envZ* showed a more accentuated mucoid phenotype than the empty vector strain (data not shown), although this change is not as evident as in the case of PMI-6.

In order to evaluate if the *envZ*-extra copies affected the initial root development of chickpea plants, pre-germinated seedlings were inoculated in water-agar plates. Interestingly, plants inoculated with PMI6pRKenvZ showed a much higher density of root hairs than plants inoculated with PMI6pRK415 (Fig 5B1 and B2). In addition, the number of lateral roots was also higher in plants inoculated with PMI6pRKenvZ (Fig 5C1 and C2). These differences were not detected for the same analysis performed with the modified EE-7 strains (data not shown).

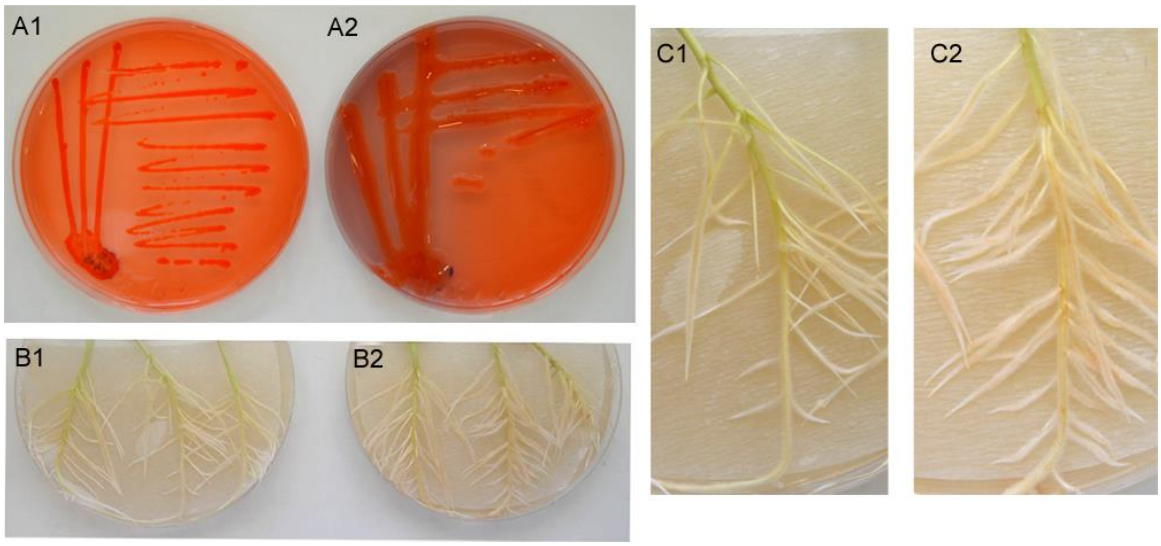

Figure 5. A – Mucoid phenotype by modified strains in YMA plates. A1 - PMI6pRK415 and A2 - PMI6pRKenvZ; B - Root hair development in chickpea plants grown in water agar medium for seven days after inoculation with B1 - PMI6pRK415 and B2 - PMI6pRKenvZ; C - Secondary roots development after inoculation with C1 - PMI6pRK415 and C2 - PMI6pRKenvZ.

5.5. Discussion

The EnvZ/OmpR two-component regulatory system regulates expression of outer membrane proteins (porins) in response to osmotic stress (Alphen and Lugtenberg 1977; Wang et al. 2012). In addition, it has been shown that mutations in *envZ* or *ompR* alter the expression levels of several other genes related with a large range of functions, as for example genes related to virulence in pathogens, fatty acid uptake, exopolysaccharide (EPS) production, peptide transportation and flagella production (Bernardini et al. 1990; Li et al. 2014; Yuan et al. 2011).

With the aim of evaluating the effects on the symbiotic performance of extra *envZ* copies, several chickpea mesorhizobia strains were modified in order to overexpress the *envZ* gene from *M. mediterraneum* UPM-Ca36^{T}.

As a first approach, to evaluate if the symbiotic parameters were improved, a pot assay was performed using chickpea plants inoculated with five mesorhizobia wild types and the corresponding modified strains. Only two strains, PMI6pRKenvZ and EE7pRKenvZ, showed a significant improvement in the symbiotic effectiveness, compared to the respective wild type and strain harboring the pRK415 plasmid. Plants inoculated with these two strains showed a higher shoot and root dry weights. Furthermore, EE7pRKenvZ induced the development of nodules with a higher average weight per nodule, compared to EE7pRK415. Previous studies revealed that

EnvZ/OmpR may regulate secretion system (SS) genes in pathogenic bacteria, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Erwinia amylovora* and *Pseudomonas syringae* (Brzostek et al. 2007; Feng et al. 2003; Feng et al. 2004; Li and Zhao 2011). Although there is no report showing the EnvZ/OmpR relation with secretion system in rhizobia, it is known that different types of SS are present in this group of bacteria and their importance in the symbiosis has been previously shown (Hubber et al. 2007; Okazaki et al. 2013; Viprey et al. 1998). For example, mutation of several T4SS genes in *M. loti* delayed nodulation on *Lotus corniculatus* (Hubber et al. 2004). *vir* mutants in *M. loti* inoculated in *Leucaena leucocephala* formed effective nodules, whereas wild type strain formed empty and small nodules. It may suggest that the SE improvement of the strains overexpression *envZ* could be due the possible regulation of the SS by EnvZ/OmpR in rhizobia.

Despite the general trend of a higher shoot dry weight for plants inoculated with the mesorhizobia strains overexpressing *envZ*, no significant differences were detected for three of the tested strains, when compared to the corresponding strains harboring the empty vector, namely V-15b, ST-2 and UPM-Ca36^T. It is noteworthy that the homologous overexpression of *envZ* in *M. mediterraneum* UPM-Ca36^T did not significantly affect the plant and nodule parameters evaluated.

In order to investigate other aspects of the symbiosis that might be affected by the presence of *envZ* extra copies, further studies were performed with EE-7 and PMI-6 strains and its transformed derivatives.

Growth curves were performed to characterize the growth kinetics of PMI-6 and EE-7 transformed strains. PMI6pRK415 strain showed a similar growth curve to the wild type strain, although with slightly lower optical density values. This was expected because the size and copy number of the plasmid could slow down the DNA replication process. Interestingly, even with the expression plasmid, the PMI6pRKenvZ strain showed a more accelerated growth than wild type and PMI6pRK415 strains. In addition, PMI-6 strain harboring pRKenvZ reached late exponential and stationary phases with higher OD values than those of the wild type and empty vector strains. These higher OD values are probably a consequence of the stronger mucoid phenotype observed for PMI6pRKenvZ. According the differences in the growth curves among the EE-7 strains were not as marked as those observed in the PMI-6 strains and the alteration on the mucoid phenotype on EE-7 strains was much less pronounced.

A successful rhizobia penetration through the infection thread requires both the continued biosynthesis of Nod factors and the synthesis of symbiotic exopolysaccharides (EPS) (Jones et al. 2007; Klein et al. 1988). EPS may be important in lowering the legume immune response during rhizobia invasion (Jones 2012; Jones et al. 2008;

Pellock et al. 2000). Since the mucoid phenotype indicates the ability of rhizobia to produce EPS (Milner et al. 1992; Sayyed et al. 2011; Staehelin et al. 2006), the highest mucoid phenotype observed in bacteria transformed with extra *envZ* copies may have improved the ability of these bacteria to infect the root hairs. A higher EPS production might be a particularly important factor accounting for the symbiotic effectiveness improvement detected for PMI6pRKenvZ, since the difference in the EPS production observed between EE-7 transformed strains was not as expressive as those observed for the PMI-6 transformed ones. Geddes et al. (2014) showed that the EPS production in response to medium acidification in *S. meliloti* is correlated with an increase in competition for nodule occupancy. *S. meliloti* 1021 overexpressing *exoR*, which encodes the enzyme responsible for the first step in succinoglycan biosynthesis, enhanced the symbiosis with *Medicago truncatula* (Jones 2012). In addition *R. leguminosarum* bv, *trifolii* strains overproducing EPS increased the shoot fresh and dry weight, the number of nodules on clovers roots and nodule occupancy (Janczarek et al. 2009). In *S. meliloti*, ExoS is a sensor kinase that together with the response regulator ChvI, functions as the positive regulator for synthesis of succinoglycan, an exopolysaccharide involved in the infection process (Wells et al. 2007) and this ExoS-ChvI two-component regulatory system is a member of the EnvZ-OmpR family (Cheng and Walker 1998). Previous studies have reported that the EnvZ/OmpR tow-component system is related with EPS production (Li et al. 2014; Pickard et al. 1994), therefore probably this system is involved in the EPS production in rhizobia, as well.

Root hair density is known to be influenced by redox conditions and phytohormones (Considine and Foyer 2014; Zhang et al. 2016) In addition, Laus et al. (2005) showed that *Rhizobium leguminosarum* EPS-deficient elicited small root hair in *Vicia sativa* plants. Therefore, the higher EPS production detected for PMI6pRKenvZ is likely to account for the higher density of root hair and secondary roots observed in plants inoculated with this strain.

With the aim of evaluating nodule development in a time-course approach, chickpea plants were inoculated and grown in hydroponic conditions. Although the previous pot assay showed no difference between the number of nodules in chickpea plants inoculated with PMI6pRKenvZ compared to plants inoculated with PMI6pRK415, the hydroponic conditions showed that the number of nodules is significantly higher in plants inoculated with the strain overexpressing *envZ* in the later period of the experiment. This apparent discrepancy between the results of two different plant assays is most likely due the shorter duration of the hydroponic assay (5 weeks), compared with the pot assay (8 weeks). These results suggest that there might be a transient higher number of nodules in plants inoculated with PMI6pRKenvZ, which may benefit the plant

in terms of N-fixation, yet in a later stage of the symbiosis that difference is not detected any longer. Besides nodule number, nodule coloration both outside and inside was inspected in nodules belonging to the several treatments. Plants inoculated with PMI6pRKenvZ showed nodules with a reddish interior, which results from the accumulation of active leghemoglobin (Downie 2005), and many of them were larger than those from plants inoculated with empty vector strain. On the other hand, most of the nodules observed in plants inoculated with the strain PMI6pRK451 were greenish, which is a typical coloration of senescent and Fix^- nodules (Roponen 1970; Van de Velde et al. 2006). Histological analysis of theses nodules confirmed a larger senescent zone and more empty cells in the nodules from plants inoculated with PMI6pRK451, while nodules resulting from PMI6pRKenvZ inoculation showed infection zones with more bacteroids. A typical senescent zone is characterized by degeneration of both symbionts that happens in the end of the fixation zone. This degradation indicates that the symbiotic interaction has finished (Timmers et al. 2000). Although these results indicate the contribution of additional *envZ* copies to the development of nodules that are more effective in fixing atmospheric nitrogen and providing these N-compounds to the host plant.

The nodulation kinetics was also evaluated in plants inoculated with EE-7 strains harboring pRK415 or pRKenvZ plasmids, for 35 days after inoculation. Although plants inoculated with EE7pRKenvZ show more nodules during the whole experiment, this difference is statistically not significant, corroborating the results in pots assay. The coloration of the interior of the nodules from both treatments was similar (light red). In this case, the improvement of SE observed in the pot assay is probably not related with the formation of more effective nodules, as seems to happen in the PMI-6 modified strain. Thus, these results suggest that extra *envZ* copies might improve the SE by different mechanisms in the two studied mesorhizobia strains.

In order to investigate in more detail the early steps of the infection process, the modified strains were transformed with fluorescent proteins (*gfp* and *rfp*) genes, inoculated in pre-germinated chickpea seeds and observed under confocal microscopy. Roots inoculated with PMI6pRKenvZGFP showed a much higher number of infection threads than the ones inoculated with the corresponding strain harboring pRK415. Analysis of co-inoculation of PMI6pRKenvZGFP and PMI6pRK415RFP also confirmed that the number of infection threads and attached bacteria on the intracellular zone were higher for the strain with extra *envZ* copies. These results suggest that EnvZ may contribute to a more effective infection and this difference at the beginning of the symbiosis process in PMI-6 may account for the higher number of nodules observed in hydroponic conditions. Upon single inoculation, no change in the number of infection

threads was detected for EE7pRKenvZ, when compared with the empty vector strain. Nevertheless, upon co-inoculation it seems that there is a higher density of bacterial cells modified with *envZ* gene attached on the intracellular zone. Several studies have shown that EnvZ/OmpR system may influence the bacteria motility, biofilm formation and flagella production (Kim et al. 2003; Li et al. 2014; Prigent-Combaret et al. 2001; Prüβ 2017; Tipton and Rather 2017; Yuan et al. 2011). Zheng et al (2015) showed that the flagellar-dependent motility in *Mesorhizobium tianshanense* is involved in the early stage of plant host interaction and a mutation causing the loss of the swimming ability lead to decreased bacterial attachment on the root hair. Others studies showed that motile bacteria are more efficient in nodulation (Soby and Bergman 1983) or more competitive on the plant root (Caetano-Anolles et al. 1988; Mellor et al. 1987) than non-motile ones. Therefore, extra *envZ* copies in PMI-6 and EE-7 could have improved their motility abilities and consequently, enhanced the infection and competitiveness of these strains. Nevertheless, this improvement in the early steps of infection does not appear to be as marked in EE7pRKenvZ and perhaps it is related with the less EPS production observed in this strain.

Extra copies of *envZ* in PMI-6 strain improved some important parameters, such as colonization and infection, number of nodules in an earlier phase of the symbiosis, mucoid phenotype (EPS) and root hair development. These same parameters evaluated in EE7pRKenvZ and EE7pRK415 did not reveal clear differences between these two strains that could account for the higher SE obtained for the strain with extra *envZ* copies, suggesting that EnvZ may influence the symbiosis process by different mechanisms.

This study represents the first report on the involvement of the EnvZ/OmpR two-component signal transduction system in the symbiosis process. Nevertheless, ExoS/ChvI, another two-component system from the same family, has been previously reported to be essential to the establishment of an effective symbiosis in *S. meliloti* (Bélanger et al. 2009; Chen et al. 2009; Soto et al. 2006). It regulates the succinoglycan (EPS I) production and flagella biosynthesis (Cheng and Walker 1998; Yao et al. 2004). A *S. meliloti exoS* mutant overproduced EPS I and did not synthesize flagella, loss of ability of swarming and swimming. Furthermore, reduced the efficiency of root hair colonization of alfalfa (Yao et al. 2004). Overall, these results suggest the involvement of TCRS in the symbiotic performance and indicate that EnvZ could be involved with the infection process and affect the EPS production in some mesorhizobia strains. As *envZ* overexpression led to different symbiotic phenotypes in the tested strains, it seems that these effects are strain dependent. In addition, for the two strains that showed a significant increase in the symbiotic effectiveness, EnvZ seems to act through different

pathways. More studies are required to better understand the exact mechanism affected by EnvZ that cause the reported phenotypes.

5.6. References

Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., & Oliveira, S. (2009). A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microbial Ecology, 58*, 930-941.

Alphen, W. V., & Lugtenberg, B. (1977). Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J Bacteriol, 131*(2), 623-630.

Bélanger, L., Dimmick, K. A., Fleming, J. S., & Charles, T. C. (2009). Null mutations in *Sinorhizobium meliloti exoS* and *chvI* demonstrate the importance of this two-component regulatory system for symbiosis. *Molecular Microbiology, 74*(5), 1223-1237. doi: 10.1111/j.1365-2958.2009.06931.x

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology, 84*(1), 188-198.

Bernardini, M. L., Fontaine, A., & Sansonetti, P. J. (1990). The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. *J Bacteriol, 172*(11), 6274-6281.

Bloemberg, G. V., Wijfjes, A. H., Lamers, G. E., Stuurman, N., & Lugtenberg, B. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microbe Interact, 13*(11), 1170-1176. doi: 10.1094/mpmi.2000.13.11.1170

Boor, K. J. (2006). Bacterial Stress Responses: What Doesn't Kill Them Can Make Them Stronger. *PLoS Biology, 4*(1), e23.

Brígido, C., Alexandre, A., & Oliveira, S. (2012). Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia. *Microbiol Res, 167*(10), 623-629.

Brígido, C., Robledo, M., Menendez, E., Mateos, P. F., & Oliveira, S. (2012). A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. *Molecular Plant-Microbe Interactions, 25*(12), 1594-1604. doi: 10.1094/mpmi-05-12-0140-r

Broughton, W. J., & Dilworth, M. J. (1971). Control of Leghaemoglobin Synthesis in Snake Beans. *Biochemical Journal, 125*(4), 1075-1080.

Brzostek, K., Brzóstkowska, M., Bukowska, I., Karwicka, E., & Raczkowska, A. (2007). OmpR negatively regulates expression of invasin in *Yersinia enterocolitica*. *Microbiology, 153*(8), 2416-2425. doi: doi:10.1099/mic.0.2006/003202-0

Caetano-Anolles, G., Wall, L. G., De Micheli, A. T., Macchi, E. M., Bauer, W. D., & Favelukes, G. (1988). Role of Motility and Chemotaxis in Efficiency of Nodulation by *Rhizobium meliloti*. *Plant Physiol, 86*(4), 1228-1235.

Chen, E. J., Fisher, R. F., Perovich, V. M., Sabio, E. A., & Long, S. R. (2009). Identification of Direct Transcriptional Target Genes of ExoS/ChvI Two-Component Signaling in *Sinorhizobium meliloti*. *J Bacteriol, 191*(22), 6833-6842. doi: 10.1128/jb.00734-09

Cheng, H.-P., & Walker, G. C. (1998). Succinoglycan Production by *Rhizobium meliloti* Is Regulated through the ExoS-ChvI Two-Component Regulatory System. *J Bacteriol, 180*(1), 20-26.

Considine, M. J., & Foyer, C. H. (2014). Redox Regulation of Plant Development. *Antioxidants & Redox Signaling, 21*(9), 1305-1326. doi: 10.1089/ars.2013.5665

Downie, J. A. (2005). Legume Haemoglobins: Symbiotic Nitrogen Fixation Needs Bloody Nodules. *Current Biology, 15*(6), R196-R198. doi: http://dx.doi.org/10.1016/j.cub.2005.03.007

Feng, X., Oropeza, R., & Kenney, L. J. (2003). Dual regulation by phospho-OmpR of *ssrA*/*B* gene expression in *Salmonella* pathogenicity island 2. *Mol Microbiol, 48*(4), 1131-1143.

Feng, X., Walthers, D., Oropeza, R., & Kenney, L. J. (2004). The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. *Mol Microbiol, 54*(3), 823-835. doi: 10.1111/j.1365-2958.2004.04317.x

Finan, T. M., Kunkel, B., De Vos, G. F., & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol, 167*(1), 66-72.

Flores-Félix, J., Menéndez, E., Marcos-García, M., Celador-Lera, L., & Rivas, R. (2015). Calcofluor white, an Alternative to Propidium Iodide for Plant Tissues Staining in Studies of Root Colonization by Fluorescent-tagged Rhizobia. *Journal of Advances in Biology & Biotechnology, 2*(1), 65-70. doi: 10.9734/jabb/2015/12444

Foo, Y. H., Gao, Y., Zhang, H., & Kenney, L. J. (2015). Cytoplasmic sensing by the inner membrane histidine kinase EnvZ. *Prog Biophys Mol Biol, 118*(3), 119-129. doi: 10.1016/j.pbiomolbio.2015.04.005

Gao, H., Zhang, Y., Han, Y., Yang, L., Liu, X., Guo, Z., . . . Yang, R. (2011). Phenotypic and transcriptional analysis of the osmotic regulator OmpR in *Yersinia pestis*. *BMC Microbiol, 11*, 39-39. doi: 10.1186/1471-2180-11-39

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M. T., . . . Velázquez, E. (2012). *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. *PLoS One, 7*(5), e38122. doi: 10.1371/journal.pone.0038122

Geddes, B. A., Gonzalez, J. E., & Oresnik, I. J. (2014). Exopolysaccharide production in response to medium acidification is correlated with an increase in competition for nodule occupancy. *Mol Plant Microbe Interact, 27*(12), 1307-1317. doi: 10.1094/mpmi-06-14-0168-r

Gibson, A. H. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. In G. H. Elkan (Ed.), *Symbiotic Nitrogen Fixation Technology* (pp. 321-363). New York: Marcel Dekker, Inc.

Hubber, A., Vergunst, A. C., Sullivan, J. T., Hooykaas, P. J., & Ronson, C. W. (2004). Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7A VirB/D4 type IV secretion system. *Mol Microbiol, 54*(2), 561-574. doi: 10.1111/j.1365-2958.2004.04292.x

Hubber, A. M., Sullivan, J. T., & Ronson, C. W. (2007). Symbiosis-induced cascade regulation of the *Mesorhizobium loti* R7A VirB/D4 type IV secretion system. *Mol Plant Microbe Interact, 20*(3), 255-261. doi: 10.1094/mpmi-20-3-0255

Janczarek, M., Jaroszuk-Ściseł, J., & Skorupska, A. (2009). Multiple copies of *rosR* and *pssA* genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*. *Antonie van Leeuwenhoek, 96*(4), 471-486. doi: 10.1007/s10482-009-9362-3

Jones, K. M. (2012). Increased production of the exopolysaccharide succinoglycan enhances *Sinorhizobium meliloti* 1021 symbiosis with the host plant *Medicago truncatula*. *J Bacteriol, 194*(16), 4322-4331. doi: 10.1128/jb.00751-12

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., & Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat Rev Microbiol, 5*(8), 619-633. doi: 10.1038/nrmicro1705

Jones, K. M., Sharopova, N., Lohar, D. P., Zhang, J. Q., VandenBosch, K. A., & Walker, G. C. (2008). Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. *Proc Natl Acad Sci U S A, 105*(2), 704-709. doi: 10.1073/pnas.0709338105

Keen, N. T., Tamaki, S., Kobayashi, D., & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene, 70*(1), 191-197. doi: 10.1016/0378-1119(88)90117-5

Kim, D.-j., Boylan, B., George, N., & Forst, S. (2003). Inactivation of *ompR* Promotes Precocious Swarming and *flhDC* Expression in *Xenorhabdus nematophila*. *J Bacteriol, 185*(17), 5290-5294. doi: 10.1128/jb.185.17.5290-5294.2003

Klein, S., Hirsch, A. M., Smith, C. A., & Signer, E. R. (1988). Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. *Mol Plant Microbe Interact, 1*(2), 94-100.

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W., & Oliveira, S. (2008). Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. *FEMS Microbiology Ecology, 66*(2), 391-400.

Laus, M. C., van Brussel, A. A. N., & Kijne, J. W. (2005). Role of cellulose fibrils and exopolysaccharides of *Rhizobium leguminosarum* in attachment to and infection of *Vicia sativa* root hairs. *Molecular Plant-Microbe Interactions, 18*(6), 533-538. doi: 10.1094/mpmi-18.0533

Li, W., Ancona, V., & Zhao, Y. (2014). Co-regulation of polysaccharide production, motility, and expression of type III secretion genes by EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora*. *Mol Genet Genomics, 289*(1), 63-75. doi: 10.1007/s00438-013-0790-4

Li, W., & Zhao, Y. (2011). Effect of EnvZ/OmpR and GrrS/GrrA systems on *Erwinia amylovora* virulence. *Phytopathology, 101*(6), S102-S102.

Mattison, K., & Kenney, L. J. (2002). Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *J Biol Chem, 277*(13), 11143-11148. doi: 10.1074/jbc.M111128200

Mellor, H. Y., Glenn, A. R., Arwas, R., & Dilworth, M. J. (1987). Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1. *Arch Microbiol, 148*(1), 34-39. doi: 10.1007/BF00429644

Mills, S. D., Ruschkowski, S. R., Stein, M. A., & Finlay, B. B. (1998). Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *ompR* and *envZ* mutants are defective for the formation of *Salmonella*-induced filaments. *Infect Immun, 66*(4), 1806-1811.

Milner, J. L., Araujo, R. S., & Handelsman, J. (1992). Molecular and symbiotic characterization of exopolysaccharide-deficient mutants of *Rhizobium tropici* strain CIAT899. *Molecular Microbiology, 6*(21), 3137-3147. doi: 10.1111/j.1365-2958.1992.tb01770.x

Nascimento, F. X., Brigido, C., Glick, B. R., Oliveira, S., & Alho, L. (2012a). *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. *Lett Appl Microbiol, 55*(1), 15-21. doi: 10.1111/j.1472-765X.2012.03251.x

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev, 67*(4), 593-656.

Nour, S. M., Cleyet-Marel, J.-C., Normand, P., & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *International Journal of Systematic Bacteriology, 45*(4), 640-648.

Okazaki, S., Kaneko, T., Sato, S., & Saeki, K. (2013). Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. *Proceedings of the National Academy of Sciences of the United States of America, 110*(42), 17131-17136. doi: 10.1073/pnas.1302360110

Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Sugiura, M., Wanner, B. L., . . . Mizuno, T. (2002). Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol, 46*(1), 281-291.

Pellock, B. J., Cheng, H. P., & Walker, G. C. (2000). Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J Bacteriol, 182*(15), 4310-4318.

Pickard, D., Li, J., Roberts, M., Maskell, D., Hone, D., Levine, M., . . . Chatfield, S. (1994). Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. *Infection and Immunity, 62*(9), 3984-3993.

Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P., & Dorel, C. (2001). Complex Regulatory Network Controls Initial Adhesion and Biofilm Formation in *Escherichia coli* via Regulation of *thecsgD* Gene. *J Bacteriol, 183*(24), 7213-7223. doi: 10.1128/jb.183.24.7213-7223.2001

Prüβ, B. M. (2017). Involvement of two-component signaling in bacterial motility and biofilm development. *J Bacteriol*. doi: 10.1128/jb.00259-17

Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F., & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis, 22*(6), 1086-1089.

Robledo, M., Jimenez-Zurdo, J. I., Soto, M. J., Velazquez, E., Dazzo, F., Martinez-Molina, E., & Mateos, P. F. (2011). Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial cellulase CelC2. *Mol Plant Microbe Interact, 24*(7), 798-807. doi: 10.1094/mpmi-10-10-0249

Roponen, I. (1970). The Effect of Darkness on the Leghemoglobin Content and Amino Acid Levels in the Root Nodules of Pea Plants. *Physiologia Plantarum, 23*(3), 452-460. doi: 10.1111/j.1399-3054.1970.tb06435.x

Sambrook, J., & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). New York: Cold Spring Harbor Laboratory.

Sayyed, R. Z., Jamadar, D. D., & Patel, P. R. (2011). Production of Exo-polysaccharide by *Rhizobium* sp. *Indian Journal of Microbiology, 51*(3), 294-300. doi: 10.1007/s12088-011-0115-4

Shin, S., & Park, C. (1995). Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. *J Bacteriol, 177*(16), 4696-4702.

Soby, S., & Bergman, K. (1983). Motility and Chemotaxis of *Rhizobium meliloti* in Soil. *Applied and Environmental Microbiology, 46*(5), 995-998.

Soto, M. J., Sanjuan, J., & Olivares, J. (2006). Rhizobia and plant-pathogenic bacteria: common infection weapons. *Microbiology, 152*(Pt 11), 3167-3174. doi: 10.1099/mic.0.29112-0

Staehelin, C., Forsberg, L. S., D'Haeze, W., Gao, M.-Y., Carlson, R. W., Xie, Z.-P., . . . Broughton, W. J. (2006). Exo-Oligosaccharides of *Rhizobium* sp. Strain NGR234 Are Required for Symbiosis with Various Legumes. *J. Bacteriol., 188*(17), 6168-6178.

Sugawara, M., Epstein, B., Badgley, B. D., Unno, T., Xu, L., Reese, J., . . . Sadowsky, M. J. (2013). Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. *Genome Biol, 14*(2), R17. doi: 10.1186/gb-2013-14-2-r17

Timmers, A. C., Soupene, E., Auriac, M. C., de Billy, F., Vasse, J., Boistard, P., & Truchet, G. (2000). Saprophytic intracellular rhizobia in alfalfa nodules. *Mol Plant Microbe Interact, 13*(11), 1204-1213. doi: 10.1094/mpmi.2000.13.11.1204

Tipton, K. A., & Rather, P. N. (2017). An *ompR-envZ* Two-Component System Ortholog Regulates Phase Variation, Osmotic Tolerance, Motility, and Virulence in *Acinetobacter baumannii* Strain AB5075. *J Bacteriol, 199*(3). doi: 10.1128/jb.00705-16

Tseng, T.-T., Tyler, B. M., & Setubal, J. C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. *BMC Microbiol, 9*(Suppl 1), S2-S2. doi: 10.1186/1471-2180-9-S1-S2

Van de Velde, W., Guerra, J. C. P., Keyser, A. D., De Rycke, R., Rombauts, S., Maunoury, N., . . . Goormachtig, S. (2006). Aging in Legume Symbiosis. A Molecular View on Nodule Senescence in Medicago truncatula. *Plant Physiol, 141*(2), 711-720. doi: 10.1104/pp.106.078691

Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M., & Lejeune, P. (1998). Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J Bacteriol, 180*(9), 2442-2449.

Viprey, V., Del Greco, A., Golinowski, W., Broughton, W. J., & Perret, X. (1998). Symbiotic implications of type III protein secretion machinery in *Rhizobium*. *Mol Microbiol, 28*(6), 1381-1389.

Wang, L. C., Morgan, L. K., Godakumbura, P., Kenney, L. J., & Anand, G. S. (2012). The inner membrane histidine kinase EnvZ senses osmolality via helix-coil transitions in the cytoplasm. *Embo j, 31*(11), 2648-2659. doi: 10.1038/emboj.2012.99

Wells, D. H., Chen, E. J., Fisher, R. F., & Long, S. R. (2007). ExoR is genetically coupled to the ExoS-ChvI two-component system and located in the periplasm of *Sinorhizobium meliloti*. *Molecular Microbiology, 64*(3), 647-664. doi: doi:10.1111/j.1365-2958.2007.05680.x

Yao, S. Y., Luo, L., Har, K. J., Becker, A., Ruberg, S., Yu, G. Q., . . . Cheng, H. P. (2004). *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both Succinoglycan and Flagellum production. *J Bacteriol, 186*(18), 6042-6049.

Yoshida, T., Cai, S., & Inouye, M. (2002). Interaction of EnvZ, a sensory histidine kinase, with phosphorylated OmpR, the cognate response regulator. *Mol Microbiol, 46*(5), 1283-1294.

Yuan, J., Wei, B., Shi, M., & Gao, H. (2011). Functional Assessment of EnvZ/OmpR Two-Component System in *Shewanella oneidensis*. *PLoS One, 6*(8), e23701. doi: 10.1371/journal.pone.0023701

Zhang, S., Huang, L., Yan, A., Liu, Y., Liu, B., Yu, C., . . . Gan, Y. (2016). Multiple phytohormones promote root hair elongation by regulating a similar set of genes in the root epidermis in Arabidopsis. *J Exp Bot, 67*(22), 6363-6372. doi: 10.1093/jxb/erw400

Zheng, H., Mao, Y., Teng, J., Zhu, Q., Ling, J., & Zhong, Z. (2015). Flagellar-Dependent Motility in *Mesorhizobium tianshanense* is Involved in the Early Stage of Plant Host Interaction: Study of an *flgE* Mutant. *Current Microbiology, 70*(2), 219-227. doi: 10.1007/s00284-014-0701-x

Chapter 6

General discussion

The conversion of atmospheric nitrogen into ammonia by symbiotic, associative and free-living bacteria, known as biological nitrogen fixation, is very important to the environment and food production. In agricultural systems, the biological nitrogen fixation process represents an alternative to the expensive nitrogen fertilizers, which are easily loss through leaching. The fixed nitrogen compensates the losses caused by the denitrification and represents an important part of the nitrogen cycle, replenishing the overall nitrogen content of the biosphere. In order to maintain crop production at high levels, nitrogen fertilizers are often supplied in large amounts. This increasing use of N-fertilizers, which constitutes the largest human interference in the nitrogen cycle, has prompted concerns regarding the emissions of nitrogen oxides, soil acidification and water eutrophication. As the world's population increases, the demand for sustainable food production has increased proportionally. As nitrogen generated by biological fixation is utilized *in situ*, it is less prone to leaching and volatilization and therefore this process contributes as an important and sustainable N-input into agriculture (Capone 2001; Dixon and Kahn 2004). In addition, some farmers use crop-rotation techniques in which they include leguminous crops, in order to fertilize the soil for future non-leguminous crops.

The use of biotechnology in agriculture has been growing over the years as a sustainable strategy for increasing animal and vegetable production (Glick 2012). Plant growth-promoting bacteria (PGPB) which include rhizobia, can be used as tools to increase the production of crops, while reducing the use of environmental damaging chemical fertilizers or pesticides (Lucy et al. 2004). Rhizobia inoculants should be effective in nitrogen fixation, persistent in soil and competitive with native populations, as well as adapted to the field environmental conditions (Stephens and Rask 2000), in order to be able to establish successful and effective symbioses.

Molecular biotechnology strategies can contribute to the improvement of rhizobia inoculants, particularly the genetic engineering of rhizobia to overexpress specific genes, directly or indirectly involved in the symbiotic process, in order to improve rhizobia performance, such as symbiotic effectiveness, nodulation efficiency, competitiveness and stress tolerance.

Using chickpea rhizobia strains as case study, the present thesis focused in better understanding how extra copies of genes directly and indirectly related with the symbiosis could improve symbiotic performance, aiming to contribute to the development of highly efficient rhizobia strains.

The NifA protein is directly involved in the nitrogen fixation in bacteroids (Novichkov et al. 2013). Encoded by *nifA*, it regulates the operon *nifHDK*, which encode the nitrogenase complex that converts atmospheric nitrogen into ammonia. It was also

reported that NifA controls other genes that are not directly involved in the nitrogen fixation process, but are related to competitiveness, nodulation efficiency, development of nodules and bacteroid persistence (Fischer 1994). As NifA regulator plays an extremely important role in the whole nitrogen fixation process, it was expected that chickpea plants inoculated with the transformed strains overexpressing this gene would show significantly higher biomass, as a consequence of a more efficient nitrogen fixation.

Overexpression of *nifA* gene in four mesorhizobia strains only improved the symbiotic effectiveness of V15bpRKnifA. In addition, plants inoculated with this strain showed a higher rate of nodules development in a hydroponic assay. Similar results were obtained with *Sinorhizobium fredii* using extra-copies of *nifA* from *Klebsiella pneumonia* that resulted in an increase in the nodulation activity and nodulation competitiveness on soybean plants (Jieping et al. 2002). Furthermore, Sanjuan and Olivares (1991) reported that multicopy plasmids carrying the *Klebsiella pneumoniae nifA* gene enhanced *S. meliloti* nodulation competitiveness on alfalfa. V15-b was also modified with extra copies of *nodD*, *envZ* or *groEL*, but the symbiotic effectiveness was not improved by the overexpression of any of these genes.

NodD is a regulator of other rhizobial nodulation genes (e.g. *nodABC*) (Cooper 2007a), thus it was expected that extra *nodD* copies in mesorhizobia strains would improve mainly the nodulation in chickpea plants. It was previously seen that *nodD* mutation in strains with only one copy frequently suppresses the nodulation (Nod^-) (e.g. *Rhizobium leguminosarum* bv. *trifolli*) (Broughton et al. 2000). Indeed, when *nodD* was overexpressed, ST2pRKnodD and PMI6pRKnodD strains showed an improvement in their ability to infect root hairs, which probably accounted for the significant increase in the rate of nodules development in plants inoculated with these strains. Moreover, nodules from plants inoculated with PMI6pRKnodD did not show senescent areas that were observed in nodules from plants inoculated with PMI6pRK415 (the corresponding control strain). Despite the fact that at the end of a 7-week pot trial, no difference in the number of nodules between PMI6pRKnodD and PMI6pRK415 was detected, plants inoculated with PMI6pRKnodD showed an expressive increase in the shoot dry weight of approximately 33%.

This is the first report on the genetic transformation of a *Mesorhizobium* strain with a symbiosis gene that significantly improved its symbiotic performance.

These plant-microbe interactions comprise several stages that require extensive gene expression reprogramming from both partners. It is known that some genes present in rhizobia, whose main function is not directly related to symbiosis, may indirectly influence this process. That is the case of several stress response genes, such as *groEL* or *clpB* (Bittner et al. 2007; Brígido et al. 2012b; Ogawa and Long 1995). For the present

study two genes involved in stress response were used to evaluate the effects of its overexpression on the symbiotic performance: the chaperone gene *groEL* and the two-component system gene *envZ*.

The ST-2 strain was the only one that showed an improvement of the symbiotic parameters when the *groEL* gene was overexpressed, such as symbiotic effectiveness and rate of nodules formation. Interestingly, as mentioned above, ST-2 also had some symbiotic features improved when harboring extra *nodD* copies. Although the main function of the GroEL chaperone is protect the bacteria against heat shock, it was reported that this protein plays a fundamental role in the formation of functional NodD and nitrogenase complex (Fischer et al. 1999; Ogawa and Long 1995). Therefore, it is tempting to suggest that there might be a similar key point determining the symbiotic performance improvement observed for both ST2pRKgroEL and ST2pRKnodD, namely an increased level of the NodD regulator. This hypothesis is supported by previous studies showing that S*inorhizobium meliloti* mutants in *groEL* gene affected the activity of NodD and formed nodules later and with a Fix- phenotype on alfalfa (Ogawa and Long 1995).

The EnvZ/OmpR two-component regulatory system is activated in response to osmotic stress and is responsible for the regulation of the expression of outer membrane porins OmpF and OmpC (Alphen and Lugtenberg 1977; Wang et al. 2012). The two-component are also associated with several cellular functions, such as virulence in pathogens, fatty acid uptake, exopolysaccharide (EPS) production, peptide transport, flagella production and regulation of type III secretion system genes in pathogenic bacteria (Bernardini et al. 1990; Brzostek et al. 2007; Feng et al. 2003; Feng et al. 2004; Li et al. 2014; Li and Zhao 2011; Mills et al. 1998; Pickard et al. 1994; Shin and Park 1995; Vidal et al. 1998; Yuan et al. 2011).

The *envZ* gene was overexpressed in five mesorhizobia strains in order to analyze its effects in the symbiotic performance. Two of those strains, PMI-6 and EE-7, showed improvement of some parameters related with the symbiosis. Interestingly, *envZ* was one of the overexpressed genes that showed the most promising results because, besides having improved the symbiotic effectiveness, the root dry weight of plants inoculated with both PMI6pRKenvZ and EE7pRKenvZ was also improved. Extra *envZ* copies increased the EPS production in PMI6pRKenvZ and the amount of root hairs and secondary roots were higher in plants inoculated with this strain. In addition, the rate of nodules formation was also increased under hydroponic conditions and the histological analysis of these nodules showed a higher number of bacteroid-occupied cells and each invaded plant cell showed a larger area occupied by bacteroids.

Unexpectedly, none of the homologous overexpression tested could improve the

UPM-Ca36^T strain.

Although some tested strains showed improvement of their symbiotic performance with the overexpression of more than one gene (PMI-6 and ST-2) and some overexpressed genes were seen to improve more than one strain (*envZ* and *nodD*), none of the selected genes improved all the tested strains and none of the tested strains showed symbiotic improvement overexpressing all the genes. Overall, these results suggest that the improvement of symbiotic performance seems to be a strain specific process.

It is noteworthy that for all the modified strains that showed an improvement in the symbiotic effectiveness, no alteration was detected in the number of nodules in the plant pot trials. However, when the nodulation of these same strains was evaluated in a time course approach, using hydroponic conditions, a higher number of nodules was obtained in all cases. The fact that these different plant trials had different durations (7 weeks for pot experiments and 33-35 days for hydroponic conditions) suggests that the higher number of nodules detected in chickpea plants inoculated with the strains carrying extra copies of a given gene, when compared to the same strain harboring the empty vector, might be a transient phase. This would mean that the strains showing SE improvement induced the formation of nodules in a faster rate, and this might be determinant for increasing plant growth, since it could represent more available N in an earlier stage of plant development.

Future perspectives

From the genes analyzed that were known to be directly related with the symbiosis process, *nodD* seems to be the one which showed more interesting and promising results. Therefore, more studies should be performed using these transformed strains, especially to understand the reason why extra copies of *nodD* only improved the symbiotic performance of some strains. Furthermore, since ST-2 showed improvement of symbiotic effectiveness while overexpressing both *nodD* and *groEL* genes, it would be interesting to investigate this known interaction between NodD and GroEL in ST-2.

The role of the *envZ* gene is well documented for *E. coli*, however to our knowledge no studies were performed regarding the EnvZ/OmpR system in rhizobia. The expression of extra copies of *envZ* besides leading to an improvement of the symbiotic effectiveness, also affected several other symbiosis related phenotypes. Therefore, additional studies are required to understand how *envZ* is related with the symbiosis. Moreover, studies aiming to evaluate the osmotic tolerance of these strains could be important, especially since the *Mesorhizobium* genomes seem to lack the two main

targets of EnvZ/OmpR regulation (*ompF* and *ompC*), suggesting that other mechanisms must be under the control of this system.

Future studies may involve the integration of extra copy of these genes in the genome of a chickpea *Mesorhizobium* strains to avoid the negative effects of the expression vector.

The precise molecular mechanisms that account for the improvement in the symbiotic effectiveness reported on the present study require further investigation, in order to better understand the genetic determinants of highly effective rhizobia strains.

References

Acharjee S, Sarmah BK (2013) Biotechnologically generating 'super chickpea' for food and nutritional security. Plant science : an international journal of experimental plant biology 207:108-16 doi:10.1016/j.plantsci.2013.02.003

Aiba H, Nakasai F, Mizushima S, Mizuno T (1989) Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in *Escherichia coli*. Journal of Biological Chemistry 264(24):14090-14094

Albala K (2007) Beans: A History. Berg Publishers, New York, NY, USA

Albright LM, Ronson CW, Nixon BT, Ausubel FM (1989) Identification of a gene linked to *Rhizobium meliloti ntrA* whose product is homologous to a family to ATP-binding proteins. Journal of bacteriology 171(4):1932-41

Alexandre A, Brígido C, Laranjo M, Rodrigues S, Oliveira S (2009) A survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. Microbial Ecology 58:930-941 doi:10.1007/s00248-009-9536-6

Alexandre A, Laranjo M, Oliveira S (2014) Global transcriptional response to heat shock of the legume symbiont *Mesorhizobium loti* MAFF303099 comprises extensive gene downregulation. DNA Res 21(2):195-206 doi:10.1093/dnares/dst050

Alexandre A, Laranjo M, Young JPW, Oliveira S (2008) *dnaJ* is a useful phylogenetic marker for alphaproteobacteria International journal of systematic and evolutionary microbiology 58(12):2839-2849

Alexandre A, Oliveira S (2011) Most heat-tolerant rhizobia show high induction of major chaperone genes upon stress. FEMS Microbiol Ecol 75:28-36

Alexandre A, Oliveira S (2013) Response to temperature stress in rhizobia. Critical Reviews in Microbiology 39(3):219-228

Alexandre A, Oliveira S (2016) Heat shock response in bacteria with large genomes: lessons from rhizobia. In: de Bruijn FJ (ed) Stress and Environmental Control of Gene Expression in Bacteria. Wiley-Blackwell Publishers

Alphen WV, Lugtenberg B (1977) Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. Journal of bacteriology 131(2):623-30

Alvarez-Morales A, Betancourt-Alvarez M, Kaluza K, Hennecke H (1986) Activation of the *Bradyrhizobium japonicum nifH* and *nifDK* operons is dependent on promoter-upstream DNA sequences. Nucleic Acids Research 14(10):4207-4227

Alvarez-Morales A, Hennecke H (1985) Expression of *Rhizobium japonicum nifH* and *nifDK* operons can be activated by the *Klebsiella pneumonia nifA* protein but not by the product of *ntrC*. Molecular & General Genetics : Mgg 199(2):306-314

B G Rolfe a, Gresshoff PM (1988) Genetic Analysis of Legume Nodule Initiation. Annual Review of Plant Physiology and Plant Molecular Biology 39(1):297-319 doi:10.1146/annurev.pp.39.060188.001501

Babst M, Hennecke H, Fischer HM (1996) Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. Molecular Microbiology 19(4):827-839

Barnett MJ, Fisher RF, Jones T, Komp C, Abola AP, Barloy-Hubler F, Bowser L, Capela D, Galibert F, Gouzy J, Gurjal M, Hong A, Huizar L, Hyman RW, Kahn D, Kahn ML, Kalman S, Keating DH, Palm C, Peck MC, Surzycki R, Wells DH, Yeh KC, Davis RW, Federspiel NA, Long SR (2001) Nucleotide sequence and predicted functions of the entire *Sinorhizobium meliloti* p*Sym*A megaplasmid. Proc Natl Acad Sci U S A 98(17):9883-9888

Batut J, Daveran-Mingot ML, David M, Jacobs J, Garnerone AM, Kahn D (1989) *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen

fixation genes both positively and negatively in *Rhizobium meliloti*. Embo j 8(4):1279-86 doi:10.1002/j.1460-2075.1989.tb03502.x

Bélanger L, Dimmick KA, Fleming JS, Charles TC (2009) Null mutations in *Sinorhizobium meliloti exoS* and *chvI* demonstrate the importance of this two-component regulatory system for symbiosis. Molecular Microbiology 74(5):1223-1237 doi:10.1111/j.1365-2958.2009.06931.x

Bergstrom LC, Qin L, Harlocker SL, Egger LA, Inouye M (1998) Hierarchical and co-operative binding of OmpR to a fusion construct containing the *ompC* and *ompF* upstream regulatory sequences of *Escherichia coli*. Genes to cells : devoted to molecular & cellular mechanisms 3(12):777-88

Beringer JE (1974a) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84 doi:10.1099/00221287-84-1-188

Beringer JE (1974b) R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology 84(1):188-198 doi:10.1099/00221287-84-1-188

Bernardini ML, Fontaine A, Sansonetti PJ (1990) The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. Journal of bacteriology 172(11):6274-81

Biswas B, Gresshoff P (2014) The Role of Symbiotic Nitrogen Fixation in Sustainable Production of Biofuels. International Journal of Molecular Sciences 15(5):7380

Bittner AN, Foltz A, Oke V (2007) Only one of five *groEL* genes is required for viability and successful symbiosis in *Sinorhizobium meliloti*. J Bacteriol 189(5):1884-1889 doi:10.1128/jb.01542-06

Bittner AN, Oke V (2006) Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. J Bacteriol 188(10):3507-3515

Black M, Moolhuijzen P, Chapman B, Barrero R, Howieson J, Hungria M, Bellgard M (2012) The Genetics of Symbiotic Nitrogen Fixation: Comparative Genomics of 14 Rhizobia Strains by Resolution of Protein Clusters. Genes 3(1):138

Bloemberg GV, Wijfjes AH, Lamers GE, Stuurman N, Lugtenberg BJ (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Molecular plant-microbe interactions : MPMI 13(11):1170-6 doi:10.1094/mpmi.2000.13.11.1170

Boor KJ (2006) Bacterial Stress Responses: What Doesn't Kill Them Can Make Them Stronger. PLoS Biology 4(1):e23

Boscari A, Van de Sype G, Le Rudulier D, Mandon K (2006) Overexpression of BetS, a *Sinorhizobium meliloti* high-affinity betaine transporter, in bacteroids from *Medicago sativa* nodules sustains nitrogen fixation during early salt stress adaptation. Molecular Plant-Microbe Interactions 19(8):896-903

Brígido C, Alexandre A, Oliveira S (2012a) Transcriptional analysis of major chaperone genes in salt-tolerant and salt-sensitive mesorhizobia. Microbiological research 167(10):623-629 doi:10.1016/j.micres.2012.01.006

Brigido C, Nascimento FX, Duan J, Glick BR, Oliveira S (2013) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Mesorhizobium* spp. reduces the negative effects of salt stress in chickpea. FEMS microbiology letters 349(1):46-53 doi:10.1111/1574-6968.12294

Brígido C, Oliveira S (2013) Most acid-tolerant chickpea mesorhizobia show induction of major chaperone genes upon acid shock. Microbial Ecology 65(1):145-153

Brígido C, Robledo M, Menendez E, Mateos PF, Oliveira S (2012b) A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants. Molecular Plant-Microbe Interactions 25(12):1594-1604 doi:10.1094/mpmi-05-12-0140-r

Broughton WJ, Dilworth MJ (1971) Control of Leghaemoglobin Synthesis in Snake Beans. Biochemical Journal 125(4):1075-1080

Broughton WJ, Jabbouri S, Perret X (2000) Keys to symbiotic harmony. Journal of bacteriology 182(20):5641-5652 doi:10.1128/JB.182.20.5641-5652.2000

Brzostek K, Brzóstkowska M, Bukowska I, Karwicka E, Raczkowska A (2007) OmpR negatively regulates expression of invasin in *Yersinia enterocolitica*. Microbiology (Reading, England) 153(8):2416-2425 doi:doi:10.1099/mic.0.2006/003202-0

Caetano-Anolles G, Wall LG, De Micheli AT, Macchi EM, Bauer WD, Favelukes G (1988) Role of Motility and Chemotaxis in Efficiency of Nodulation by *Rhizobium meliloti*. Plant physiology 86(4):1228-35

Cai SJ, Inouye M (2002) EnvZ-OmpR Interaction and Osmoregulation in *Escherichia coli*. Journal of Biological Chemistry 277(27):24155-24161 doi:10.1074/jbc.M110715200

Capone DG (2001) Marine nitrogen fixation: what's the fuss? Current opinion in microbiology 4(3):341-8

Carranca C, de Varennes A, Rolston D (1999) Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the N-15 isotope dilution technique. European Journal of Agronomy 10(1):49-56

Chen EJ, Fisher RF, Perovich VM, Sabio EA, Long SR (2009) Identification of Direct Transcriptional Target Genes of ExoS/ChvI Two-Component Signaling in *Sinorhizobium meliloti*. Journal of bacteriology 191(22):6833-6842 doi:10.1128/jb.00734-09

Chen W-M, Moulin L, Bontemps C, Vandamme P, Béna G, Boivin-Masson C (2003) Legume Symbiotic Nitrogen Fixation by beta-Proteobacteria Is Widespread in Nature. Journal of bacteriology 185(24):7266-7272

Chen WX, Wang E, Wang SY, Li YB, Chen XQ (1995) Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root-nodule bacterium isolated from an arid saline environment in Xinjiang, Peoples-Republic-of-China. International Journal of Systematic Bacteriology 45(1):153-159

Chen WX, Wang ET, Kuykendall D (2005) Genus VI. *Mesorhizobium*. Bergeys Manual of Systematic Bacteriology. vol vol. 2 (The Proteobacteria). Springer, New York, pp pp. 403-408

Cheng H-P, Walker GC (1998) Succinoglycan Production by *Rhizobium meliloti* Is Regulated through the ExoS-ChvI Two-Component Regulatory System. Journal of bacteriology 180(1):20-26

Chengtao Y, Guanqiao Y, Shanjiong SS, Jiabi Z (2004) Functional difference between *Sinorhizobium meliloti* NifA and *Enterobacter cloacae* NifA. Science in China Series C, Life sciences 47(1):44-51 doi:10.1360/02yc0268

Choi EY, Ahn GS, Jeon KW (1991) Elevated levels of stress proteins associated with bacterial symbiosis in amaeba-proteus and soybean root nodules cells. Biosystems 25(3):205-212 doi:10.1016/0303-2647(91)90006-7

Considine MJ, Foyer CH (2014) Redox Regulation of Plant Development. Antioxidants & Redox Signaling 21(9):1305-1326 doi:10.1089/ars.2013.5665

Cooper JE (2004) Multiple Responses of Rhizobia to Flavonoids During Legume Root Infection. Adv Bot Res 41:1-62 doi:10.1016/S0065-2296(04)41001-5

Cooper JE (2007a) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J Appl Microbiol 103 doi:10.1111/j.1365-2672.2007.03366.x

Cooper JE (2007b) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. Journal of Applied Microbiology 103(5):1355-1365 doi:doi:10.1111/j.1365-2672.2007.03366.x

Crossman LC, Castillo-Ramírez S, McAnnula C, Lozano L, Vernikos GS, Acosta JL, Ghazoui ZF, Hernández-González I, Meakin G, Walker AW, Hynes MF, Young JPW, Downie JA, Romero D, Johnston AWB, Dávila G, Parkhill J, González V (2008) A Common Genomic Framework for a Diverse Assembly of Plasmids in the Symbiotic Nitrogen Fixing Bacteria. PLOS ONE 3(7):e2567 doi:10.1371/journal.pone.0002567

D'Haeze W, Holsters M (2002) Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology 12(6):79R-105R doi:10.1094 / MPMI -20-2-0129

da-Silva JR, Alexandre, A, Brígido, C, Oliveira, S (2017) Can stress response genes be used to improve the symbiotic performance of rhizobia? AIMS Microbiology 3(3):365-382

de Lajudie P, Willems A, Nick G, Moreira F, Molouba F, Hoste B, Torck U, Neyra M, Collins MD, Lindström K, Dreyfus B, Gillis M (1998) Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. International Journal of Systematic Bacteriology 48(2):369-382

de Philip P, Batut J, Boistard P (1990) *Rhizobium meliloti* Fix L is an oxygen sensor and regulates *R. meliloti nifA* and *fixK* genes differently in *Escherichia coli*. Journal of bacteriology 172(8):4255-62

del Cerro P, Rolla-Santos AAP, Gomes DF, Marks BB, Pérez-Montaño F, Rodríguez-Carvajal MÁ, Nakatani AS, Gil-Serrano A, Megías M, Ollero FJ, Hungria M (2015) Regulatory *nodD1* and *nodD2* genes of *Rhizobium tropici* strain CIAT 899 and their roles in the early stages of molecular signaling and host-legume nodulation. BMC Genomics 16(1):251 doi:10.1186/s12864-015-1458-8

Den Herder G, Parniske M (2009) The unbearable naivety of legumes in symbiosis. Current opinion in plant biology 12(4):491-9 doi:10.1016/j.pbi.2009.05.010

Dénarié J, Debellé F, Promé JC (1996) *Rhizobium* Lipo-Chitooligosaccharide Nodulation Factors: Signaling Molecules Mediating Recognition and Morphogenesis. Annual Review of Biochemistry 65(1):503-535

Denison RF, Harter BL (1995) Nitrate Effects on Nodule Oxygen Permeability and Leghemoglobin (Nodule Oximetry and Computer Modeling). Plant physiology 107(4):1355-1364

Dixon R, Kahn D (2004) Genetic Regulation of Biological Nitrogen Fixation. Nature Reviews Microbiology 2(8):621-631

Djordjevic MA (2004) *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. PROTEOMICS 4(7):1859-1872

Djordjevic MA, Chen HC, Natera S, Van Noorden G, Menzel C, Taylor S (2003a) A global analysis of protein expression profiles in Sinorhizobium meliloti: discovery of new genes for nodule occupancy and stress adaptation. Mol Plant Microbe Interact 16 doi:10.1094/mpmi.2003.16.6.508

Djordjevic MA, Chen HC, Natera S, van Noorden G, Menzel C, Taylor S, Renard C, Geiger O, Weiller GF (2003b) A global analysis of protein expression profiles in *Sinorhizobium meliloti*: Discovery of new genes for nodule occupancy and stress adaptation. Molecular Plant-Microbe Interactions 16(6):508-524

Downie JA (1998) Functions of rhizobial nodulation genes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The Rhizobiaceae Molecular Biology of Model Plant-Associated Bacteria. Kluwer Academic Publishers, Dordrecht, pp 387-402

Downie JA (2005) Legume Haemoglobins: Symbiotic Nitrogen Fixation Needs Bloody Nodules. Current Biology 15(6):R196-R198 doi:http://dx.doi.org/10.1016/j.cub.2005.03.007

Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. Fems Microbiology Reviews 34(2):150-170 doi:10.1111/j.1574-6976.2009.00205.x

Dutta R, Qin L, Inouye M (1999) Histidine kinases: diversity of domain organization. Molecular Microbiology 34(4):633-640 doi:10.1046/j.1365-2958.1999.01646.x

Dziejman M, Mekalanos JJ (1995) Two-Component Signal Transduction and Its Role in the Expression of Bacterial Virulence Factors Two-Component Signal Transduction. American Society of Microbiology

E. Newton W (2007) Physiology, Biochemistry, and Molecular Biology of Nitrogen Fixation,

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13(4):17r-27r doi:10.1093/glycob/cwg047

Eliziário F (2015) Análise e Sobre-expressão de Genes de Simbiose de Rizóbios de Grão-de-bico. Master thesis, Évora University

Euzéby JP (1997) List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol(47):590-592

FAO (2009) How to feed the world 2050. Food and Agriculture Organization, Rome, Italy, p 35

FAOSTAT (2014) Food and Agriculture Organization. PUblisher. http://www.fao.org/faostat/en/#data/QC Accessed 18/09/2017 2017

Farkas A, Maroti G, Durgo H, Gyorgypal Z, Lima RM, Medzihradszky KF, Kereszt A, Mergaert P, Kondorosi E (2014) Medicago truncatula symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. Proc Natl Acad Sci U S A 111(14):5183-8 doi:10.1073/pnas.1404169111

Fayet O, Ziegelhoffer T, Georgopoulos C (1989) The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. Journal of bacteriology 171(3):1379-1385

Feng X, Oropeza R, Kenney LJ (2003) Dual regulation by phospho-OmpR of *ssrA*/*B* gene expression in *Salmonella* pathogenicity island 2. Mol Microbiol 48(4):1131-43

Feng X, Walthers D, Oropeza R, Kenney LJ (2004) The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. Mol Microbiol 54(3):823-35 doi:10.1111/j.1365-2958.2004.04317.x

Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. Nature Biotechnology 21(11):1266-1267

Finan TM, Kunkel B, De Vos GF, Signer ER (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. Journal of bacteriology 167(1):66-72 doi:10.1128/jb.167.1.66-72.1986

Fischer HM (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiological Reviews 58(3):352-386

Fischer HM, Alvarez-Morales A, Hennecke H (1986) The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of nif gene expression and formation of determinate symbiosis. Embo j 5(6):1165-73

Fischer HM, Babst M, Kaspar T, Acuña G, Arigoni F, Hennecke H (1993) One member of a *gro*ESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. The Embo Journal 12(7):2901-2912

Fischer HM, Schneider K, Babst M, Hennecke H (1999) GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum*. Archives of microbiology 171(4):279-289

Flores-Félix J, Menéndez E, Marcos-García M, Celador-Lera L, Rivas R (2015) Calcofluor white, an Alternative to Propidium Iodide for Plant Tissues Staining in Studies of Root Colonization by Fluorescent-tagged Rhizobia. Journal of Advances in Biology & Biotechnology 2(1):65-70 doi:10.9734/jabb/2015/12444

Foo YH, Gao Y, Zhang H, Kenney LJ (2015) Cytoplasmic sensing by the inner membrane histidine kinase EnvZ. Prog Biophys Mol Biol 118(3):119-29 doi:10.1016/j.pbiomolbio.2015.04.005

Forst SA, Delgado J, Inouye M (1989) DNA-binding properties of the transcription activator (OmpR) for the upstream sequences of *ompF* in *Escherichia coli* are altered by *envZ* mutations and medium osmolarity. Journal of bacteriology 171(6):2949-2955 doi:10.1128/jb.171.6.2949-2955.1989

Fournier J, Timmers ACJ, Sieberer BJ, Jauneau A, Chabaud M, Barker DG (2008) Mechanism of Infection Thread Elongation in Root Hairs of *Medicago truncatula* and Dynamic Interplay with Associated Rhizobial Colonization. Plant physiology 148(4):1985-1995 doi:10.1104/pp.108.125674

Fred EB (1932) Root nodule bacteria and leguminous plants. University of Wisconsin Press., Madison.USA.

Gage DJ (2004) Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. Microbiol Mol Biol Rev 68(2):280-300

Gao H, Zhang Y, Han Y, Yang L, Liu X, Guo Z, Tan Y, Huang X, Zhou D, Yang R (2011) Phenotypic and transcriptional analysis of the osmotic regulator OmpR in *Yersinia pestis*. BMC microbiology 11:39-39 doi:10.1186/1471-2180-11-39

Gao R, Stock AM (2009) Biological insights from structures of two-component proteins. Annual review of microbiology 63:133-54 doi:10.1146/annurev.micro.091208.073214

García-Fraile P, Carro L, Robledo M, Ramírez-Bahena M-H, Flores-Félix J-D, Fernández MT, Mateos PF, Rivas R, Igual JM, Martínez-Molina E, Peix Á, Velázquez E (2012) *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. PLOS ONE 7(5):e38122 doi:10.1371/journal.pone.0038122

Garcia M, Dunlap J, Loh J, Stacey G (1996) Phenotypic characterization and regulation of the *nolA* gene of *Bradyrhizobium japonicum*. Molecular plant-microbe interactions : MPMI 9(7):625-36

Garg N, Geetanjali (2007) Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. Agron Sustain Dev 27 doi:10.1051/agro:2006030

Geddes BA, Gonzalez JE, Oresnik IJ (2014) Exopolysaccharide production in response to medium acidification is correlated with an increase in competition for nodule occupancy. Molecular plant-microbe interactions : MPMI 27(12):1307-17 doi:10.1094/mpmi-06-14-0168-r

Gerahty N, Caetano-Anollés G, Joshi PA, Gresshoff PM (1992) Anatomical analysis of nodule development in soybean reveals an additional autoregulatory control point. Plant Science 85(1):1-7 doi:https://doi.org/10.1016/0168-9452(92)90087-3

Geurts R, Bisseling T (2002) Rhizobium Nod Factor Perception and Signalling. The Plant Cell 14(Suppl):s239-s249 doi:10.1105/tpc.002451

Gibson AH (1987) Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. In: Elkan GH (ed) Symbiotic Nitrogen Fixation Technology. Marcel Dekker, Inc, New York, pp 321-363

Gilles-Gonzalez MA, Ditta GS, Helinski DR (1991) A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature 350(6314):170-2 doi:10.1038/350170a0

Glick BR (2012) Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica 2012:1-15

Glick BR (2015) Beneficial Plant-Bacterial Interactions. Springer, Waterloo

Gong Z, Zhu J, Yu G, Zou H (2007) Disruption of *nifA* gene influences multiple cellular processes in *Sinorhizobium meliloti*. J Genet Genomics 34(9):783-9 doi:10.1016/s1673-8527(07)60089-7

Gong ZY, He ZS, Zhu JB, Yu GQ, Zou HS (2006) *Sinorhizobium meliloti nifA* mutant induces different gene expression profile from wild type in Alfalfa nodules. Cell research 16(10):818-829 doi:10.1038/sj.cr.7310096

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant physiology 131(3):872-7 doi:10.1104/pp.017004

Gresshoff PM, Delves AC (1986) Plant Genetic Approaches to Symbiotic Nodulation and Nitrogen Fixation in Legumes. In: Blonstein AD, King PJ (eds) A Genetic Approach to Plant Biochemistry. Springer Vienna, Vienna, pp 159-206

Guisbert E, Herman C, Lu CZ, Gross CA (2004) A chaperone network controls the heat shock response in *E. coli*. Genes Dev 18(22):2812-21 doi:10.1101/gad.1219204

Haag AF, Arnold MF, Myka KK, Kerscher B, Dall'Angelo S, Zanda M, Mergaert P, Ferguson GP (2013) Molecular insights into bacteroid development during *Rhizobium*-legume symbiosis. FEMS Microbiol Rev 37(3):364-83 doi:10.1111/1574-6976.12003

Hall MN, Silhavy TJ (1981) Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. Journal of molecular biology 151(1):1-15

Harlocker SL, Bergstrom L, Inouye M (1995) Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. The Journal of biological chemistry 270(45):26849-56

Head CG, Tardy A, Kenney LJ (1998) Relative binding affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* regulatory sites. Journal of molecular biology 281(5):857-70 doi:10.1006/jmbi.1998.1985

Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311(1-2):1-18 doi:10.1007/s11104-008-9668-3

Hertig C, Li RY, Louarn AM, Garnerone AM, David M, Batut J, Kahn D, Boistard P (1989) *Rhizobium meliloti* regulatory gene *fixJ* activates transcription of *R. meliloti nifA* and *fixK* genes in *Escherichia coli*. Journal of bacteriology 171(3):1736-8

Hoffman BM, Lukoyanov D, Yang ZY, Dean DR, Seefeldt LC (2014) Mechanism of nitrogen fixation by nitrogenase: the next stage. Chemical reviews 114(8):4041-62 doi:10.1021/cr400641x

Honma MA, Ausubel FM (1987) *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. Proceedings of the National Academy of Sciences 84(23):8558-8562

Hsing W, Silhavy TJ (1997) Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. Journal of bacteriology 179(11):3729-35 doi:10.1128/jb.179.11.3729-3735.1997

Huang KJ, Igo MM (1996) Identification of the bases in the *ompF* regulatory region, which interact with the transcription factor OmpR. Journal of molecular biology 262(5):615-28 doi:10.1006/jmbi.1996.0540

Hubber A, Vergunst AC, Sullivan JT, Hooykaas PJ, Ronson CW (2004) Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7A VirB/D4 type IV secretion system. Mol Microbiol 54(2):561-74 doi:10.1111/j.1365-2958.2004.04292.x

Hubber AM, Sullivan JT, Ronson CW (2007) Symbiosis-induced cascade regulation of the *Mesorhizobium loti* R7A VirB/D4 type IV secretion system. Molecular plant-microbe interactions : MPMI 20(3):255-61 doi:10.1094/mpmi-20-3-0255

Hungria M, Campo R, Chueire L, Grange L, Megías M (2001) Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil. Biology and Fertility of Soils 33(5):387-394 doi:10.1007/s003740100338

Hungria M, Johnston AW, Phillips DA (1992) Effects of flavonoids released naturally from bean (*Phaseolus vulgaris*) on *nodD*-regulated gene transcription in *Rhizobium leguminosarum* bv. *phaseoli*. Molecular plant-microbe interactions : MPMI 5(3):199-203

Hungria M, Stacey G (1997) Molecular signals exchanged between host plants and rhizobia: Basic aspects and potential application in agriculture. Soil Biology and Biochemistry 29(5):819-830 doi:http://dx.doi.org/10.1016/S0038-0717(96)00239-8

Hungria M, Vargas MAT (2000) Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Research 65(2-3):151-164

Hydroworld (2009) Renewable Fuels: Manufacturing Ammonia from Hydropower. PUblisher. http://www.hydroworld.com/articles/hr/print/volume-28/issue-7/articles/renewable-fuels-manufacturing.html Accessed 28/09/2017 2017

Issa AA, Abd-Alla MH, Ohyama T (2014) Nitrogen Fixing Cyanobacteria: Future Prospect. In: Ohyama T (ed) Advances in Biology and Ecology of Nitrogen Fixation. InTech, Rijeka, p Ch. 02

Janczarek M, Jaroszuk-Ściseł J, Skorupska A (2009) Multiple copies of *rosR* and *pssA* genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*. Antonie van Leeuwenhoek 96(4):471-486 doi:10.1007/s10482-009-9362-3

Janczarek M, Rachwał K, Marzec A, Grządziel J, Palusińska-Szysz M (2015) Signal molecules and cell-surface components involved in early stages of the legume-*rhizobium* interactions. Applied Soil Ecology 85:94-113 doi:10.1016/j.apsoil.2014.08.010

Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggaard-Nielsen H, Alves BJR, Morrison MJ (2012) Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agronomy for Sustainable Development 32(2):329-364 doi:10.1007/s13593-011-0056-7

Jieping Z, Xiaomi D, Ling X, Jiabi Z, Shanjiong S, Guanqiao Y (2002) Extra-copy *nifA* enhances the nodulation efficiency of *Sinorhizobium fredii*. Chinese Science Bulletin 47(7):565-567 doi:10.1360/02tb9130

Jones KM (2012) Increased production of the exopolysaccharide succinoglycan enhances *Sinorhizobium meliloti* 1021 symbiosis with the host plant *Medicago truncatula*. Journal of bacteriology 194(16):4322-31 doi:10.1128/jb.00751-12

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat Rev Microbiol 5(8):619-33 doi:10.1038/nrmicro1705

Jones KM, Sharopova N, Lohar DP, Zhang JQ, VandenBosch KA, Walker GC (2008) Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. Proc Natl Acad Sci U S A 105(2):704-9 doi:10.1073/pnas.0709338105

Jukanti AK, Gaur PM, Gowda CL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. The British journal of nutrition 108 Suppl 1:S11-26 doi:10.1017/s0007114512000797

Kaminski PA, Batut J, Boistard P (1998) A Survey of Symbiotic Nitrogen Fixation by Rhizobia. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The Rhizobiaceae: Molecular Biology of Model Plant-Associated Bacteria. Kluwer Academic Publishers, Dordrecht, pp 432-460

Karunakaran R, Ramachandran VK, Seaman JC, East AK, Mouhsine B, Mauchline TH, Prell J, Skeffington A, Poole PS (2009) Transcriptomic Analysis of *Rhizobium leguminosarum* Biovar *viciae* in Symbiosis with Host Plants *Pisum sativum* and *Vicia cracca*. Journal of bacteriology 191(12):4002-4014 doi:10.1128/JB.00165-09

Kato M, Aiba H, Tate S, Nishimura Y, Mizuno T (1989) Location of phosphorylation site and DNA-binding site of a positive regulator, OmpR, involved in activation of the osmoregulatory genes of *Escherichia coli*. FEBS letters 249(2):168-72

Keen NT, Tamaki S, Kobayashi D, Trollinger D (1988) Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70(1):191-197 doi:10.1016/0378-1119(88)90117-5

Kidaj D, Wielbo J, Skorupska A (2012) Nod factors stimulate seed germination and promote growth and nodulation of pea and vetch under competitive conditions. Microbiological research 167(3):144-50 doi:10.1016/j.micres.2011.06.001

Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425(6953):78-81

Kim D-j, Boylan B, George N, Forst S (2003) Inactivation of *ompR* Promotes Precocious Swarming and *flhDC* Expression in *Xenorhabdus nematophila*. Journal of bacteriology 185(17):5290-5294 doi:10.1128/jb.185.17.5290-5294.2003

Klein S, Hirsch AM, Smith CA, Signer ER (1988) Interaction of *nod* and *exo Rhizobium meliloti* in alfalfa nodulation. Molecular plant-microbe interactions : MPMI 1(2):94-100

Kondorosi E, Gyuris J, Schmidt J, John M, Duda E, Hoffmann B, Schell J, Kondorosi A (1989) Positive and negative control of nod gene expression in *Rhizobium meliloti* is required for optimal nodulation. The EMBO Journal 8(5):1331-1340

Krause A, Doerfel A, Gottfert M (2002) Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. Molecular plant-microbe interactions : MPMI 15(12):1228-35 doi:10.1094/mpmi.2002.15.12.1228

Kuzma MM, Hunt S, Layzell DB (1993) Role of Oxygen in the Limitation and Inhibition of Nitrogenase Activity and Respiration Rate in Individual Soybean Nodules. Plant physiology 101(1):161-169

Labidi M, Laberge S, Vezina LP, Antoun H (2000) The *dnaJ* (hsp40) locus in *Rhizobium leguminosarum* bv. *phaseoli* is required for the establishment of an effective symbiosis with *Phaseolus vulgaris*. Molecular Plant-Microbe Interactions 13(11):1271-1274

Laranjo M, Alexandre A, Oliveira S (2014) Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. Microbiological research 169(1):2-17 doi:10.1016/j.micres.2013.09.012

Laranjo M, Alexandre A, Rivas R, Velázquez E, Young JPW, Oliveira S (2008) Chickpea rhizobia symbiosis genes are highly conserved across multiple *Mesorhizobium* species. FEMS Microbiol Ecol 66(2):391-400 doi:10.1111/j.1574-6941.2008.00584.x

Laranjo M, Machado J, Young JPW, Oliveira S (2004) High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. FEMS Microbiol Ecol 48(1):101-107

Laranjo M, Oliveira S (2011) Tolerance of *Mesorhizobium* type strains to different environmental stresses. Antonie van Leeuwenhoek 99(3):651-662

Laranjo M, Young JPW, Oliveira S (2012) Multilocus sequence analysis reveals multiple symbiovars within *Mesorhizobium* species. Systematic and Applied Microbiology 35(6):359-367 doi:10.1016/j.syapm.2012.06.002

Laus MC, van Brussel AAN, Kijne JW (2005) Role of cellulose fibrils and exopolysaccharides of *Rhizobium leguminosarum* in attachment to and infection of *Vicia sativa* root hairs. Molecular Plant-Microbe Interactions 18(6):533-538 doi:10.1094/mpmi-18.0533

lBosworth AH, Williams MK, Albrecht KA, Kwiatkowski R, Beynon J, Hankinson TR, Ronson CW, Cannon F, Wacek TJ, Triplett EW (1994) Alfalfa Yield Response to Inoculation with Recombinant Strains of *Rhizobium meliloti* with an Extra Copy of *dct*ABD and/or Modified *nif*A Expression. Applied and Environmental Microbiology 60(10):3815-3832

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344(6268):781-4 doi:10.1038/344781a0

Li W, Ancona V, Zhao Y (2014) Co-regulation of polysaccharide production, motility, and expression of type III secretion genes by EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora*. Mol Genet Genomics 289(1):63-75 doi:10.1007/s00438-013-0790-4

Li W, Zhao Y (2011) Effect of EnvZ/OmpR and GrrS/GrrA systems on *Erwinia amylovora* virulence. Phytopathology 101(6):S102-S102

Limpens E, Bisseling T (2009) Nod Factor Signal Transduction in the Rhizobium–Legume Symbiosis. In: Emons AMC, Ketelaar T (eds) Root Hairs. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 249-276

Limpens E, van Zeijl A, Geurts R (2015) Lipochitooligosaccharides modulate plant host immunity to enable endosymbioses. Annu Rev Phytopathol 53:311-34 doi:10.1146/annurev-phyto-080614-120149

Lopez-Baena FJ, Vinardell JM, Perez-Montano F, Crespo-Rivas JC, Bellogin RA, Espuny Mdel R, Ollero FJ (2008) Regulation and symbiotic significance of nodulation outer proteins secretion in *Sinorhizobium fredii* HH103. Microbiology (Reading, England) 154(Pt 6):1825-36 doi:10.1099/mic.0.2007/016337-0

Lopez O, Morera C, Miranda_Rios J, Girard L, Romero D, Soberon M (2001) Regulation of gene expression in response to oxygen in *Rhizobium etli*: role of *FnrN* in *fixNOQP* expression and in symbiotic nitrogen fixation. Journal of bacteriology 183(24):6999-7006

Lucy M, Reed E, Glick BR (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86(1):1-25 doi:10.1023/B:ANTO.0000024903.10757.6e

Lund PA (2009) Multiple chaperonins in bacteria - why so many? Fems Microbiology Reviews 33(4):785-800 doi:10.1111/j.1574-6976.2009.00178.x

Macchiavelli RE, Brelles-Marino G (2004) Nod factor-treated *Medicago truncatula* roots and seeds show an increased number of nodules when inoculated with a limiting population of *Sinorhizobium meliloti*. Journal of experimental botany 55(408):2635-2640 doi:10.1093/jxb/erh261

Machado D, Krishnan HB (2003) *nodD* alleles of *Sinorhizobium fredii* USDA191 differentially influence soybean nodulation, *nodC* expression, and production of exopolysaccharides. Current Microbiology 47(2):134-137 doi:10.1007/s00284-002-3972-6

MacLean AM, Finan TM, Sadowsky MJ (2007) Genomes of the symbiotic nitrogen-fixing bacteria of legumes. Plant physiology 144(2):615-622 doi:10.1104/pp.107.101634

Maj D, Wielbo J, Marek-Kozaczuk M, Martyniuk S, Skorupska A (2009) Pretreatment of clover seeds with nod factors improves growth and nodulation of Trifolium pratense. Journal of chemical ecology 35(4):479-87 doi:10.1007/s10886-009-9620-x

Manen JF, Simon P, Van Slooten JC, Osterås M, Frutiger S, Hughes GJ (1991) A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. The Plant Cell 3(3):259-270 doi:10.1105/tpc.3.3.259

Martínez-Salazar JM, Sandoval-Calderon M, Guo XW, Castillo-Ramirez S, Reyes A, Loza MG, Rivera J, Alvarado-Affantranger X, Sanchez F, Gonzalez V, Davila G, Ramirez-Romero MA (2009) The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses. Microbiology (Reading, England) 155:386-397 doi:10.1099/mic.0.021428-0

Martinez M, Palacios JM, Imperial J, Ruiz-Argueso T (2004) Symbiotic autoregulation of *nifA* expression in *Rhizobium leguminosarum* bv. *viciae*. Journal of bacteriology 186(19):6586-6594

Mattison K, Kenney LJ (2002) Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. The Journal of biological chemistry 277(13):11143-8 doi:10.1074/jbc.M111128200

Mellor HY, Glenn AR, Arwas R, Dilworth MJ (1987) Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1. Archives of microbiology 148(1):34-39 doi:10.1007/BF00429644

Mills SD, Ruschkowski SR, Stein MA, Finlay BB (1998) Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *ompR* and *envZ* mutants are defective for the formation of *Salmonella*-induced filaments. Infect Immun 66(4):1806-11

Milner JL, Araujo RS, Handelsman J (1992) Molecular and symbiotic characterization of exopolysaccharide-deficient mutants of *Rhizobium tropici* strain CIAT899.

Molecular Microbiology 6(21):3137-3147 doi:10.1111/j.1365-2958.1992.tb01770.x
Minchin FR, James EK, Becana M (2008) Oxygen Diffusion, Production Of Reactive Oxygen And Nitrogen Species, And Antioxidants In Legume Nodules. In: Dilworth MJ, James EK, Sprent JI, Newton WE (eds) Nitrogen-fixing Leguminous Symbioses. Springer Netherlands, Dordrecht, pp 321-362
Minder AC, Narberhaus F, Babst M, Hennecke H, Fischer HM (1997) The *dnaKJ* operon belongs to the sigma(32)-dependent class of heat shock genes in *Bradyrhizobium japonicum*. Molecular & General Genetics 254(2):195-206
Mitsui H, Sato T, Sato Y, Ito N, Minamisawa K (2004) *Sinorhizobium meliloti* RpoH(1) is required for effective nitrogen-fixing symbiosis with alfalfa. Molecular Genetics and Genomics 271(4):416-425
Moscatiello R, Alberghini S, Squartini A, Mariani P, Navazio L (2009) Evidence for calcium-mediated perception of plant symbiotic signals in aequorin-expressing *Mesorhizobium loti*. BMC microbiology 9(1):206 doi:10.1186/1471-2180-9-206
Moulin L, Klonowska A, Caroline B, Booth K, Vriezen JA, Melkonian R, James EK, Young JP, Bena G, Hauser L, Land M, Kyrpides N, Bruce D, Chain P, Copeland A, Pitluck S, Woyke T, Lizotte-Waniewski M, Bristow J, Riley M (2014) Complete Genome sequence of *Burkholderia phymatum* STM815(T), a broad host range and efficient nitrogen-fixing symbiont of Mimosa species. Stand Genomic Sci 9(3):763-74 doi:10.4056/sigs.4861021
Moussaid S, Domínguez-Ferreras A, Muñoz S, Aurag J, Berraho EB, Sanjuán J (2015) Increased trehalose biosynthesis improves *Mesorhizobium ciceri* growth and symbiosis establishment in saline conditions. Symbiosis 67(1):103-111 doi:10.1007/s13199-015-0338-y
Nandwani R, Dudeja SS (2009) Molecular diversity of a native mesorhizobial population of nodulating chickpea (*Cicer arietinum* L.) in Indian soils. Journal of basic microbiology 49(5):463-70 doi:10.1002/jobm.200800355
Narberhaus F (2010) Translational control of bacterial heat shock and virulence genes by temperature-sensing mRNAs. RNA Biol 7(1):84-89
Nascimento F, Brígido C, Alho L, Glick BR, Oliveira S (2012b) Enhanced chickpea growth-promotion ability of a *Mesorhizobium* strain expressing an exogenous ACC deaminase gene. Plant and Soil 353(1):221-230 doi:10.1007/s11104-011-1025-2
Nascimento FX, Brigido C, Glick BR, Oliveira S, Alho L (2012a) *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. Lett Appl Microbiol 55(1):15-21 doi:10.1111/j.1472-765X.2012.03251.x
Nelson MS, Sadowsky MJ (2015) Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. Frontiers in Plant Science 6:491 doi:10.3389/fpls.2015.00491
Newcomb W, Sippell D, Peterson RL (1979) The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. Canadian Journal of Botany 57(23):2603-2616 doi:10.1139/b79-309
Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67(4):593-656
Nogales J, Campos R, BenAbdelkhalek H, Olivares J, Lluch C, Sanjuan J (2002) *Rhizobium tropici* genes involved in free-living salt tolerance are required for the establishment of efficient nitrogen-fixing symbiosis with *Phaseolus vulgaris*. Molecular Plant-Microbe Interactions 15(3):225-232
Nomura M, Arunothayanan H, Dao TV, Le HTP, Kaneko T, Sato S, Tabata S, Tajima S (2010a) Differential protein profiles of Bradyrhizobium japonicum USDA110 bacteroid during soybean nodule development. Soil Science and Plant Nutrition 56(4):579-590 doi:10.1111/j.1747-0765.2010.00500.x

Nomura M, Arunothayanan H, Van Dao T, Le HT-P, Kaneko T, Sato S, Tabata S, Tajima S (2010b) Differential protein profiles of *Bradyrhizobium japonicum* USDA110 bacteroid during soybean nodule development. Soil Science & Plant Nutrition 56(4):579-590 doi:10.1111/j.1747-0765.2010.00500.x

Nour SM, Cleyet-Marel J-C, Normand P, Fernandez MP (1995) Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. International Journal of Systematic Bacteriology 45(4):640-648 doi:10.1099/00207713-45-4-640

Nour SM, Fernandez MP, Normand P, Cleyet-Marel J-C (1994) *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). International Journal of Systematic Bacteriology 44(3):511-522

Novichkov PS, Kazakov AE, Ravcheev DA, Leyn SA, Kovaleva GY, Sutormin RA, Kazanov MD, Riehl W, Arkin AP, Dubchak I, Rodionov DA (2013) RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. BMC Genomics 14:745 doi:10.1186/1471-2164-14-745

Nukui N, Minamisawa K, Ayabe S-I, Aoki T (2006) Expression of the 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Gene Requires Symbiotic Nitrogen-Fixing Regulator Gene *nifA2* in *Mesorhizobium loti* MAFF303099. Appl Environ Microbiol 72(7):4964-4969

Ogawa J, Long SR (1995) The *Rhizobium meliloti groELc* locus is required for regulation of early nod genes by the transcription activator *nodD*. Genes & Development 9(6):714-729

Okazaki S, Kaneko T, Sato S, Saeki K (2013) Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. Proc Natl Acad Sci U S A 110(42):17131-17136 doi:10.1073/pnas.1302360110

Oke V, Long SR (1999) Bacteroid formation in the *Rhizobium*-legume symbiosis. Current opinion in microbiology 2(6):641-646

Oke V, Rushing BG, Fisher EJ, Moghadam-Tabrizi M, Long SR (2001) Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*. Microbiology (Reading, England) 147(Pt 9):2399-408 doi:10.1099/00221287-147-9-2399

Oldroyd GE, Downie JA (2004) Calcium, kinases and nodulation signalling in legumes. Nature reviews Molecular cell biology 5(7):566-76 doi:10.1038/nrm1424

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annual review of genetics 45:119-44 doi:10.1146/annurev-genet-110410-132549

Oldroyd GED (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nature Reviews Microbiology 11(4):252-263 doi:10.1038/nrmicro2990

Oldroyd GED, Downie JM (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes Annual Review of Plant Biology. Annual Review of Plant Biology, vol 59, pp 519-546

Ono Y, Mitsui H, Sato T, Minamisawa K (2001) Two RpoH homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti*. Molecular and General Genetics 264(6):902-912

Oshima T, Aiba H, Masuda Y, Kanaya S, Sugiura M, Wanner BL, Mori H, Mizuno T (2002) Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. Mol Microbiol 46(1):281-91

Paço A, Brígido C, Alexandre A, Mateos PF, Oliveira S (2016) The Symbiotic Performance of Chickpea Rhizobia Can Be Improved by Additional Copies of the *clpB* Chaperone Gene. PLOS ONE 11(2):e0148221 doi:10.1371/journal.pone.0148221

Park H, Inouye M (1997) Mutational analysis of the linker region of EnvZ, an osmosensor in *Escherichia coli*. Journal of bacteriology 179(13):4382-4390

Park H, Saha SK, Inouye M (1998) Two-domain reconstitution of a functional protein histidine kinase. Proc Natl Acad Sci U S A 95(12):6728-6732

Parkinson JS, Kofoid EC (1992) Communication modules in bacterial signaling proteins. Annual review of genetics 26:71-112 doi:10.1146/annurev.ge.26.120192.000443

Peck MC, Fisher RF, Bliss R, Long SR (2013) Isolation and characterization of mutant *Sinorhizobium meliloti* NodD1 proteins with altered responses to luteolin. Journal of bacteriology 195(16):3714-23 doi:10.1128/jb.00309-13

Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ (2015) Bacterial Associations with Legumes. Critical Reviews in Plant Sciences 34(1-3):17-42 doi:10.1080/07352689.2014.897899

Pellock BJ, Cheng HP, Walker GC (2000) Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. Journal of bacteriology 182(15):4310-8

Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves BJR, Urquiaga S, Boddey RM, Dakora FD, Bhattarai S, Maskey SL, Sampet C, Rerkasem B, Khan DF, Hauggaard-Nielsen H, Jensen ES (2009) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. Symbiosis 48(1):1-17 doi:10.1007/BF03179980

Perez-Montano F, Guasch-Vidal B, Gonzalez-Barroso S, Lopez-Baena FJ, Cubo T, Ollero FJ, Gil-Serrano AM, Rodriguez-Carvajal MA, Bellogin RA, Espuny MR (2011) Nodulation-gene-inducing flavonoids increase overall production of autoinducers and expression of N-acyl homoserine lactone synthesis genes in rhizobia. Research in microbiology 162(7):715-23 doi:10.1016/j.resmic.2011.05.002

Pérez-Montaño F, Jiménez-Guerrero I, Del Cerro P, Baena-Ropero I, López-Baena FJ, Ollero FJ, Bellogín R, Lloret J, Espuny R (2014) The Symbiotic Biofilm of *Sinorhizobium fredii* SMH12, Necessary for Successful Colonization and Symbiosis of *Glycine max* cv Osumi, Is Regulated by Quorum Sensing Systems and Inducing Flavonoids via NodD1. PLoS ONE 9(8):e105901 doi:10.1371/journal.pone.0105901

Perret X, Staehelin C, Broughton WJ (2000) Molecular basis of symbiotic promiscuity. Microbiology and Molecular Biology Reviews 64(1):180-201

Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer H-M, Hennecke H (2007a) Genome-Wide Transcript Analysis of *Bradyrhizobium japonicum* Bacteroids in Soybean Root Nodules. Molecular Plant-Microbe Interactions 20(11):1353-1363 doi:10.1094/MPMI-20-11-1353

Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer HM, Hennecke H (2007b) Genome-wide transcript analysis of Bradyrhizobium japonicum bacteroids in soybean root nodules. Molecular Plant-Microbe Interactions 20(11):1353-1363 doi:10.1094/mpmi-20-11-1353

Philippot L, Hallin S (2011) Towards food, feed and energy crops mitigating climate change. Trends in Plant Science 16(9):476-480 doi:10.1016/j.tplants.2011.05.007

Pickard D, Li J, Roberts M, Maskell D, Hone D, Levine M, Dougan G, Chatfield S (1994) Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. Infection and Immunity 62(9):3984-3993

Podleśny J, Wielbo J, Podleśna A, Kidaj D (2014) The pleiotropic effects of extract containing rhizobial Nod factors on pea growth and yield. Central European Journal of Biology 9(4):396-409 doi:10.2478/s11535-013-0277-7

Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, Dorel C (2001) Complex Regulatory Network Controls Initial Adhesion and Biofilm Formation in *Escherichia coli* via Regulation of *thecsgD* Gene. Journal of bacteriology 183(24):7213-7223 doi:10.1128/jb.183.24.7213-7223.2001

Prüβ BM (2017) Involvement of two-component signaling in bacterial motility and biofilm development. Journal of bacteriology doi:10.1128/jb.00259-17

Rampersaud A, Harlocker SL, Inouye M (1994) The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. The Journal of biological chemistry 269(17):12559-66

Recourt K, van Brussel AA, Driessen AJ, Lugtenberg BJ (1989) Accumulation of a nod gene inducer, the flavonoid naringenin, in the cytoplasmic membrane of *Rhizobium leguminosarum* biovar *viciae* is caused by the pH-dependent hydrophobicity of naringenin. Journal of bacteriology 171(8):4370-4377

Reina-Bueno M, Argandona M, Nieto JJ, Hidalgo-Garcia A, Iglesias-Guerra F, Delgado MJ, Vargas C (2012) Role of trehalose in heat and desiccation tolerance in the soil bacterium *Rhizobium etli*. BMC microbiology 12:207

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil 321(1):305-339 doi:10.1007/s11104-009-9895-2

Rivas R, Laranjo M, Mateos PF, Oliveira S, Martinez-Molina E, Velázquez E (2007) Strains of *Mesorhizobium amorphae* and *Mesorhizobium tianshanense*, carrying symbiotic genes of common chickpea endosymbiotic species, constitute a novel biovar (*ciceri*) capable of nodulating *Cicer arietinum*. Letters in Applied Microbiology 44(4):412-418 doi:doi:10.1111/j.1472-765X.2006.02086.x

Rivas R, Velázquez E, Valverde A, Mateos PF, Martínez-Molina E (2001) A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. Electrophoresis 22(6):1086-1089

Robledo M, Jimenez-Zurdo JI, Soto MJ, Velazquez E, Dazzo F, Martinez-Molina E, Mateos PF (2011) Development of functional symbiotic white clover root hairs and nodules requires tightly regulated production of rhizobial cellulase CelC2. Molecular plant-microbe interactions : MPMI 24(7):798-807 doi:10.1094/mpmi-10-10-0249

Rodpothong P, Sullivan JT, Songsrirote K, Sumpton D, Cheung KW, Thomas-Oates J, Radutoiu S, Stougaard J, Ronson CW (2009) Nodulation gene mutants of *Mesorhizobium loti* R7A-*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. Molecular plant-microbe interactions : MPMI 22(12):1546-54 doi:10.1094/mpmi-22-12-1546

Rodrigues C, Laranjo M, Oliveira S (2006) Effect of heat and pH stress in the growth of chickpea mesorhizobia. Current Microbiology 53(1):1-7

Rodriguez-Quinones F, Maguire M, Wallington EJ, Gould PS, Yerko V, Downie JA, Lund PA (2005) Two of the three *groEL* homologues in *Rhizobium leguminosarum* are dispensable for normal growth. Archives of microbiology 183(4):253-265

Roponen I (1970) The Effect of Darkness on the Leghemoglobin Content and Amino Acid Levels in the Root Nodules of Pea Plants. Physiologia Plantarum 23(3):452-460 doi:10.1111/j.1399-3054.1970.tb06435.x

Rouws LF, Simoes-Araujo JL, Hemerly AS, Baldani JI (2008) Validation of a Tn5 transposon mutagenesis system for *Gluconacetobacter diazotrophicus* through characterization of a flagellar mutant. Archives of microbiology 189(4):397-405 doi:10.1007/s00203-007-0330-x

Rubio LM, Ludden PW (2008) Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. Annual review of microbiology 62:93-111 doi:10.1146/annurev.micro.62.081307.162737

Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis. Proc Natl Acad Sci U S A 108 Suppl 2:10800-7 doi:10.1073/pnas.1100304108

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory, New York

Sanjuan J, Olivares J (1989) Implication of *nif*A in regulation of genes located on a *Rhizobium meliloti* cryptic plasmid that affect nodulation efficiency. Journal of bacteriology 171(8):4154-4161 doi: 10.1128/jb.171.8.4154-4161.1989

Sanjuan J, Olivares J (1991) Multicopy plasmids carrying the *Klebsiella pneumoniae nifA* enhance *Rhizobium meliloti* nodulation competitiveness on alfalfa. Molecular Plant-Microbe Interactions 4(4):365-369 doi:10.1094/mpmi-4-365

Sarma AD, Emerich DW (2005) Global protein expression pattern of *Bradyrhizobium japonicum* bacteroids: A prelude to functional proteomics. PROTEOMICS 5(16):4170-4184 doi:10.1002/pmic.200401296

Sarma AD, Emerich DW (2006) A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. Proteomics 6(10):3008-28 doi:10.1002/pmic.200500783

Sayyed RZ, Jamadar DD, Patel PR (2011) Production of Exo-polysaccharide by *Rhizobium* sp. Indian Journal of Microbiology 51(3):294-300 doi:10.1007/s12088-011-0115-4

Schell MA (1993) Molecular biology of the LysR family of transcriptional regulators. Annual review of microbiology 47:597-626 doi:10.1146/annurev.mi.47.100193.003121

Schetgens RMP, Hontelez JGJ, van den Bos RC, van Kammen A (1985) Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. Molecular and General Genetics MGG 200(3):368-374 doi:10.1007/BF00425719

Schlaman HRM, Phillips DA, Kondorosi E (1998) Genetic Organization and Transcriptional Regulation of Rhizobial Nodulation Genes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The Rhizobiaceae: Molecular Biology of Model Plant-Associated Bacteria. Springer Netherlands, Dordrecht, pp 361-386

Schumann W (2016) Regulation of bacterial heat shock stimulons. Cell Stress Chaperones 21(6):959-968 doi:10.1007/s12192-016-0727-z

Shin S, Park C (1995) Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. Journal of bacteriology 177(16):4696-702

Shvaleva A, Coba de la Peña T, Rincón A, Morcillo CN, García de la Torre VS, Lucas MM, Pueyo JJ (2010) Flavodoxin overexpression reduces cadmium-induced damage in alfalfa root nodules. Plant and Soil 326(1):109-121 doi:10.1007/s11104-009-9985-1

Soby S, Bergman K (1983) Motility and Chemotaxis of *Rhizobium meliloti* in Soil. Applied and Environmental Microbiology 46(5):995-998

Soto MJ, Dominguez-Ferreras A, Perez-Mendoza D, Sanjuan J, Olivares J (2009) Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. Cellular Microbiology 11(3):381-388 doi:10.1111/j.1462-5822.2009.01282.x

Soto MJ, Sanjuan J, Olivares J (2006) Rhizobia and plant-pathogenic bacteria: common infection weapons. Microbiology (Reading, England) 152(Pt 11):3167-74 doi:10.1099/mic.0.29112-0

Soupène E, Foussard M, Boistard P, Truchet G, Batut J (1995) Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. Proc Natl Acad Sci U S A 92(9):3759-63

Spaink HP (2000) Root nodulation and infection factors produced by rhizobial bacteria. Annual review of microbiology 54 doi:10.1146/annurev.micro.54.1.257

Spaink HP, Sheeley DM, van Brussel AA, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. Nature 354(6349):125-30 doi:10.1038/354125a0

Sprent JI (2009) Evolution of Nodulation Legume Nodulation. Wiley-Blackwell, pp 51-63

Staehelin C, Forsberg LS, D'Haeze W, Gao M-Y, Carlson RW, Xie Z-P, Pellock BJ, Jones KM, Walker GC, Streit WR, Broughton WJ (2006) Exo-Oligosaccharides

of *Rhizobium* sp. Strain NGR234 Are Required for Symbiosis with Various Legumes. J Bacteriol 188(17):6168-6178
Stephens JHG, Rask HM (2000) Inoculant production and formulation. Field Crops Research 65(2-3):249-258
Stepkowski T, Czaplinska M, Miedzinska K, Moulin L (2003) The variable part of the *dnaK* gene as an alternative marker for phylogenetic studies of rhizobia and related alpha Proteobacteria. Systematic and Applied Microbiology 26(4):483-494
Suarez R, Wong A, Ramirez M, Barraza A, Orozco MdC, Cevallos MA, Lara M, Hernandez G, Iturriaga G (2008) Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. Molecular Plant-Microbe Interactions 21(7):958-966 doi:10.1094/mpmi-21-7-0958
Sugawara M, Epstein B, Badgley BD, Unno T, Xu L, Reese J, Gyaneshwar P, Denny R, Mudge J, Bharti AK, Farmer AD, May GD, Woodward JE, Medigue C, Vallenet D, Lajus A, Rouy Z, Martinez-Vaz B, Tiffin P, Young ND, Sadowsky MJ (2013) Comparative genomics of the core and accessory genomes of 48 *Sinorhizobium* strains comprising five genospecies. Genome Biol 14(2):R17 doi:10.1186/gb-2013-14-2-r17
Sullivan JT, Brown SD, Ronson CW (2013) The NifA-RpoN Regulon of *Mesorhizobium loti* Strain R7A and Its Symbiotic Activation by a Novel LacI/GalR-Family Regulator. Plos One 8(1) doi:e5376210.1371/journal.pone.0053762
Sullivan JT, Brown SD, Yocum RR, Ronson CW (2001) The bio operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. Microbiology (Reading, England) 147(5):1315-1322
Summers ML, Elkins JG, Elliott BA, McDermott TR (1998) Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. Molecular Plant-Microbe Interactions 11(11):1094-1101 doi:10.1094/mpmi.1998.11.11.1094
Taíz L, Zieger E (2004) Plant Physiology, 3 edn, Porto Alegre, Brazil
Talbi C, Sanchez C, Hidalgo-Garcia A, Gonzalez EM, Arrese-Igor C, Girard L, Bedmar EJ, Delgado MJ (2012) Enhanced expression of *Rhizobium etli cbb*(3) oxidase improves drought tolerance of common bean symbiotic nitrogen fixation. Journal of experimental botany 63(14):5035-43 doi:10.1093/jxb/ers101
Tena W, Wolde-Meskel E, Degefu T, Walley F (2017) Genetic and phenotypic diversity of rhizobia nodulating chickpea (*Cicer arietinum* L.) in soils from southern and central Ethiopia. Can J Microbiol 63(8):690-707 doi:10.1139/cjm-2016-0776
Terpolilli JJ, Hood GA, Poole PS (2012) What determines the efficiency of N(2)-fixing *Rhizobium*-legume symbioses? Adv Microb Physiol 60:325-89 doi:10.1016/B978-0-12-398264-3.00005-X
Terpolilli JJ, O'Hara GW, Tiwari RP, Dilworth MJ, Howieson JG (2008) The model legume *Medicago truncatula* A17 is poorly matched for N2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. The New phytologist 179(1):62-6 doi:10.1111/j.1469-8137.2008.02464.x
Theunis M, Kobayashi H, Broughton WJ, Prinsen E (2004) Flavonoids, NodD1, NodD2, and *nod*-box NB15 modulate expression of the *y4wEFG* locus that is required for indole-3-acetic acid synthesis in *Rhizobium* sp. strain NGR234. Molecular plant-microbe interactions : MPMI 17(10):1153-61 doi:10.1094/mpmi.2004.17.10.1153
Timmers AC, Soupene E, Auriac MC, de Billy F, Vasse J, Boistard P, Truchet G (2000) Saprophytic intracellular rhizobia in alfalfa nodules. Molecular plant-microbe interactions : MPMI 13(11):1204-13 doi:10.1094/mpmi.2000.13.11.1204
Tipton KA, Rather PN (2017) An *ompR-envZ* Two-Component System Ortholog Regulates Phase Variation, Osmotic Tolerance, Motility, and Virulence in *Acinetobacter baumannii* Strain AB5075. Journal of bacteriology 199(3) doi:10.1128/jb.00705-16

Tschauner K, Hornschemeyer P, Muller VS, Hunke S (2014) Dynamic interaction between the CpxA sensor kinase and the periplasmic accessory protein CpxP mediates signal recognition in E. coli. PLoS One 9(9):e107383 doi:10.1371/journal.pone.0107383

Tseng T-T, Tyler BM, Setubal JC (2009) Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC microbiology 9(Suppl 1):S2-S2 doi:10.1186/1471-2180-9-S1-S2

Tsung K, Brissette RE, Inouye M (1989) Identification of the DNA-binding domain of the OmpR protein required for transcriptional activation of the *ompF* and *ompC* genes of *Escherichia coli* by in vivo DNA footprinting. The Journal of biological chemistry 264(17):10104-9

Uchiumi T, Ohwada T, Itakura M, Mitsui H, Nukui N, Dawadi P, Kaneko T, Tabata S, Yokoyama T, Tejima K, Saeki K, Omori H, Hayashi M, Maekawa T, Sriprang R, Murooka Y, Tajima S, Simomura K, Nomura M, Suzuki A, Shimoda Y, Sioya K, Abe M, Minamisawa K (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. Journal of bacteriology 186(8):2439-48

Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. Field Crops Research 65(2):211-228 doi:https://doi.org/10.1016/S0378-4290(99)00088-X

van Brussel AA, Bakhuizen R, van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJ, Kijne JW (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. Science (New York, NY) 257(5066):70-2 doi:10.1126/science.257.5066.70

van Brussel AA, Tak T, Boot KJ, Kijne JW (2002) Autoregulation of root nodule formation: signals of both symbiotic partners studied in a split-root system of *Vicia sativa* subsp. nigra. Molecular plant-microbe interactions : MPMI 15(4):341-9 doi:10.1094/mpmi.2002.15.4.341

Van de Velde W, Guerra JCP, Keyser AD, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S (2006) Aging in Legume Symbiosis. A Molecular View on Nodule Senescence in Medicago truncatula. Plant physiology 141(2):711-720 doi:10.1104/pp.106.078691

van Dillewijn P, Martinez-Abarca F, Toro N (1998) Multicopy vectors carrying the *Klebsiella pneumoniae nifA* gene do not enhance the nodulation competitiveness of *Sinorhizobium meliloti* on alfalfa. Molecular plant-microbe interactions : MPMI 11(8):839-42 doi:10.1094/mpmi.1998.11.8.839

Vance CP (2001) Symbiotic Nitrogen Fixation and Phosphorus Acquisition. Plant Nutrition in a World of Declining Renewable Resources. Plant physiology 127:390-397 doi:http://dx.doi.org/10.1104/pp.010331

Vasse J, de Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. Journal of bacteriology 172(8):4295-306 doi:10.1128/jb.172.8.4295-4306.1990

Via VD, Zanetti ME, Blanco F (2016) How legumes recognize rhizobia. Plant Signal Behav 11(2):e1120396 doi:10.1080/15592324.2015.1120396

Vidal O, Longin R, Prigent-Combaret C, Dorel C, Hooreman M, Lejeune P (1998) Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. Journal of bacteriology 180(9):2442-9

Vijn I, Martinez-Abarca F, Yang WC, das Neves L, van Brussel A, van Kammen A, Bisseling T (1995) Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. The Plant journal : for cell and molecular biology 8(1):111-9 doi:10.1046/j.1365-313X.1995.08010111.x

Vinardell JM, Lopez-Baena FJ, Hidalgo A, Ollero FJ, Bellogin R, del Rosario Espuny M, Temprano F, Romero F, Krishnan HB, Pueppke SG, Ruiz-Sainz JE (2004) The effect of FITA mutations on the symbiotic properties of *Sinorhizobium fredii* varies

in a chromosomal-background-dependent manner. Archives of microbiology 181(2):144-54 doi:10.1007/s00203-003-0635-3

Vinuesa P, Silva C, Lorite MJ, Izaguirre-Mayoral ML, Bedmar EJ, Martínez-Romero E (2005) Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from rrs, *atpD*, *recA* and *nifH* sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. Systematic and Applied Microbiology 28(8):702-716

Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X (1998) Symbiotic implications of type III protein secretion machinery in *Rhizobium*. Mol Microbiol 28(6):1381-9

Vitousek PM, Menge DNL, Reed SC, Cleveland CC (2013) Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philosophical Transactions of the Royal Society B: Biological Sciences 368(1621)

Wang LC, Morgan LK, Godakumbura P, Kenney LJ, Anand GS (2012) The inner membrane histidine kinase EnvZ senses osmolality via helix-coil transitions in the cytoplasm. Embo j 31(11):2648-59 doi:10.1038/emboj.2012.99

Wei G-H, Yang X-Y, Zhang J-W, Gao J-M, Ma Y-Q, Fu Y-Y, Wang P (2007) Rhizobialide: A New Stearolactone Produced by *Mesorhizobium* sp. CCNWGX022, a Rhizobial Endophyte from *Glycyrrhiza uralensis*. Chemistry & Biodiversity 4(5):893-898 doi:10.1002/cbdv.200790077

Weiss C, Jebara F, Nisemblat S, Azem A (2016) Dynamic Complexes in the Chaperonin-Mediated Protein Folding Cycle. Frontiers in Molecular Biosciences 3:80 doi:10.3389/fmolb.2016.00080

Wells DH, Chen EJ, Fisher RF, Long SR (2007) ExoR is genetically coupled to the ExoS-ChvI two-component system and located in the periplasm of *Sinorhizobium meliloti*. Molecular Microbiology 64(3):647-664 doi:doi:10.1111/j.1365-2958.2007.05680.x

Wittenberg JB (1974) Facilitated oxygen diffusion. The role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. The Journal of biological chemistry 249(13):4057-66

Yao SY, Luo L, Har KJ, Becker A, Ruberg S, Yu GQ, Zhu JB, Cheng HP (2004) *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both Succinoglycan and Flagellum production. Journal of bacteriology 186(18):6042-6049

Yeh KC, Peck MC, Long SR (2002) Luteolin and GroESL modulate in vitro activity of NodD. Journal of bacteriology 184(2):525-530

Yoshida T, Cai S, Inouye M (2002) Interaction of EnvZ, a sensory histidine kinase, with phosphorylated OmpR, the cognate response regulator. Mol Microbiol 46(5):1283-94

Young JP, Crossman L, Johnston A, Thomson N, Ghazoui Z, Hull K, Wexler M, Curson A, Todd J, Poole P, Mauchline T, East A, Quail M, Churcher C, Arrowsmith C, Cherevach I, Chillingworth T, Clarke K, Cronin A, Davis P, Fraser A, Hance Z, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Sanders M, Simmonds M, Whitehead S, Parkhill J (2006) The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biology 7(4):R34

Yuan J, Wei B, Shi M, Gao H (2011) Functional Assessment of EnvZ/OmpR Two-Component System in *Shewanella oneidensis*. PLOS ONE 6(8):e23701 doi:10.1371/journal.pone.0023701

Zahran HH (1999) *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews 63(4):968-989

Zhang F, Smith DL (1996) Genistein accumulation in soybean (Glycine max L Merr) root systems under suboptimal root zone temperatures. Journal of Experimental Botany 47(299):785-792 doi:10.1093/jxb/47.6.785

Zhang JJ, Liu TY, Chen WF, Wang ET, Sui XH, Zhang XX, Li Y, Li Y, Chen WX (2012) *Mesorhizobium muleiense* sp. nov., nodulating with *Cicer arietinum* L. International journal of systematic and evolutionary microbiology 62(Pt 11):2737-42 doi:10.1099/ijs.0.038265-0

Zhang S, Huang L, Yan A, Liu Y, Liu B, Yu C, Zhang A, Schiefelbein J, Gan Y (2016) Multiple phytohormones promote root hair elongation by regulating a similar set of genes in the root epidermis in Arabidopsis. Journal of experimental botany 67(22):6363-6372 doi:10.1093/jxb/erw400

Zhao CT, Wang ET, Chen WF, Chen WX (2008) Diverse genomic species and evidences of symbiotic gene lateral transfer detected among the rhizobia associated with *Astragalus* species grown in the temperate regions of China. FEMS microbiology letters 286(2):263-73 doi:10.1111/j.1574-6968.2008.01282.x

Zheng H, Mao Y, Teng J, Zhu Q, Ling J, Zhong Z (2015) Flagellar-Dependent Motility in *Mesorhizobium tianshanense* is Involved in the Early Stage of Plant Host Interaction: Study of an *flgE* Mutant. Current Microbiology 70(2):219-227 doi:10.1007/s00284-014-0701-x

Zhu Y, Qin L, Yoshida T, Inouye M (2000) Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain. Proceedings of the National Academy of Sciences 97(14):7808-7813 doi:10.1073/pnas.97.14.7808

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