Tolerance of chickpea mesorhizobia to acid and salt stress

Clarisse Cordeiro Brígido

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

Supervisor: Professor Solange Oliveira

ÉVORA, 2012

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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To my beloved Grandparents

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Thesis publications

The present work is based on the following manuscripts:

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

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Other publications

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Nascimento F., **Brígido C.**, Glick B.R., Oliveira S., Alho L. "*Mesorhizobium ciceri* LMS-1 expressing an exogenous ACC deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints" Letters in Applied Microbiology*,* 55**:**15-21.

Abstract

The main objectives of this thesis were to evaluate the tolerance of a collection of native Portuguese chickpea rhizobia to abiotic stresses, namely acidity and salinity, and to investigate the molecular bases of acidity and salinity tolerance. Additionally, the evaluation of the symbiotic performance of ACC deaminase-transformed strains under salinity was performed. The involvement of the chaperone ClpB in the response to abiotic stresses and in the symbiosis with chickpea was investigated by gene deletion in a *Mesorhizobium* strain.

Chickpea rhizobia were assigned to several *Mesorhizobium* species. In both stress conditions, tolerant and sensitive rhizobia were found, including moderately acidophilic isolates. The analysis of the expression of the chaperone genes *dnaK* and *groESL* suggests their involvement in acid tolerance. ACC deaminase-transformed rhizobia strains showed an improvement of their symbiotic performance under salinity. The characterization of the *ClpB* knockout mutant indicated that ClpB is involved in the nodulation process.

Resumo

Tolerância de rizóbios de grão-de-bico à acidez e salinidade

Os principais objetivos desta tese foram a avaliação da tolerância a stresses abióticos, nomeadamente acidez e salinidade, de uma coleção de rizóbios portugueses nativos de grão-de-bico, e investigar as bases moleculares da tolerância a ambos os stresses. Adicionalmente, avaliou-se a eficiência simbiótica de estirpes transformadas com o gene da ACC desaminase em condições de salinidade. Investigou-se ainda o envolvimento da chaperone ClpB na resposta a stresses abióticos e na simbiose com grão-de-bico através da deleção do gene.

Os rizóbios de grão-de-bico pertencem a diferentes espécies de *Mesorhizobium*. Encontraram-se rizóbios tolerantes e sensíveis a ambos os stresses, incluindo isolados moderadamente acidófilos. A análise da expressão dos genes de chaperones *dnaK* e *groESL* sugere o seu envolvimento na tolerância à acidez. Estirpes de rizóbio transformadas com o gene da ACC desaminase apresentaram uma melhoria da sua eficiência simbiótica em condições salinas. A caracterização do mutante ClpB de *Mesorhizobium* indicou que esta chaperone está envolvida no processo de nodulação.

Abbreviations

ACC – 1-aminocyclopropane-1-carboxylate

ADP – adenosine diphosphate

ATP – adenosine triphosphate

bp – base pair

BSA – bovine serum albumin

bv. – biovar

DAPD – direct amplified polymorphic DNA

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

EDTA – Ethylenediamine tetraacetic acid

g – gravity acceleration

Gha – Giga hectares

Hsp- heat shock protein

kb – kilobases

kDa – kiloDalton

Mbp – megabase pairs

Mha – million hectares

min – minute

mM –milimolar

MOPS – 3-(N-morpholino)propanesulfonic acid

mRNA – messenger ribonucleic acid

PCR – polymerase chain reaction

RAPD – random amplified polymorphic DNA

RFLP – restriction fragment length polymorphism

RNA – ribonucleic acid

sec – seconds

SSC – sodium chloride / sodium citrate

TBS – Tris buffered saline buffer

TE – Tris/EDTA buffer

Tg – teragram

Tris – tris hydroxymethyl aminomethane

U – unit

v/v – volume per volume

w/v – weight per volume

Nucleotide Bases

A – Adenine

C – Cytosine

G –Guanine

T – Thymine

Genes

16S rRNA – 16S ribosomal RNA

acdS – 1-aminocyclopropane-1-carboxylate deaminase

atpD – ATP synthase subunit β

clpB – Chaperone ClpB

dnaJ – Co-chaperone DnaJ

dnaK – Chaperone DnaK

gfp – Green fluorescent protein

groEL – Chaperone GroEL

groES – Co-chaperone GroES

nifH – Nitrogenase Iron protein

nodC – N-acetylglucosaminyl transferase

recA – DNA recombinase A

List of Tables

Table 1.1 Description of the species belonging to the *Mesorhizobium* genus, in terms of type strain, country and host of origin, as well as the respective reference. 9

Table 2.1 Characteristics of soils used to obtain chickpea isolates. 51

Table 2.2 Rhizobia isolates used in the present study. Province of origin, 16S rRNA gene accession number, species cluster defined from the 16S rRNA gene sequence analysis, plasmid number and symbiotic effectiveness (SE) values are indicated for each isolate. 52

Table 2.3 Bacterial strains and gene accession numbers (GenBank) used in this work. 61

Table 3.1 List of the isolates used in the present study (from Chapter 2). 84

Table 4.1 Bacterial strains and plasmids used in this study. 104

Table 5.1 List of the isolates used in the present study (from chapter 2). 123

Table 6.1 Plasmids and strains used in the present study. 145

Table 6.2 Symbiotic performance in chickpea plants after inoculation with wild-type LMS-1 or Δ*clpB* mutant strain. Results obtained from a plant growth assay using chickpea plants inoculated with *M. ciceri* LMS-1 and *M. ciceri* LMS-1 Δ*clpB* strains under controlled conditions in a growth chamber. 159

List of Figures

Figure 1.1 Illustration of the main steps in the infection process of the legume-rhizobia symbiosis (from Djordjevic and Weinman, 1991). 14

Figure 1.2 Illustration of endocytosis of bacteria and bacteroids differentiation (from Jones *et al.*, 2007). 15

Figure 1.3 Illustration of the process of protein folding in *E. coli* cytoplasm (from Kolaj *et al.*, 2009). 22

Figure 1.4 Disaggregation of bacterial aggregates by ClpB in combination with the DnaK system (adapted from Diemand and Lupas 2006). 24

Figure 2.1 Neighbor-joining tree showing the phylogenetic relationship of chickpea 33 rhizobia isolates and strains, based on partial 16S rRNA gene analysis (alignment length 578 bp). 56

Figure 2.2 Distribution of the 121 chickpea rhizobia isolates by species clusters, as defined from de 16S rRNA gene-based phylogeny (A). Map of the provinces of Portugal and Madeira Island showing the distribution of isolates in each province, according to their cluster. Pie charts sizes are proportional to the number of isolates in each province (B). 58

Figure 2.3 Example of an agarose gel showing plasmid profiles of chickpea rhizobia isolates. 59

Figure 2.4 Number of isolates in each cluster according to their plasmid number. 60

Figure 2.5 Neighbor-joining tree showing the phylogenetic relationship between chickpea rhizobia isolates and other rhizobia strains, based on partial *nifH* gene analysis (alignment length 472 bp). 64

Figure 2.6 Neighbor-joining tree showing the phylogenetic relationship of chickpea isolates and type strains, based on *nodC* gene analysis (alignment length 486 bp). 65

Figure 2.7 Neighbor-joining tree showing the phylogenetic relationship of chickpea isolates and type strains, based on *acdS* gene analysis (alignment length 542 bp). 66

Figure 3.1 Growth of chickpea mesorhizobia under different salt stress conditions: 1.5% NaCl (open diamonds) and 3% NaCl (closed circles). 86

Figure 3.2 CA biplot of the relationship between provinces of origin of the isolates and tolerance to 1.5% NaCl. 88

Figure 3.3 Comparison of transcriptional analysis of the *dnaK* gene (a) and the *groESL* operon (b) between a salt-tolerant and a salt-sensitive isolate submitted to salt shock. 89

Figure 3.4 Expression levels of *dnaK* and *groESL* genes after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the four species clusters (A-D). 90

Figure 3.5 Expression levels of *clpB* gene after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the Cluster B. 92

Figure 4.1 Results obtained from plant growth assay using chickpea plants inoculated with transformed and native mesorhizobia salt-tolerant and salt-sensitive strains under control and salinity conditions. 109

Figure 4.2 Results obtained from plant growth experiments using chickpea plants inoculated with *acdS*-transformed and no-transformed mesorhizobia salt-tolerant and salt-sensitive strains under control and salinity conditions 111

Figure 5.1 Growth of chickpea mesorhizobia under acid and alkaline stress conditions: pH 5 (squares) and pH 9 (circles). 127

Figure 5.2 CA biplot of the relationship between origin soil pH and tolerance to acid pH of the isolates. 128

Figure 5.3 CA biplot of the relationship between origin soil pH and the isolates species clusters. 129

Figure 5.4 Comparison of transcriptional analysis of the *dnaK* gene (a) and the *groESL* operon (b) between acid-tolerant and acid-sensitive isolates submitted to acidic shock. 131

Figure 5.5 Expression levels of *dnaK* and *groESL* genes after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the four species clusters. 132

Figure 6.1 Alignment of ClpB proteins amino acid sequences from bacteria belonging to *Mesorhizobium* genus, *E. coli* and *Thermus thermophilus*. 153

Figure 6.2 Southern hybridization of total DNA from *M. ciceri* LMS-1 (A), *ΔclpB* mutant (B) and complemented strain (C) digested with HindIII restriction enzyme. 154

Figure 6.3 Growth curves of wild-type (open triangles) and Δ*clpB* mutant (open circles) strains under different conditions during 54 hours. 156

Figure 6.4 Western blot analysis of ClpB production levels with polyclonal antibody against *E. coli* ClpB (1:500). Total protein lysates (15 µg per lane) were obtained from wild-type (1-3) and Δ*clpB* mutant (4-6) strains under control and heat shock conditions. 157

Figure 6.5 A-Nodulation kinetics of chickpea plants inoculated with either wild-type or Δ*clpB* mutant strain during 35 days after inoculation. B-Analysis of *nodC* gene expression by semi-quantitative RT-PCR during control and heat shock conditions in *M. ciceri* LMS-1 and Δ*clpB* mutant 160

Figure 6.6 Bright field micrographs of 2µm histological sections of representative embedded nodules, stained with Toluidine Blue, formed by the wild-type *M. ciceri* LMS-1 (A), or the Δ*clpB* mutant strains (B-F). 162

Figure 6.7 Confocal laser scanning micrographs of propidium iodide-stained roots inoculated with GFP-tagged LMS-1 and Δ*clpB* mutant strains showing the initial infection process in chickpea roots. Images from chickpea root and root hairs were obtained 4 days after inoculation with either wild-type (A) or Δ*clpB* mutant (B) strains. 163

Table of Contents

ACKNOWLEDGMENTS	**V**
ABSTRACT	**IX**
RESUMO	**XI**
ABBREVIATIONS	**XIII**
LIST OF TABLES	**XV**
LIST OF FIGURES	**XVI**
Chapter 1 – State of the art	**1**
1.1 The importance of nitrogen fixation	2
1.2 Mechanisms of biological nitrogen fixation	3
1.3 Symbiotic Nitrogen Fixation	4
1.4 Rhizobia	5
The *Mesorhizobium* genus	8
1.5 Host legume	10
Chickpea (*Cicer arietinum* L.)	11
1.6 The legume-rhizobia nodulation process	12
1.7 Effects of environmental stresses in symbiosis	15
Salt stress	16
Acid stress	18
1.8 Molecular chaperones	21
Protein folding	21
Protein disaggregation	23
1.9 Major molecular chaperones in rhizobia	24
Major molecular chaperones in the legume-rhizobia symbiosis	27
1.10 Aims of the study	28
1.11 References	29

Chapter 2 - Isolation and characterization of native chickpea rhizobia	**43**
2.1 Summary	44
2.2 Introduction	45
2.3 Materials and Methods	47
Isolates collection	47
DNA extraction and manipulation	47
Amplification of the 16S rRNA, *nifH*, *nodC* and *acdS* genes	47
Phylogenetic analysis	48
Plasmid profiles	49
Symbiotic effectiveness (SE)	49
Statistical analysis	49
2.4 Results	50
Isolates collection	50
Phylogeny based on the 16S rRNA gene analysis	51
Plasmid profiles	59
Symbiotic effectiveness	60
Phylogeny analysis of *nifH*, *nodC* and *acdS* genes	63
2.5 Discussion	67
2.6 References	72
Chapter 3 - Tolerance of chickpea mesorhizobia to salinity	**77**
3.1 Summary	78
3.2 Introduction	79
3.3 Materials and Methods	81
Bacterial isolates	81
Salt stress tolerance	81
Statistical analysis	81
RNA extraction and northern hybridization	82
3.4 Results	85
Salt stress tolerance	85

Transcriptional analysis of the major chaperone genes upon salt shock 88
3.5 Discussion 92
3.6 References 96

Chapter 4 - Evaluation of the symbiotic performance of *acdS*-transformed mesorhizobia under salt stress **99**

4.1 Summary 100
4.2 Introduction 101
4.3 Materials and Methods 103
Bacterial Growth Conditions 103
Triparental Mating 104
ACC deaminase activity assay 105
Chickpea seed sterilization 105
Evaluation of the chickpea plants tolerance to salt 106
Plant growth assay under control and salt stress 106
Statistical analysis 107
4.4 Results 107
4.5 Discussion 112
4.6 References 116

Chapter 5 - Tolerance of chickpea mesorhizobia to acidity **119**

5.1 Summary 120
5.2 Introduction 121
5.3 Materials and Methods 122
Bacterial isolates 122
pH stress tolerance 124
Statistical analysis 124
RNA extraction and northern hybridization 125
5.4 Results 126
Evaluation of mesorhizobia tolerance to acidic and alkaline stresses 126
Transcriptional analysis of the major chaperones genes upon acidic shock 130
5.5 Discussion 133

5.6 References 137

Chapter 6 - Evaluation of the role of chaperone ClpB in chickpea mesorhizobia stress tolerance and symbiosis **141**

6.1 Summary 142

6.2 Introduction 143

6.3 Materials and Methods 145

Plasmids, bacterial strains and growth conditions 145

DNA methods and construction of LMS-1 derivative strains 146

Analysis of stress tolerance 147

Immunoblotting 148

Analysis of the Symbiotic Phenotype 149

Analysis of *nodC* gene expression by semi-quantitative RT-PCR 150

Statistical analysis 151

Microscopy 151

6.4 Results 152

Analysis of the *clpB* gene sequence from *Mesorhizobium ciceri* LMS-1 152

Confirmation of the Δ*clpB* knockout mutant construction 154

Δ*clpB* mutant phenotype under environmental stresses 155

Evaluation of the symbiotic performance of the Δ*clpB* mutant strain 158

The Δ*clpB* mutant shows a delay in nodule development 159

Initial interaction of the Δ*clpB* mutant with chickpea roots 162

6.5 Discussion 164

6.6 References 169

Chapter 7 – General Discussion **175**

Future perspectives 185

References 186

“This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”- Winston Churchill

Chapter 1

State of the art

To increase crops productivity to meet the demands of the growing human population and simultaneously maintain sustainable agricultural practices is a challenging task. The increase in land area affected by abiotic stresses due to climate changes and agricultural practices makes this task even more challenging.

The legume-rhizobium symbiotic association plays an important role in agriculture sustainability. The knowledge of the molecular biology of the response to stress of these symbiotic systems can certainly be helpful for the development of more efficient rhizobia inoculants.

1.1 The importance of nitrogen fixation

The Earth's population is expected to reach 10 billion by 2035 (Bockman *et al.*, 1990; Waggoner, 1994). It is clear that to maintain the current level of protein and caloric intake over the next 20 years unprecedented increases in crop production are required. Furthermore, the climate changes and anthropogenic activities, such as urban development, road construction, industrial processes, mining and inadequate agricultural practices, are resulting in the eutrophication and pollution of soils and fresh water resources, soil degradation, loss of soil fertility, and desertification (McLauchlan, 2006; Spiertz, 2010; Gordon *et al.*, 2010). So, augment in crops production will need to be achieved despite a significant deterioration of much prime agricultural lands and will require the utilization of large areas now considered marginal.

Nitrogen is an essential nutrient for plant growth, however, its availability is one of the major limiting factors for most crop species, since plants cannot fix nitrogen. Nitrogen can be provided by the use of chemical nitrogen fertilizers. The increase in the use of the chemical nitrogen fertilizers was registered as 10-fold from 1950 to 1990, mainly in cereal grain yields in developed countries (Waggoner, 1994). Moreover, the demand for nitrogen increased greatly in the last 5 years, especially in the emerging countries, such as India and China in Asia, and Brazil and Argentina in Latin America (FAO, 2011). However, the use of nitrogen fertilizers accelerates the depletion of large amounts of fossil fuels, nonrenewable energy resources, and it contributes substantially to environmental pollution through atmospheric emission and leaching of nitrogenous compounds to ground or surface water (Bohlool *et al.*, 1992; Peoples and Crasswell, 1992; Velthof *et al.*, 2009). Consequently the massive use of the chemical nitrogen fertilizers is a serious problem for sustainability.

Sustainable agriculture is defined as agriculture that is managed toward greater resource efficiency and conservation while maintaining an environment favorable for evolution of all species (Bohlool *et al.*, 1992). From this perspective, the biological nitrogen fixation (BNF), that is the assimilation of atmospheric nitrogen

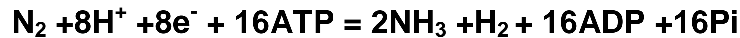
in forms of organic compounds by microorganisms, is one of the most important contributions to the agricultural sustainability. BNF is a sustainable source of nitrogen in cropping systems, as fixed-nitrogen can be used directly by the plant and it is less susceptible to volatilization, denitrification and leaching, avoiding pollution problems in soil and water (Jensen and Hauggaard-Nielsen, 2003; Garg and Geetanjali, 2007). The total BNF is estimated to be twice as much as the total nitrogen fixation by non-biological processes (Bezdicek and Kennedy, 1998). Thus, BNF in agrosystems reduces the need for chemical nitrogen fertilizers and consequently reduces global warming and water contamination (Bohlool *et al*., 1992).

1.2 Mechanisms of biological nitrogen fixation

The major conversion of atmospheric nitrogen into ammonia is accomplished by microorganisms in the BNF process (Peters *et al*., 1995). Nitrogen-fixing microorganisms, named diazotrophs, have a central role in almost all aspects of nitrogen availability and thus in life support on Earth. All these microorganisms are prokaryotes, including some Archaea and Bacteria. BNF can be accomplished by diazotrophs in free-living or in symbiotic associations (with plants or other organisms).

The conversion of dinitrogen into ammonia is catalyzed in all diazotrophs by the nitrogenase enzyme complex in an ATP-dependent manner. This enzyme complex is composed by two components that are named according to their main functional subunits, dinitrogenase reductase (Fe protein) and the dinitrogenase (Mo-Fe protein) (Hageman and Burris, 1978). The nitrogenase complex is encoded by the *nif* (H, D, K, Y, B, Q, E, N, X, U, S, V, W, Z) genes. For example, the *nifDK* genes are structural genes that encode the NifD/K (α and β subunits of the dinitrogenase) and *nifH* gene codes for a subunit of the nitrogenase complex NifH (γ_2 homodimeric azoferredoxin) (for review see Dixon and Kahn, 2004).

The biological nitrogen fixation can be represented by the following equation:



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

The reactions occur while atmospheric nitrogen (N_2) is bound to the nitrogenase. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduces the Mo-Fe protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process, HN=NH is reduced to $H_2N\text{-}NH_2$, and this in turn is reduced to $2NH_3$.

The nitrogenase enzyme complex is highly sensitive to oxygen, due to the fact that oxygen reacts with the iron component of the proteins. Nevertheless, the free-living aerobic bacteria, such as *Azotobacter* species, developed several mechanisms to overcome such limitation in soils, for example by maintaining a very low level of oxygen in their cells or by producing extracellular polysaccharides (Dalton and Postgate, 1969; Yates, 1970). In the symbiotic nitrogen-fixing organisms, such as *Rhizobium*, the plant leghemoglobin regulate the supply of oxygen to the nodule tissues in order to maintain the low oxygen level within the nodules (Fischer, 1994).

1.3 Symbiotic Nitrogen Fixation

Despite the importance of all nitrogen-fixing microorganisms, rhizobia, soil bacteria that are able to establish symbiosis with legumes, are the most important nitrogen-fixing agents in agricultural systems and are the best studied. This particular symbiotic relationship plays an important role in agriculture in grain and forage legumes, such as bean, pea and chickpea, increasing crop productivity without the requirement of chemical nitrogen fertilizers and thus contributing to the sustainable agriculture as well as for the pollution reduction (Freiberg *et al.*, 1997; Stephens and Rask, 2000). Furthermore, this association is also important as legume crops can improvement the soil nitrogen availability to other crops, for instance cereals, as well as in land remediation (Aslam *et al.*, 2003).

The specific symbiotic association between rhizobia and leguminous plants results in the formation of specialized structures, called root nodules, where bacteria can convert dinitrogen into ammonia and supply it to the host plant in exchange for carbohydrates (Young, 1992). This prokaryotic-eukaryotic intimacy is based on a complex molecular crosstalk between both partners, which is initiated by the secretion of flavonoids and other compounds to the rhizosphere by legume plants, inducing the rhizobial lipo-chito-oligosaccharides, the so-called Nod factors (Cooper, 2007). A complete and efficient nitrogen fixation in legume-rhizobia symbiosis requires the coordinate interaction of several major classes of genes present in rhizobia: the *nif* genes and *fix* genes (Kaminski *et al*., 1998) for atmospheric nitrogen fixation, and the *nod*, *nol* and *noe* genes for nodulation (Downie, 1998).

Associations of nitrogen-fixing microorganisms and non-legumes are also important in agriculture and natural life. For example, actinorhizal symbiosis, undertaken by *Frankia* bacteria with non-legume angiosperm families (Benson and Dawson, 2007), is not only beneficial for soil erosion control, but also in wood production with economic importance. On the other hand, the photosynthetic cyanobacteria can also fix nitrogen. They also form symbiotic associations with other organisms, such as the water fern *Azolla* and cycads. Nitrogen-fixing cyanobacteria are an important symbiont of coral reefs (Lesser *et al*., 2004).

1.4 Rhizobia

Rhizobia are gram-negative soil bacteria able to establish a nitrogen-fixing symbiotic relationship with legumes plants (Young, 1992). Once the symbiosis is established, the rhizobia convert atmospheric N_2 into ammonia to their legume host plant. These bacteria may be considered as plant growth promoting bacteria (PGPB), since they directly affect plant growth and development. PGPB are beneficial soil bacteria, which may facilitate plant growth and development both

directly and indirectly (Glick, 1995; Park *et al.*, 2005; Vassilev *et al.*, 2006). PGPB may use one or more of the following mechanisms: fix atmospheric nitrogen and supply it to plants; synthesize siderophores which can sequester iron from the soil and provide it to plant cells; synthesize phytohormones such as auxins, cytokinins and gibberellins, which can act to enhance various stages of plant growth; solubilize minerals such as phosphorus, making them more readily available for plant growth; and synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can lower plant ethylene levels (see Garcia de Salamone *et al.*, 2005; Lugtenberg and Kamilova, 2009; Spaepen *et al.*, 2009; Glick, 2010; Vilchez and Manzanera, 2011).

In general, rhizobia are aerobic or facultative anaerobic, rod shaped, and do not produce endospores. Rhizobia cells are mobile with one polar or sub-polar flagellum with two to six peripheral flagella (Jordan, 1984). Rhizobia can exist in two fundamentally different modes, namely as free-living saprophytic heterotrophs or as legume-host-specific nitrogen fixing symbionts. In soil ecosystem, rhizobia may be present as free-living on the order of 10 to 10^6 microorganisms per gram per soil, depending on the season, culture's history and agriculture practices. On the other hand, when in symbiosis with their hosts, nodules can contain more than 10^{10} cells per gram (McDermott *et al.*, 1987). At the end of the growing season, nodule senescence leads to the release of a large number of rhizobia into soil.

These bacteria were described for the first time by Martinus Beijerinck from a leguminous plant root nodule (Beijerinck, 1888) and since then their study increased greatly. More than 90 species comprising 12 genera were described to date, and this number is increasing as a result of the high diversity of leguminous plants being investigated. Most of these bacterial species belong to the *α-Proteobacteria* class including species of *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Ensifer* (Young, 2003) (formely *Sinorhizobium*) and *Mesorhizobium* (Jarvis *et al.*, 1997). Until recently, the rhizobia species were restricted to the *α-Proteobacteria* class,

however, rhizobia from *β-Proteobacteria* have been described. β-Proteobacteria comprises three genera containing rhizobia species, namely *Burkholderia* (Moulin *et al.*, 2001), *Cupriavidus* (Chen *et al.*, 2001) and *Herbaspirillum* (Valverde *et al.*, 2003). Nevertheless, both classes comprise non-nodulating legume bacteria, such as *Rhizobium larrymoorei* (Young *et al.*, 2001).

Rhizobial genomes are composed by core and accessory elements. The core genome includes the housekeeping genes, which are required for proper functioning of the cell, as well as other genes necessary for the maintenance and basic metabolism. The phylogeny based on the housekeeping genes constitutes a good biological basis for *rhizobium* taxonomy (Martens *et al.*, 2007; 2008; Vinuesa *et al.*, 2008). On the other hand, the accessory genome is responsible for special features, not essential for functioning, such as symbiosis or other kinds of ecological niche adaptation (Young *et al.*, 2006; Lindström *et al.*, 2006; Maclean *et al.*, 2007).

Complete sequencing of some rhizobial genomes has allowed the perception of rhizobial genome complexity. Until April 2012, more than 35 rhizobial complete genomes were ongoing submission or were already available. Significant differences are observed within genomes from the rhizobial species belonging to the α-*Proteobacteria* class. The variation in chromosome size and plasmid numbers is high. For example, *Bradyrhizobium japonicum* USDA110 possesses a chromosome with a size of 9.11Mbp and no plasmids while *Rhizobium etli* CFN42 has a chromosome of 4.38Mbp and six plasmids ranging on size from 0.184Mbp to 0.642Mbp in size. Another interestingly feature brought out with the systematic sequencing is the symbiosis genes location. For example, in the *Mesorhizobium* genus, namely *M. loti* R7A and *M. sp.* MAFF303099, the symbiosis genes are on a chromosomal "symbiosis island" (Kaneko *et al.*, 2000; Sullivan *et al.*, 2002). In contrast, *Rhizobium* species show the symbiosis genes located on plasmids, usually designated by p*Sym*.

The *Mesorhizobium* genus

The *Mesorhizobium* genus is included in the *Rhizobiales* order (Jarvis *et al.*, 1997). Nowadays, this genus consists of a total of 22 species (Table 1.1). However, this number is increasing mostly due to the identification of microsymbionts from newly studied wild legumes.

Although the *Mesorhizobium* genus is the most recently described rhizobial genus within the *Rhizobiales* order, it is one of the genus that comprise the highest number of nodulating rhizobial species. *Mesorhizobium* type strains were isolated from legume root nodules, with the exception of *M. thiogangeticum*, which was obtained from a legume rhizosphere (Ghosh and Roy, 2006).

Within the *Mesorhizobium* genus, the 16S rRNA gene sequence used for phylogenetic studies shows a high similarity between species. For example, some *Mesorhizobium* species are 100% identical in terms of the comparable 16S rRNA gene sequence, as the recently described *M. metallidurans* and *M. gobiense*. In the genus *Mesorhizobium*, other genes have been used for phylogenetic purposes, such as *dnaK* (Stepkowski *et al.*, 2003), *atpD* and *recA* (Vinuesa *et al.*, 2005). More recently, the *dnaJ* gene suggested as a phylogenetic marker at the level of phylum (*Proteobacteria*) and class (*α-Proteobacteria*), can also be used for identification of mesorhizobia isolates with more resolution than the 16S rRNA gene (Alexandre *et al.*, 2008).

Three nodulating mesorhizobia strains are completely sequenced, namely *M. sp.* MAFF303099, *M. opportunistum* LMG 24607 and *M. ciceri* bv. *biserrulae* WSM1271. Nevertheless, the complete genome annotation of a few *Mesorhizobium* strains, such as *M. australicum* LMG 24608, *M. huakuii* 7653R and *M. alhagi* CCNWXJ12-2*,* is ongoing submission. This will allow to compare genes related to the symbiotic lifestyle and to study the evolution of the legumes-mesorhizobia symbiosis.

Table 1.1 Description of the species belonging to the *Mesorhizobium* genus, in terms of type strain, country and host of origin, as well as the respective reference.

Species	Type strain	Origin	Original host	Reference
M. albiziae	CCBAU 61158	China	*Albizia kalkora*	Wang *et al.* (2007)
M. alhagi	CCNWXJ12-2	China	*Alhagi sparsifolia*	Chen *et al.* (2010)
M. amorphae	ACCC 19665	China	*Amorpha fruticosa*	Wang *et al.* (1999)
M. australicum	LMG 24608	Australia	*Biserrula pelecinus*	Nandasena *et al.* (2009)
M. camelthorni	ACCC14549	China	*Alhagi sparsifolia*	Chen *et al.* (2011)
M. caraganae	CCBAU 11299	China	*Caragana spp.*	Guan *et al.* (2008)
M. chacoense	LMG 19008	Argentina	*Prosopis alba*	Velázquez *et al*. (2001)
M. ciceri	UPM-Ca7	Spain	*Cicer arietinum*	Nour *et al*. (1994)
M. gobiense	CCBAU 83330	China	*Oxytropis glabra*	Han *et al*. (2008)
M. huakuii	CCBAU 2609	China	*Astragalus sinicus*	Chen *et al*. (1991)
M. loti	NZP 2213	New Zealand	*Lotus corniculatus*	Jarvis *et al*. (1982)
M. mediterraneum	UPM-Ca36	Spain	*Cicer arietinum*	Nour *et al*. (1995)
M. metallidurans	STM 2683	France	*Anthyllis vulneraria*	Vidal *et al*. (2009)
M. opportunistum	LMG 24607	Australia	*Biserrula pelecinus*	Nandasena *et al*. (2009)
M. plurifarium	ORS 1032	Senegal	*Acacia senegal*	de Lajudie *et al*. (1998)
M. robiniae	ACCC 14543	China	*Robinia pseudoacacia*	Zhou *et al*. (2010)
M. septentrionale	HAMBI 2582	China	*Astragalus adsurgens*	Gao *et al*. (2004)
M. shangrilense	CCBAU 65327	China	*Caragana spp.*	Lu *et al*. (2009)
M. tarimense	CCBAU 83306	China	*Lotus frondosus*	Han *et al*. (2008)
M. temperatum	HAMBI 2583	China	*Astragalus adsurgens*	Gao *et al*. (2004)
M. tianshanense	A-1BS	China	*Glycyrrhiza pallidiflora*	Chen *et al*. (1995)
M. thiogangeticum	LMG 22697	India	*Clitoria ternatea**	Ghosh and Roy (2006)

**M. thiogangeticum* was isolated from this species rhizosphere.

1.5 Host legume

Legumes are important crops for animal and human food, constituting the largest source of vegetable protein in human diets and livestock feed. The legumes are an important and diverse family of angiosperms and are divided into three subfamilies, Mimosoideae, Caesalpinoideae and Papilionoideae. Most cultivated legumes, such as common bean (*Phaseolus vulgaris*), soybean (*Glicine max*) and chickpea (*Cicer arietinum*), belong to the Papilionoideae subfamily, which is the largest one in total number of genera. Moreover, over 90% of the legumes belonging to the Papilionoideae and Mimosoideae subfamily can be nodulated whereas only 30% of the legumes members of the Caesalpinoideae subfamily are known to be nodulated (Doyle, 2001). The production of legumes worldwide is approximately 250Mha and through the symbiotic relationship about 90 Tg of atmospheric nitrogen per year is fixed (Kinzig and Socolow, 1994).

The legume-*rhizobium* specificity can be determined in both partners perspective. The molecular signalling exchange between both partners is fundamental for an effective legume-*rhizobium* symbiosis and can determine the specificity of this symbiotic relationship. Production of Nod factors or lipo-chito-oligosaccharide signalling molecules by the prokaryotic partner is activated by the release of plant phenolic signals, mainly flavonoids, into the rhizosphere. The phenolic flavonoid compounds partly determine the specificity of the symbiotic relationship as each rhizobium species responds to specific flavonoids. Another determinant of host-symbiont specificity is attributed to the different Nod factors substituents attached to the oligosaccharide backbone (Dénarié *et al*., 1996; Oldroyd, 2001).

Most rhizobia species interact with only a few select legumes, but some have been shown to have a broad host range (Pueppke and Broughton, 1999). For example, the strain *Ensifer* sp. NGR234 is able to nodulate over 120 plant genera, including the non-legume *Parasponia andersonii*. This feature may rely on the family of Nod factors secreted, which are more diverse than in all other rhizobia known (Schmeisser *et al*., 2009) and in the concentration of Nod factors released by the NGR234 that is much higher than usual. More recently, *M.*

opportunistum WSM2075 was isolated from *Biserrula pelecinus* root nodules, but the symbiotic genes of this organism provide a broader range of hosts for nodulation, including also *Astragalus adsurgens, A. membranaceus, Lotus peregrinus* and *Macroptilium atropurpureum* (Nandasena *et al*., 2009).

On the other hand, there are legumes species that can be nodulated by several rhizobia species and others that are very restrict for nodulation and only accept as microsymbionts a reduced number of species. For example, *Phaseolus vulgaris* is known as a promiscuous host, since it can be nodulated by rhizobia belonging to diverse genera (such as *Bradyrhizobium*, *Rhizobium* and *Ensifer*) while *Cicer arietinum* is considered a restrict host, because it is nodulated only by *Mesorhizobium* species. Nevertheless, the host range depends on the legume cultivar used and conditions tested (Martinez-Romero, 2003).

Chickpea (*Cicer arietinum* L.)

Chickpea (*Cicer arietinum*) was one of the earliest grain crops cultivated by humans. Today, chickpea ranks third (FAO, 2008) in world production among food legumes following beans (*Phaseolus* spp.) and field pea (*Pisum sativum*).

Total annual world chickpea production is 8.4 million tonnes, and the major chickpea producing countries include India (65% of annual production), Pakistan (10%), Turkey (7%), Iran (3%), Myanmar (2%), Mexico (1.5%) and Australia (1.5%). In Portugal, chickpea production is evaluated in 650 tonnes (FAO, 2008).

Chickpea seed is a protein-rich supplement to cereal-based diets, especially critical in developing countries where people cannot afford animal protein. Chickpea is also a low-input requiring crop, deriving over 70% of its nitrogen requirement through symbiotic nitrogen fixation and providing benefits for following cereal crops (Siddique *et al*., 2005).

Two rhizobia species were described as chickpea microsymbionts, namely *Mesorhizobium ciceri* (Nour *et al*., 1994) and *Mesorhizobium mediterraneum*

(Nour *et al.*, 1995). Since then, several studies addressed chickpea rhizobia diversity using different approaches. Phenotypic and/or molecular characterization of chickpea rhizobia isolated from diverse areas worldwide were conducted (Maâtallah *et al.*, 2002; Laranjo *et al.*, 2004; Rivas *et al.*, 2006; L'Taief *et al.*, 2007; Nandwani and Dudeja, 2009). Molecular characterization has been performed using RAPD fingerprinting, 16S rRNA sequencing or RFLP (Maâtallah *et al.*, 2002; Rivas *et al.*, 2006; L´Taief *et al.*, 2007; Nandwani and Dudeja, 2009) or DAPD fingerprint (Laranjo *et al.*, 2004). These data showed high diversity of species able to nodulate chickpea but all from *Mesorhizobium* genus. Despite the fact that *Ensifer meliloti* strains isolated from Tunisian soils were able to induce nodule formation in chickpea plants, these nodules were ineffective (Romdhane *et al.*, 2007).

On the other hand, the phylogenetic analysis of two symbiosis genes (*nifH* and *nodC*) of chickpea rhizobia, including *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* type strains as well as Portuguese and Spanish isolates, showed a high similarity, suggesting that the symbiosis genes were horizontally transferred (Rivas *et al.*, 2007; Laranjo *et al.*, 2008).

1.6 The legume-rhizobia nodulation process

The legume-rhizobia symbiosis results in the formation of root nodules that provide an environment suitable for nitrogen fixation by rhizobia. In general, the formation of symbiotic N_2-fixing nodules requires two developmental processes: bacterial infection and nodule organogenesis (for more details see Oldroyd *et al.*, 2011). Although these processes can be separated genetically, they must be coordinated in both spatial and temporal manner to ensure nodule formation at the site of bacterial infection. Herein, the description of the most common nodulation processes are detailed in below and schematized in Fig. 1.1 and 1.2.

In broad terms, leguminous plants excrete, through their roots, specific chemical substances that promote rhizobial proliferation in rizosphere (Fig. 1.1). Among

others, phenolic flavonoid compounds are the most relevant compounds involved in the interaction with symbiotic bacteria (Redmond *et al*., 1986). Flavonoid perception attracts the bacteria to the root and activates *nod* (nodulation) gene expression, via the bacteria activator NodD (Lindström *et al*., 2002). NodD activates transcription of *nod* boxes promoters, and represents the first level of host-specific recognition (Schultze and Kondorosi, 2008). The *nod* genes expression lead to the production of strain-specific lipo-chito-oligosaccharides, also called as Nod factors (NF) (Spaink, 2000). NFs have an oligosaccharide backbone of N-acetyl-D-glucosamine units with a fatty acyl- group attached to the non-reducing sugar, which may undergo additional modifications in the length and degree of saturation of the fatty acid group depending on the species of rhizobia (Oldroyd and Downie, 2008). Thus, modifications on NFs structure may determine the host recognition and therefore NFs are considered the second level of host-specific recognition (Perret *et al*., 2000). For instance, *nodC* is involved in the first step of the synthesis of Nod factors and is important in determining the length of the chitin oligosaccharide chain, which is one of the host determinant factors (Kamst *et al*., 1997).

The presence of compatible rhizobia species and their corresponding NF are enough to trigger nodule development. Normally, the tip of the emerging root hair is the primary target for infection by rhizobia. Attachment of rhizobia to root hairs stimulates root hair deformation and also promotes cortical cell divisions (Fig. 1.1 B).

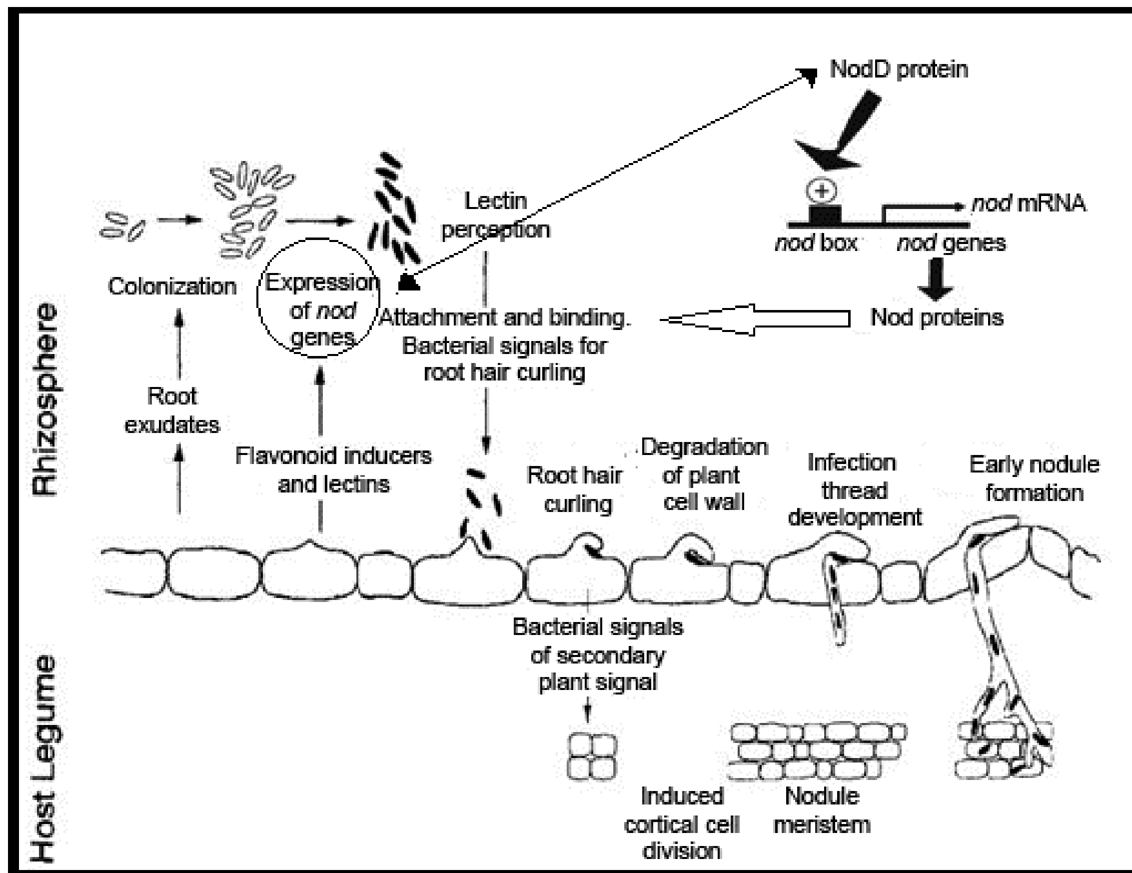


Figure 1.1 Illustration of the main steps in the infection process of the legume-rhizobia symbiosis (from Djordjevic and Weinman, 1991).

Rhizobial infection can occur through root hairs, via cracks in the epidermis and by interstitial infections between epidermal cells (for review see Oldroyd and Downie, 2008). However, the most common via of infection is the formation of infection threads (IT) in growing root hairs, and this involves re-differentiation of the root-hair cell to enable it to make the IT (Fig. 1.1). The IT grows through the root hair into the root cortex and the newly induced dividing cells. Bacteria are released from near the growing tip of the IT into an infection droplet in the host cell cytoplasm. Through a process resembling endocytosis, the bacteria are surrounded by a plant-derived membrane, termed the peribacteroid membrane, which in turns forms the symbiosome (Fig 1.2) (Udvardi and Day, 1997, for review see Jones *et al*., 2007).

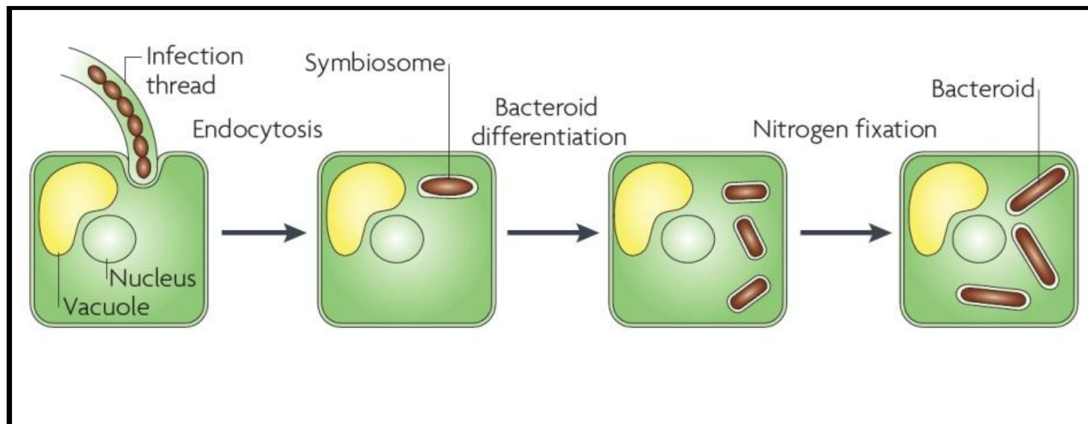


Figure 1.2 Illustration of endocytosis of bacteria and bacteroids differentiation (from Jones *et al*., 2007).

The membrane-enveloped bacteria continue to divide within the host cells before they differentiate into bacteroids and start to fix nitrogen (Fig. 1.2) (Roth and Stacey, 1989a, b). Atmospheric nitrogen is converted into ammonia by bacteroids and is subsequently assimilated into the plant following its conversion to glutamine by glutamine synthase. Within the nodule interior and the neighboring plant cells, essential nutrients are exchange between bacteroids and plant cells.

1.7 Effects of environmental stresses in the symbiosis

The soil environment is constantly changing, which can be relatively stressful for both macro- and microorganisms. Changes such as fluctuations in pH, temperature, salinity and nutrient availability greatly influence the growth, survival and metabolic activity of soil microorganism and plants, and thus interfere with their ability to enter into symbiotic interactions (Zahran, 1999). So, microbes and plants have to evolve to adapt to the constant changing and often inhospitable soil environment. Nevertheless, the dual mode of existence of rhizobia, as free-living or in symbiosis, gives them advantages in terms of survival and persistence over most other soil bacteria.

It is predictable that any environmental factor that negatively influences either the growth of rhizobia or the host plant itself has an impact on symbiotic nitrogen fixation. However, these factors can directly influence the plant growth during post-nodulation events and consequently the efficient functioning of the nitrogenase enzyme complex, or indirectly affect the nodulation process itself and thus affect nitrogen fixation. Herein, the negative impacts of acid and salt stresses in rhizobia and consequently in symbiosis are addressed.

Salt stress

Soil salinity affects about 800 Mha of arable lands worldwide (Munns and Tester 2008), and this area is expanding. Salinity affects agricultural production in arid and semiarid regions, where rainfall is limited and is not sufficient to transport salts from the plant root zone (Tester and Davenport, 2003).

Salt stress affects both bacteria and plants in two ways: it induces ionic stress due to the high concentration of ions and also osmotic stress through the change in the solute concentration around the cells, producing water deficit and desiccation. In general, legumes are more sensitive to salinity than their rhizobial counterparts, and consequently the symbiosis is more sensitive to salt stress than the free-living rhizobia. Salt stress may inhibit the initial steps of the symbiosis (nodule initiation, nodule infection, and development) but it also has a depressive effect on nitrogen fixation (Zahran, 1999). The detrimental effects of salt stress on inoculum viability, nodulation and nitrogen fixation have been reported for many *Rhizobium* spp. strains (Israel *et al.*, 1988). It has been reported that fast growing rhizobia are more salt-tolerant than slow-growing rhizobia (Zahran, 1999).

Rhizobia subjected to salt stress may undergo morphological alterations, leading to changes in cell morphology and size or modifications in the pattern of extracellular polysaccharides (EPS) (Lloret *et al.*, 1998; Vanderlinde *et al.*, 2010) and lipopolysaccharides (LPS) (Sousi *et al.*, 2001; Vanderlinde *et al.*, 2009). The

latter responses may have an impact on the symbiotic interaction because EPS and LPS are necessary to establish symbiosis and for the development of root nodules.

Many microbes, including rhizobia, use distinct mechanisms for osmotic adaptation upon salt stress (Zahran, 1999). In general, the metabolism of *Rhizobium* is slowed down under osmotic stress, due to the repression of genes implicated in the tricarboxylic acid cycle, in the uptake of carbon supply, in glycogen metabolism, in the respiratory chains, and in ribosome composition (Domínguez-Ferreras *et al*., 2006). Furthermore, alteration of the expression of genes encoding chaperones and elongation factors as well as genes involved in cell division are observed in rhizobia during salt stress (Wei *et al*., 2004; Miller-Williams *et al*., 2006).

Intracellular accumulation of low-molecular-weight organic solutes (osmolytes), including amino acids, sugars, and polyamines, or the accumulation of ions, to equilibrate internal and external osmotic concentrations has been observed in some species of rhizobia when subjected to salt stress (Talibart *et al*., 1997; Pichereau *et al*., 1998; Gouffi *et al*., 1999; Rüberg *et al*., 2003). Some compatible solutes can be used as either nitrogen or carbon sources for growth, suggesting that their catabolism may be regulated to prevent degradation during salt stress. For example, *E. meliloti* overcomes salt stress-induced growth inhibition by accumulating compatible solutes, glutamate and proline (Rüberg *et al*., 2003; Domínguez-Ferreras *et al*., 2006). The *glgA2*, *glgB2*, and *glgX* genes involved in glycogen metabolism are expressed at higher levels during exposure to salt stress, indicating that glycogen accumulates during salt stress (Domínguez-Ferreras *et al*., 2006).

The transport systems of ions may also be involved in rhizobia response to salt stress due to the intracellular accumulation of potassium and some polyamines (Zahran, 1999; Vriezen *et al*., 2007). Nogales *et al*. (2002) identified a *kup* gene specifying an inner-membrane protein involved in potassium uptake, which confers salt tolerance in *Rhizobium tropici.* Another identified transporter involved

in the early response to osmotic stress was BetS (betaine/proline transporter) in *E. meliloti* (Boscari *et al.*, 2002).

Several genes such as *ntrY* (nitrogen regulator), *noeJ* (mannose-1-phosphate guanylytransferanse), *alaS* (alanyl-tRNA synthase), *dnaJ* (heat-shock chaperone), *greA* (transcription elongation factor), *omp*10 (outer membrane lipoprotein), *relA* ((p)ppGpp synthetase) and *nuoL* (NADH dehydronase I chain L protein) were identified as being involved in salt stress response in rhizobia (Nogales *et al.*, 2002; Wei *et al.*, 2004). Despite that several genes have been identified in rhizobia response to salinity, the tolerance mechanisms of rhizobia to overcome salt stress remains unknown, mainly due to the fact that response and adaptation to salinity stress is a complex phenomenon involving many physiological and biochemical processes that likely reflect changes in gene expression.

Acid stress

Agricultural practices and climate changes increase the amount of land affected by acidity, and thus limit legume crop productivity. Worldwide, more than 1.5 Gha of acid soils limit agriculture production (Graham and Vance, 2000) and as much as 25% of the Earth's croplands are impacted by problems associated with soil acidity (Munns, 1986).

Legumes and their rhizobia exhibit varied response to soil acidity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when depending on symbiotic nitrogen fixation (Rice *et al.*, 1977; Brockwell *et al.*, 1991; Bordeleau and Prévost, 1994). However, differences in acid soil tolerance by legumes have been reported. Some species, like lucerne (*Medicago sativa*), are extremely sensitive to acidity (Rice *et al.*, 1977), while others, such as *Lotus tenuis* tolerate relatively low soil pH (Miñón *et al.*, 1990).

Rhizobia differ in their response to acidity when grown in liquid culture. Some are moderately acid tolerant such as *Mesorhizobium ciceri* (Laranjo and Oliveira,

2011), *Mesorhizobium loti* and *R. tropici* (Wood *et al.*, 1988) while others, like *E. meliloti*, are very sensitive to acidity (Brockwell *et al.*, 1991; Tiwari *et al.*, 1992). Nevertheless, different strains of the same species may also vary widely in their pH tolerance (Glenn and Dilworth, 1994).

Rhizobia can be more sensitive to low pH than their legume host. Indeed, it is in many cases the inability of the microsymbiont to persist under acidic conditions that reduces the effectiveness of the symbiosis. Brockwell *et al.* (1991) reported a nearly 10^{-3} decrease in the number of *E. meliloti* in soils with a $pH < 6$ compared to those with a $pH > 7.0$. Therefore, soil acidity is one of the main environmental factors limiting the establishment of the *rhizobium*-legume symbiosis, mainly because acidity limits rhizobia survival and persistence in soil, leading to a reduction or inexistent nodulation (Zahran, 1999). So, the selection of rhizobial strains tolerant to low pH may improve the acid tolerance of the legume through an efficient symbiotic nitrogen fixation under acidity conditions. However, the relationship between soil acidity and rhizobia competitiveness, and ability to survive in acid soils is not always straight forward.

Although some acid-tolerant rhizobia strains have been identified (Wood *et al.*, 1988; Vinuesa *et al.*, 2005; Laranjo and Oliveira, 2011), the mechanisms that they use to survive and grow under acidic conditions have not been fully elucidated and therefore the molecular basis for differences in pH tolerance among strains of rhizobia is still not clear. Several reports point out that possible mechanisms involved in pH tolerance are the regulation of cytoplasmic pH (Chen *et al.*, 1993; Graham *et al.*, 1994), proton exclusion and/or extrusion (Chen *et al.*, 1993), exopolysaccharide production (Cunningham and Munns, 1984), and changes in the hydrophobicity of the plasma membrane (Graham *et al.*, 1994). In several bacteria, like *R. leguminosarum* bv trifolii these mechanisms have been described as being an adaptive response to pH (O'Hara and Glenn, 1994), while in others, such as in the acid tolerant *R. tropici* UMR 1899 these mechanisms seem to be constitutive (Graham *et al.*, 1994).

Acidic soils are often associated with increased aluminium and manganese toxicity and reduced calcium availability, since these metals become more soluble at lower pH (Hungria and Vargas, 2000; Reeve *et al*., 2002). Rhizobia exhibit an adaptive acid tolerance response that is influenced by calcium (Glenn *et al*., 1999). These effects of calcium under acid conditions might be explained by calcium stabilization of various cellular components, or by direct or indirect calcium effects on gene expression. Furthermore, when rhizobia are exposed to acidic conditions, the presence of a carbon source and the rhizobial ability to take it up enhances their survival (Clarke *et al*., 1993; Steele *et al*., 1999). The activation of glutathione synthesis might be essential for tolerance to acid stress (Muglia *et al*., 2007). Furthermore, acidity can affect the size and morphology of rhizobia and their potassium content (Watkin *et al*., 2003).

More recently, using molecular techniques, several genes, such as *actA, actP, exoR, lpiA, actR, actS,* and *phrR*, were shown to be essential for rhizobia growth at low pH. For instance, the protein products of these genes include ActA (an apolipoprotein acyl transferase) (Tiwari *et al*., 1996a), ActS (a histidine kinase 'sensor') (Tiwari *et al*., 1996b), ActR (a response regulator) (Tiwari *et al*., 1996b) and ActP (a CPx heavy metaltransporting ATPase) (Reeve *et al*., 2002); LpiA (a putative transmembrane protein (Reeve *et al*., 2006) or exopolysaccharide biosynthesis (*exoR*) (Dilworth *et al*., 2001). Vinuesa *et al*. (2003), using a *Tn*5-mutagenesis approach, isolated and characterized the pH-responsive genes, *lpiA* and *atvA*, from *Rhizobium* tropici CIAT899. The microarray data of *E. meliloti* wild-type and sigma *rpoH1* mutant, upon acid stress, pointed out several genes as being involved in acid-response (de Lucena *et al*., 2010). This study also allowed identification of genes related with distinct function, for example *ibpA*, *grpE* and *groEL5*, as known to be involved in heat shock, *tufA* and *rplC*, as genes involved in translation.

1.8 Molecular chaperones

To be functional, proteins must adopt a defined three-dimensional structure called the native fold. However, folded proteins are at permanent risk of unfolding, especially under environmental stress conditions (Jahn and Radford, 2005). Bacteria have evolved several mechanisms that ensure protein folding and promote homeostasis under stress conditions (Frydman, 2001). One of the mechanisms relies on the activation of proteins such as molecular chaperones, proteases, and regulatory factors (Hartl *et al*., 2011).

The collectively termed molecular chaperones are complex machineries that include several conserved protein families and are recognized by their action on the guidance of proteins to their “proper” fate but do not remain associated with the final product (Wickner *et al*., 1999). Members of these protein families are often known as stress proteins or heat-shock proteins (HSPs), as they are upregulated under conditions of stress in which the concentrations of aggregation-prone folding intermediates increase. Several different classes of structurally unrelated chaperones exist in cells, forming cooperative pathways and networks, which guarantee the optimal functionality of proteins during their life-time (Hartl *et al*., 2011).Two main functions attributed to several molecular chaperones comprise folding (including *de novo* folding and refolding of stress-denatured proteins) and disaggregation (Fig 1.3).

Protein Folding

In the bacterial cytosol, the folding of new proteins is assisted by three major molecular chaperone complexes well characterized in *E. coli*: Trigger factor (TF), DnaK-DnaJ-GrpE and GroEL-GroES complexes (Genevaux *et al*., 2004; Hartl and Hayer-Hartl, 2002). TF is the first chaperone to interact with nascent polypeptide chains coming from the bacterial ribosome (Pfanner, 1999).

DnaK–DnaJ–GrpE and GroEL–GroES are multicomponent molecular machines that promote folding through ATP- and cofactor-regulated binding and release cycles. They typically recognize hydrophobic amino-acid side chains exposed by non-native proteins and may functionally cooperate with ATP-independent chaperones, such as IbpA and IbpB. The two systems act sequentially, whereby DnaK system interacts upstream with nascent and newly synthesized polypeptides and the GroESL function downstream in the final folding of those proteins that fail to reach native state by cycling on DnaK system alone (Frydman *et al*., 1994; Kolaj *et al*., 2009).

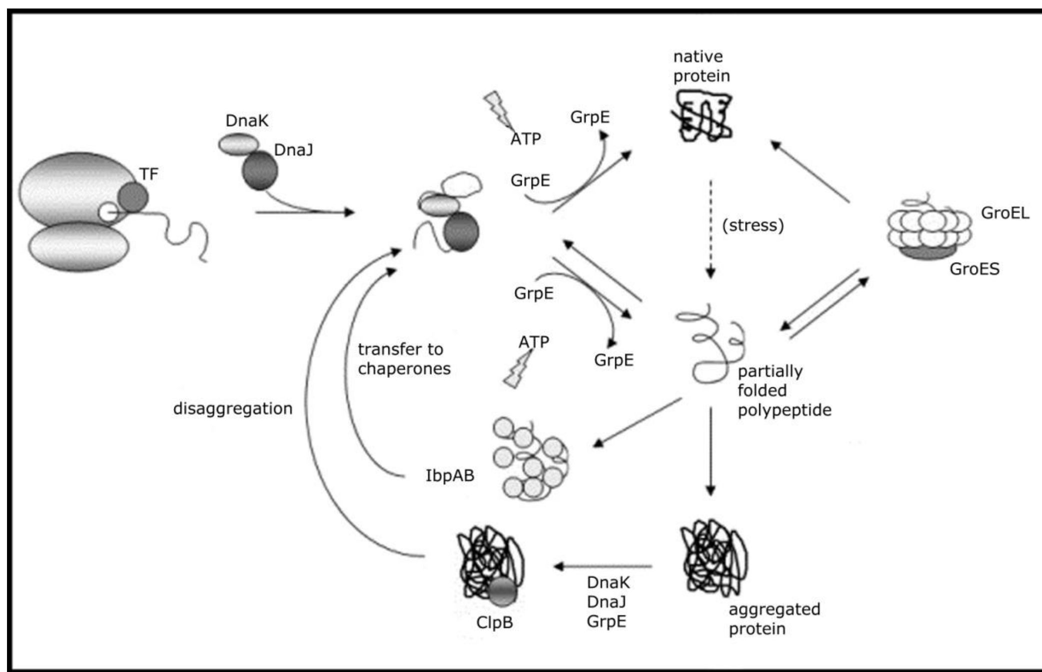


Figure 1.3 Illustration of the process of protein folding in *E. coli* cytoplasm (from Kolaj *et al*., 2009).

DnaK binds to solvent-exposed hydrophobic regions in unfolded polypeptide chains, assisting the folding and preventing misfolding and/or aggregation in an ATP-driven process that is regulated by the co-chaperone DnaJ and the nucleotide-release cofactor GrpE (Genevaux *et al*., 2004). Hydrolysis of ATP to ADP is strongly accelerated by DnaJ, leading to lid closure and stable peptide

binding, and DnaJ also interacts directly with unfolded polypeptides and can recruit DnaK to protein substrates (Kampinga and Craig, 2010).

The folding activity of GroEL requires the cooperation of the co-chaperone GroES to form the GroESL complex in the presence of ATP (Chaudhuri *et al*., 2009). The apical domains of GroEL present hydrophobic amino-acid residues for substrate binding in the ring centre. Subsequent folding depends on global substrate encapsulation by GroES. The complex GroESL can assist the protein folding by two different ways: a *cys-* folding action or a *trans-* folding mechanism. Nevertheless, independently of the mechanism, for many proteins, multiple binding-release cycles are necessary in order to obtain correct folding (Farr *et al*., 2003; Chaudhuri *et al*., 2009).

Protein Disaggregation

Partially folded or misfolded proteins are problematic because they tend to aggregate in a concentration-dependent manner. In bacteria, a network of chaperones and proteases carries out the processing of aggregated protein. The key elements recognized in protein disaggregation are DnaK and ClpB. ClpB belongs to the superfamily AAA^+ proteins, but contrary to the other proteins included in the superfamily AAA^+, the activity of ClpB does not consist in the generic proteolytic action, but it is specifically involved in protein aggregate disintegration (Dougan *et al*., 2002). Interestingly, the ClpB activity in fully protein disaggregation is achieved in collaboration with the DnaK–DnaJ–GrpE system (Fig. 1.4) (Acébron *et al*., 2009). The DnaK system acts previously or together with ClpB (Fig. 1.4). Initially, DnaJ associates with the aggregate and drives DnaK to the aggregate surface, and this mediates the binding of ClpB to the aggregate (Fig 1.4 (i)). ClpB operates by an ATP-dependent mechanism. The disaggregation process occurs by translocating proteins from the aggregate through their central channel (Fig. 1.4 (ii)) and releasing them in an unfolded state into the solution (Fig. 1.4 (iii)). Once released, proteins may refold

spontaneously or require the involvement of other chaperones including the DnaK system (Fig. 1.4 (iv)).

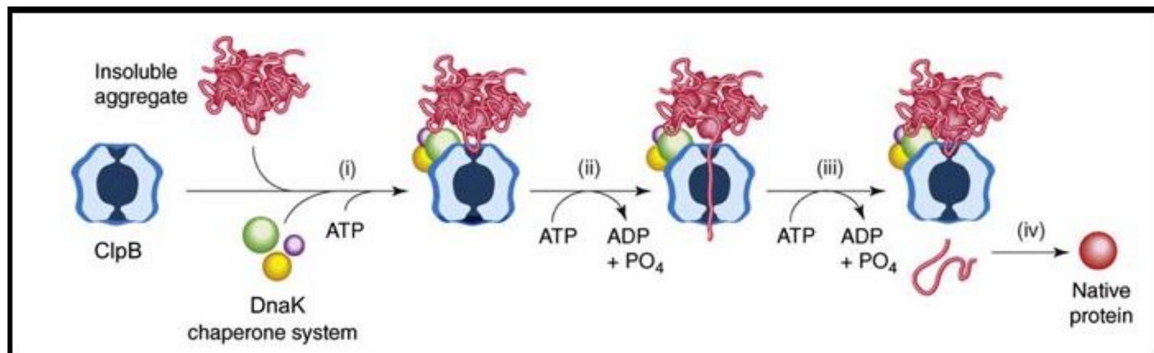


Figure 1.4 Disaggregation of bacterial aggregates by ClpB in combination with the DnaK system (adapted from Diemand and Lupas, 2006).

It is possible that ClpB function as a disaggregation machine may rely on the ATP hydrolysis at the two nucleotide-binding domains of each monomer. Furthermore, the probable mechanism of disaggregation–refolding by ClpB involves the extraction of polypeptides from aggregates by forced unfolding, translocation through ClpB central pore and release into cellular milieu for spontaneous or chaperone-mediated refolding (Diemand and Lupas, 2006; Doyle and Wickner, 2009).

1.9 Major molecular chaperones in rhizobia

The DnaK-DnaJ-GrpE and GroESL systems are the best characterized molecular chaperone systems in *E. coli*. In contrast, ClpB system is not fully understood (Doyle and Wickner, 2009). In rhizobia, these major chaperones systems are less studied and understood.

Several copies of the *groEL* gene are found in rhizobia genomes. *Bradyrhizobium japonicum*, *Ensifer meliloti*, *Mesorhizobium sp.* MAFF303099, *Rhizobium etli* and *Rhizobium leguminosarum,* all have three or more *groEL* homologues (Fischer *et al*., 1993; Rusanganwa and Gupta, 1993; Ogawa and Long, 1995; Kaneko *et al*.,

2000; Galibert *et al.*, 2001; Young *et al.*, 2006). Despite the similar degree in functionality within the multiple *groESL* copies, these copies have different regulation systems and are differentially induced in rhizobia.

B. japonicum shares similar regulation mechanisms of heat shock genes described for *E. coli* (positive regulation by the σ^{32} factor) and *B. subtilis* (negative regulation by the CIRCE system) (Babst *et al.*, 1996). Only *groESL1* is σ^{32} dependent while the *groESL4,5* are under control of a CIRCE element (Fischer *et al.*, 1993; Babst *et al.*, 1996; Minder *et al.*, 2000). A third mechanism regulates the *groESL3,* which is under the control of the NifA activator and σ^{54} (Fischer, 1994). Both CIRCE and σ^{32} dependent regulation were found in *R. leguminosarum* and *E. meliloti* (Mitsui *et al.*, 2004; Bittner and Oke, 2006; Gould *et al.*, 2007).

The multiple *groESL* copies are also differentially induced. For example, in *B. japonicum*, *groESL1,4,5* are heat inducible and *groESL3* is induced by low oxygen conditions (Fischer *et al.*, 1993, Babst *et al.*, 1996). In *R. leguminosarum* only one of the three *groEL* homologues is needed for normal growth and corresponds to the highly expressed one (Rodriguez-Quinones *et al.*, 2005). In *E. meliloti*, two of the five *groESL* copies are heat inducible (Mitsui *et al.*, 2004; Bittner and Oke, 2006) and each one is regulated by a distinct mechanism. One operon was found to be involved in the regulation of early *nod* genes (Ogawa and Long, 1995).

In rhizobia, the DnaKJ system has been less studied than the GroESL system. As in most bacteria, also in rhizobia the *dnaK* gene is found as a single copy gene. In contrast, its co-chaperone *dnaJ* is often found in several copies in the genome.

The *dnaKJ* operon was characterized in *B. japonicum* and was found to be under the control of σ^{32} factor (Minder *et al.*, 1997). Although for some organisms the *dnaK* gene does not seem to be essential for normal growth, in *B. japonicum*, it seems to be essential since no *dnaK* knockout mutant could be obtained. In

contrast, *dnaJ* mutants were successfully obtained in different rhizobia species. *Rizobium tropici dnaJ* mutants showed higher sensitivity to salt stress condition while *B. japonicum dnaJ* mutants displayed slower growth at high temperatures (Minder *et al*., 1997; Nogales *et al*., 2002).

The ClpB chaperone has been scarcely studied in rhizobia. Analysis of the complete genomes available shows that the *clpB* gene is found mostly as a single copy gene in rhizobia. It seems that *clpB* gene is under regulation of the *rpoH*1 gene in *E. meliloti* (Mitsui *et al*., 2004; de Lucena *et al*., 2010). Transcriptional analyses of *E. meliloti* cells upon heat shock and acid stress showed up-regulation of *clpB* in both stress conditions, suggesting that this chaperone may be involved in stress response in rhizobia (Sauviac *et al*., 2007; de Lucena *et al*., 2010).

Although all these molecular chaperones are known to be involved in the heat shock response in many organisms, several studies suggest their involvement in other types of stresses due to their ability to help denatured or aggregated proteins to reach their native conformation, and thus their functional state (Thomas and Baneyx, 1998). In rhizobia, the DnaKJ and GroESL are associated to heat shock response. However, little is known about the involvement of these molecular chaperones in response to salinity or acidity conditions. A few transcriptomic studies pointed out their possible involvement in rhizobia tolerance to acid or salt stresses (Domínguez-Ferreras *et al*., 2006; Hellweg *et al*., 2009; de Lucena *et al*., 2010)

Major molecular chaperones in the legume-rhizobia symbiosis

Transcriptomic and proteomic analyses of rhizobia in symbiosis with host plants suggest the involvement of chaperones, such as ClpB and GroESL, in the symbiosis (Djordjevic *et al.*, 2003; 2004; Karunakaran *et al.*, 2009).

However, the involvement of these molecular chaperones in the nitrogen-fixing simbiosis seems to be controversial, probably due to their involvement in different stages in the symbiosis process of rhizobia. For example, *dnaJ* is required for effective symbiosis of *R. leguminosarum* bv. *phaseoli* (Labidi *et al.*, 2000), however in *B. japonicum* the symbiotic performance of *dnaJ* mutants was not altered (Minder *et al.*, 1997). 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. In *E. meliloti,* the DnaK chaperone is required for optimum symbiotic function (Summers *et al.*, 1998).

Furthermore, in *E. meliloti,* a *groESL*5 mutant strain is able of normal symbiotic nitrogen fixation (Mitsui *et al.*, 2004). From the five *groESL* operons in the *E. meliloti* genome only one operon (*groEL1*) was found to be involved in symbiosis (Ogawa and Long, 1995). All single mutants in *E. meliloti* are viable but double mutants are depleted in their symbiotic phenotype (Fischer *et al.*, 1999; Bittner *et al.*, 2007). Similarly, *B. japonicum* mutants that individually lack one *groEL* gene do not change the symbiotic phenotype (Fischer *et al.*, 1993) while double mutation on *groEL*3 and *groEL*4 genes affects the symbiotic performance (Fischer *et al.*, 1999). These two copies are the ones most abundant in GroEL pool in bacteroids (Fischer *et al.*, 1993) and are required for the formation of a functional nitrogenase in *B. japonicum* (Fischer *et al.*, 1999).

In contrast to the other two major chaperone systems mentioned above, there are no reports on the characterization of the role of chaperone ClpB in the symbiosis process.

1.10 Aims of the study

Due to the rapid increase of the area of damaged soils, mainly due to acidity and salinity, the demand for isolation and development of rhizobial strains tolerant to either stress conditions is imperative. On the other hand, and in spite of the studies conducted during the last decades, the molecular bases of the tolerance of legume-rhizobia systems to environmental stresses remains largely unknown.

The present study was focused in the following main objectives:

- To evaluate the tolerance of a collection of native Portuguese chickpea rhizobia to acid and salt stress;

- To investigate the molecular bases of acid- and salt-tolerance in rhizobia, namely the transcriptional levels of major chaperone systems genes, *dnaKJ* and *groESL,* upon acid or salt-shock;

- To investigate the symbiotic performance under salt stress of chickpea rhizobia expressing an exogenous ACC deaminase gene;

- To investigate the role of the chaperone ClpB in the tolerance to environmental stresses as well as in the symbiosis, through *clpB* gene deletion in a chickpea *Mesorhizobium* strain.

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Chapter 2

Isolation and characterization of native chickpea rhizobia

The study of a large collection of rhizobia isolates serves for diverse proposals, including ecological studies, search for inoculants with economic and agricultural interest and eventually to find new species. Moreover, isolates characterization can bring new insights regarding their relationships with the host and environment. This chapter describes the isolation and characterization of a large collection of native Portuguese chickpea rhizobia. C. Brígido isolated and characterized a subset of the rhizobia collection.

This chapter is based on the following manuscripts:

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

Nascimento, F.X., Brígido, C., Glick, B.R., Oliveira, S. (2012) “ACC deaminase genes are conserved between *Mesorhizobium* species able to nodulate the same host plant” FEMS Microbiology Letters, 336:26-37.

2.1 Summary

Several *Mesorhizobium* species are able to induce effective nodules in chickpea, one of the most important legumes worldwide. Our aims were to examine the diversity and biogeography of chickpea rhizobia considering Portugal as a case study. In addition, the phylogeny of the symbiosis genes, *nifH* and *nodC*, as well as the ACC deaminase (*acdS*) genes of chickpea rhizobia isolates was examined. One hundred and twenty one isolates were obtained from continental Portugal and Madeira Island. The 16S rRNA gene phylogeny revealed that isolates are highly diverse, grouping with most known *Mesorhizobium* type strains. Interestingly, only 37% of the isolates grouped with *M. ciceri* or *M. Mediterraneum,* the formerly described specific chickpea microsymbionts. Although chickpea is nodulated by many different species, phylogenetic analysis of the symbiosis genes revealed that they share common symbiosis genes (*nifH* and *nodC*), confirming the suggestion of lateral transfer of symbiosis genes across different species. Moreover, *acdS* genes were detected in 10 of 12 mesorhizobia type strains as well as in 17 of the 17 chickpea mesorhizobia isolates. The phylogenetic analysis indicates that strains belonging to different *Mesorhizobium* species, but nodulating the same host plant, have similar *acdS* genes, suggesting that *acdS* genes were horizontally acquired. An association between province of origin and species cluster of the isolates was found. A correlation was found between species cluster and origin soil pH of the isolates, suggesting that pH is a key environmental factor, which influences the species geographic distribution. To our knowledge, this is one of the few surveys on chickpea rhizobia and the first systematic assessment of indigenous rhizobia in Portugal.

2.2 Introduction

Chickpea (*Cicer arietinum*) is the third most important legume crop worldwide, after dry bean and pea (FAO, 2008). Nevertheless, few studies addressed the genetic diversity of native chickpea rhizobia (Kuykendall *et al.*, 1993; Nour *et al.*, 1994a; Aouani *et al.*, 2001; Maâtallah *et al.*, 2002b). In Portugal, only a small area has been studied (Laranjo *et al.*, 2001; Laranjo *et al.*, 2002, Laranjo *et al.*, 2004).

Jarvis *et al.* (1997) included rhizobia that nodulate chickpea in the genus *Mesorhizobium*. Two species were first identified as specific chickpea microsymbionts: *M. ciceri* (Nour *et al.*, 1994b) and *M. mediterraneum* (Nour *et al.*, 1995). Chickpea has been considered a narrow-host range legume (Broughton and Perret, 1999). Nevertheless, recent studies have shown that chickpea is able to establish symbioses with several species of *Mesorhizobium*, namely *M. amorphae*, *M. loti*, and *M. tianshanense*, however, these carry symbiosis genes (*nodC* and *nifH*) identical to those carried by *M. ciceri* and *M. mediterraneum* (Rivas *et al.*, 2007; Laranjo *et al.*, 2008).

Bacterial phylogeny has relied on the sequence analysis of single core genes, mainly the 16S rRNA gene (Menna *et al.*, 2006) but also on other housekeeping genes, such as *atpD* and *recA* (Young and Park, 2007). Still, in the last decade, analysis of the 16S rRNA gene has been, by far, the most widely used approach to define molecular phylogeny and taxonomy of bacteria (Gevers and Coenye, 2007; Sun *et al.*, 2008). The 16S rRNA gene is the only sequence available for most bacterial species, including type strains. Thus, the 16S rRNA gene is a useful tool for placing any new isolate among its closer taxonomic relatives. Despite the fact that species of *Mesorhizobium* genus share a high similarity in terms of 16S rRNA gene sequence, which limits mesorhizobia chickpea isolates affiliation, the 16S rRNA sequence can be useful for a broad identification of a high number of isolates.

The plant hormone ethylene is known for its inhibitory effects in various aspects of nodule formation and development (Guinel and Geil, 2002). Nevertheless, some rhizobial strains utilize different mechanisms for lowering ethylene levels such as the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which is responsible for the cleavage of ACC (the immediate precursor of ethylene in plants) into ammonia and α-ketobutyrate (Honma and Shimomura, 1978). The prevalence of ACC deaminase genes in rhizobia has been studied primarily in *Rhizobium* spp. (Ma *et al*., 2003; Duan *et al.,* 2009). In these studies, many *Rhizobium* spp. have been found to possess an *acdS* gene and produce ACC deaminase under free-living conditions (Duan *et al*., 2009). The first report on *acdS* gene presence in *Mesorhizobium* was obtained following the complete sequencing of *M. sp.* MAFF303099 (Kaneko *et al*., 2000). Subsequently, the presence of an *acdS* gene in the symbiosis island of *M. loti* R7A was also reported (Sullivan *et al.,* 2002). Despite the presence of ACC deaminase in some *Mesorhizobium* strains, not much is known about the environmental distribution of *acdS* genes in this bacterial genus.

The aims of this study were to examine the biogeography of rhizobia able to nodulate chickpea, to investigate the presence of a predominant chickpea rhizobia species and to identify the most efficient species in the symbiosis, considering Portugal as a case study. A survey on chickpea rhizobia was carried out in continental and insular Portugal. Genetic diversity of native isolates was examined through molecular phylogeny based on 16S rRNA gene sequences and by plasmid profiles analysis. Symbiotic effectiveness of chickpea native isolates was estimated. Additionally, the phylogeny of the symbiosis genes (*nifH* and *nodC*) as well as the *acdS* genes of some *Mesorhizobium* type strains and chickpea rhizobia isolates was examined.

2.3 Materials and Methods

Isolates collection

More than 45 soil sampling sites were collected of the 11 provinces of continental Portugal and from the Madeira and Azores Islands. Only one of the soil sampling sites was known as been used for chickpea cultivation, namely ENMP site, which is an experimental agricultural field.

Chickpea seeds (Chk 3226) were surface-sterilized with calcium hypochlorite 14%, washed with sterile distilled water, and pre-germinated in water-agar. Seeds were sown in sterilized pots containing the soil samples. Plants were maintained in the plant growth chamber under controlled conditions for 8 weeks. Nodules were harvested and isolates were obtained as described by Somasegaran and Hoben (1994). Isolates were re-inoculated, under sterile and controlled conditions, in order to confirm their ability to nodulate chickpea.

DNA extraction and manipulation

Rhizobial strains and isolates were grown in 5 mL of tryptone yeast (Beringer, 1974) medium at 28°C for 2 to 4 days. The bacterial cultures were centrifuged at 16000 x g for 1 minute and used for genomic DNA extraction using the E.Z.N.A bacterial DNA kit (Omega) following the manufacturer's suggested protocol or according to the protocol described by Rivas *et al.* (2001).

Amplification of the 16S rRNA, *nifH*, *nodC* and *acdS* genes

The 16S rRNA gene was amplified for each isolate using primers Y1 (Young *et al.*, 1991) and Y3 (Laranjo *et al.*, 2004), corresponding to positions 20 to 1507 in *Escherichia coli.* Amplification reaction was carried out as previously reported (Laranjo *et al.*, 2004).

The *nifH* gene was amplified by PCR using primers and conditions previously described by Laranjo *et al.* (2008). For amplification of *nodC* gene, primers and conditions described by Rivas *et al.* (2007) were used. To amplify the *acdS* gene of mesorhizobia type strains and chickpea mesorhizobia isolates, PCR primers were designed based on the *M. sp.* MAFF303099 and *M. ciceri* bv. *biserrulae* WSM1271 *acdS* gene sequences, resulting in primers *acdS*-F2 (5'-CAAGCTGCGCAAGCTCGAATA-3') and *acdS*-R6 (5'-CATCCCTTGCATCGATTTGC-3'). The *acdS* gene was amplified using the following program: 3 min of initial denaturation at 95ºC, 35 cycles of 1 min denaturation at 94ºC, followed by 1 min and 30 sec of primer annealing at 49ºC and 1 minute of elongation at 72ºC, and a final elongation step of 5 minutes at 72ºC. A fragment with 760 bp-long was the expected amplification product. Polymerase chain reaction (PCR) products were purified using GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare) or ExoSAP-It (USB) following the manufacturer's instructions.

Phylogenetic analysis

Nucleotide sequences were analyzed and edited using BioEdit Sequence Alignment Editor (version 7.0.4.1) (Hall, 1999). Alignments were generated using Clustal W (Thompson *et al*., 1994). The 16S rRNA, *nifH*, *nodC* and *acdS* gene sequences obtained were compared with those from GenBank database. Phylogenetic and molecular evolutionary analyzes were performed using MEGA5 (version 5.05) software (Tamura *et al*., 2011) using both neighbor-joining and maximum likelihood methods. The best evolutionary model of nucleotide substitutions was determined for each phylogenetic analysis. Bootstrap analysis was based on 1000 replicates.

Plasmid profiles

The plasmid profiles were analyzed by horizontal agarose gel electrophoresis using a two-comb system, as described by Laranjo *et al.* (2001).

Symbiotic effectiveness (SE)

Plant growth chamber trials were performed under controlled conditions in order to evaluate the symbiotic effectiveness (SE) of the isolates (Laranjo *et al*., 2002). Pre-germinated chickpea seeds, obtained as described before, were sown in sterilized vermiculite and inoculated with a bacterial suspension of each isolate grown in yeast mannitol agar (Vincent, 1970). Uninoculated plants were used as negative control and uninoculated plants supplemented with nitrogen (140 ppm nitrogen as KNO_3, in the nutrient solution) were used as positive control. Three replicates were used for each treatment. Plants were collected after 8 weeks and several parameters were measured, such as shoot dry weight, root dry weight, number of nodules, and nodules dry weight. Symbiotic effectiveness (SE) was determined as the ratio of shoot dry weight (SDW) of inoculated plants minus SDW of non-inoculated control plants and SDW of non-inoculated nitrogen-supplemented control plants minus SDW of non-inoculated control plants (Gibson, 1987). Symbiotic effectiveness is presented as percentages.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Relationships between categorical variables were determined using the chi-square test of association. Relationships between a continuous variable and an unordered categorical variable were tested using analysis of variance (one-way ANOVA). Results are presented as the test statistic (χ^2), degrees of freedom (*df*), and probability of equal or greater deviation (*P*). In the case of no homogeneity of variances, the Kruskal–Wallis test, as well as the

Welch test, was used instead of the ANOVA, in order to investigate a possible relationship between soil pH of the sampling sites and species group of the isolates. Different post hoc tests (Tamhane, Dunnett T3 and Games-Howell) were conducted to search for categories that differ significantly from others. Correspondence analysis (CA) was used as an explorative method to study associations and to reveal interdependencies between two variables (Benzécri, 1973). Visualization using CA is based on representing χ^2 distances among variables.

2.4 Results

Isolation and characterization of native chickpea rhizobia from the following origin sites were performed by Ana Alexandre, M. Laranjo or S. Rodrigues: Beja, Caldas da Rainha, Coimbra, Elvas, ENMP, Évora, Leiria, Lamego, Guarda, Portalegre, Setúbal, Sintra and Viseu.

Isolates collection

Although several sampled sites were collected, only in 26 of the total sampling sites were confirmed as containing rhizobia able to nodulate chickpea. Moreover, no nodules were obtained with any of the soil samples collected from Minho province and Azores Islands. A total of 121 chickpea rhizobia isolates were obtained from 26 sites, covering ten provinces of continental Portugal and Madeira Islands. These isolates were used for further studies. Soil characteristics of each site are shown in Table 2.1.

Table 2.1 Characteristics of soils used to obtain chickpea isolates.

Soils	Phosphorus (ppm)	Nitrogen (ppm)	Potassium (ppm)	Texture	pH	O.M. (%)	E.C. (µmhos/ cm)
Alenquer	ND	ND	ND	ND	ND	ND	ND
Aveiro	44	9	66	Medium	7.08	5.3	50
Aveiro II	200	68	740	Medium	6.07	4.8	120
Beja	108	28	197	Medium	8.20	ND	ND
Bragança	444	681	710	Medium	6.69	4.7	430
Caldas da Rainha	82	131	170	Medium	6.83	3.2	300
Castelo Branco	248	132	216	Medium	6.46	6.6	80
Coimbra	108	66	224	Medium	5.66	2.4	80
Elvas	48	64	143	Medium	6.20	ND	ND
ENMP	529	12	137	Medium	7.90	ND	ND
Évora	56	47	55	Coarse	5.10	ND	ND
Guarda	476	74	1060	Coarse	7.37	5.1	110
Lamego	563	53	580	Medium	6.58	5.8	60
Leiria	182	57	356	Medium	8.19	2.0	120
Portalegre	26	87	152	Medium	5.25	3.7	110
Portimão	744	194	1600	Medium	8.66	3.1	290
Portimão I	318	153	180	Medium	7.20	3.7	130
Porto II	40	35	340	Medium	6.37	8.3	70
Praia do Alemão	16	7	86	Coarse	7.80	1.2	74
Salir	46	9	50	Coarse	8.90	1.5	156
Santarém	140	101	530	Medium	7.83	4.7	110
Serra d' Água	74	42	560	Medium	7·63	1.7	70
Setúbal	631	18	350	Medium	8.06	ND	ND
Sintra	808	352	910	Medium	7.83	4.1	20
Telhado	210	86	178	Medium	7.32	2.8	70
Viseu	12	11	126	Medium	5.94	2.4	50

Soil sample analyses were performed in the Laboratório Químico Agricola of the University of Évora, Portugal. O.M- Organic matter, E.C. Electrical conductivity; ND-not determined

Phylogeny based on the 16S rRNA gene analysis

Genbank accession numbers for the 16S rRNA gene sequences of all isolates are shown in Table 2.2 and compared to those available on GenBank (Table 2.3).

Table 2.2 Rhizobia isolates used in the present study. Province of origin, 16S rRNA gene accession number, species cluster defined from the 16S rRNA gene sequence analysis, plasmid number and symbiotic effectiveness (SE) values are indicated for each isolate.

Province	Origin	Isolate	16S rRNA gene accesion number	Species cluster	Plasmid number	SE (%)
Trás-os Montes e Alto Douro	Bragança	BR-8	EU652123	B	2	45
		BR-9	EU652124	B	1	43
		BR-15	EU652125	B	2	21
		BR-16	EU652126	B	2	35
		BR-28	EU652127	B	0	48
	Lamego	LM-1	EU652128	A	1	14
		LM-9	EU652129	A	1	55
		LM-13	EU652130	A	1	11
		LM-18	EU652131	B	1	61
		LM-21	EU652132	A	1	22
Douro Litoral	Porto	PII-1	EU652133	B	3	58
		PII-2	EU652134	B	2	71
		PII-3	EU652106*	B	2	47
		PII-4	EU652135	B	2	31
Beira Litoral	Aveiro	A3	EU652136	A	0	36
		A8b	EU652107*	A	0	0
	Aveiro II	AII5	EU652137	A	2	26
		AII7	EU652138	A	2	32
	Coimbra	C-1	EF504313*	A	1	47
		C-3	EU652108*	A	1	15
		C-7	EU652139	A	1	14
		C-9	EU652140	A	2	20
		C-13	EU652109*	A	1	49
		C-14	EU652110*	A	2	32
		C-15	EU652141	A	1	20
		C-23	EU652142	A	1	23
		C-24	EU652143	A	1	39
		C-25	EU652144	A	2	21
		C-27b	EF504314*	A	1	62
	Leiria	L-19	EU652111*	A	0	48
Beira Baixa	Castelo Branco	CB-10	EU652150	B	0	56
		CB-19	EU652151	B	0	30
		CB-23	EU652152	B	4	52
		CB-30	EU652153	B	4	45
		CB-38	EU652154	B	4	61
		CB-75	EU652155	B	0	38
	Telhado	T-3	EU652157	A	1	32
		T-4	EU652158	A	1	86
		T-5	EU652159	A	0	56
		T-7	EU652160	A	1	54
		T-8	EU652114*	A	1	31
Beira Alta	Guarda	G-1	EU652145	B	0	34
		G-4	EU652146	B	0	41
		G-10	EU652147	B	0	48
		G-24	EU652148	B	0	58
		G-55	EU652149	B	0	88

(Table 2.2 continued)

Province	Origin	Isolate	16S rRNA gene accesion number	Species cluster	Plasmid number	SE (%)
Beira Alta	Viseu	V-5b	EU652112*	A	1	65
		V-15b	EF504315*	A	0	23
		V-18	EF504316*	A	0	67
		V-20	EF504317*	A	1	67
		V-25b	EU652113*	A	1	69
Estremadura	Alenquer	AL-13	JN191652	A	1	15
	Salir	SL-1	JN191657	C	0	21
		SL-2	JN191658	D	0	5
		SL-3	JN191659	A	0	26
		SL-5	JN191660	C	0	30
		SL-6	JN191661	A	2	33
		SL-7	JN191662	C	0	39
		SL-9	JN191663	A	0	5
	Caldas da Rainha	CR-3	EU652161	C	0	77
		CR-16	EU652162	C	0	79
		CR-18	EU652163	C	0	41
		CR-29	EU652164	C	0	55
		CR-32	EU652115*	C	0	57
	Setúbal	ST-2	AY225401*	C	0	4(c)
		ST-5	EU652165	C	0	21
		ST-8	EU652166	C	0	7
		ST-20	EU652167	C	0	43
		ST-33	EU652168	C	0	44
	Sintra	S-1	EU652169	D	3	53
		S-8	EU652116*	B	1	83
		S-15	EU652170	B	0	79
		S-24	JN191653	B	0	100
		S-26	EU652171	B	0	68
Ribatejo	Santarém	STR-2	EU652117*	A	1	40
		STR-4	EU652172	A	1	50
		STR-10	EU652173	A	1	28
		STR-14	EU652118*	C	1	64
		STR-16	EU652174	C	2	49
Alto Alentejo	Elvas	75	AY225386*	B	1(a)	35(c)
		78	AY225387*	A	1(a)	63(c)
		79	DQ787130	B	1(a)	47(a)
		83	DQ787131	A	1(a)	49(a)
		85	AY225388*	A	1(a)	60(c)
		CV-1	DQ787132	A	0(b)	28(b)
		CV-11	DQ787133	A	0(b)	21(b)
		CV-16	AY225389*	B	1(b)	42(c)
		CV-18	AY225390*	A	1(b)	72(c)
	ENMP	EE-2	AY225396*	D	2(b)	36(c)
		EE-7	AY225397*	B	0(b)	84(c)
		EE-12	AY225398*	B	1	10(c)
		EE-14	AY225399*	D	4(b)	32(c)
		EE-29	AY225400*	D	4	21(c)

(Table 2.2 continued)

Province	Origin	Isolate	16S rRNA gene accesion number	Species cluster	Plasmid number	SE (%)
Alto Alentejo	Évora	87	DQ787134	A	1	ND
		89a	DQ787135	A	4	ND
		90	AY225391*	A	1	49(c)
		92	DQ787136	A	2	42(a)
		93	AY225392*	C	0	27(c)
		94	AY225393*	A	1	33(c)
		96	DQ787137	A	2	ND
		98	AY225394*	A	2	72(c)
		101	DQ787138	A	2	ND
		102	AY225395*	A	0(a)	54(c)
	Portalegre	PT-35	EU652119*	A	1	56
Baixo Alentejo	Beja	6b	AY225381*	D	2(a)	76(c)
		7a	AY225382*	B	2	39(c)
		27	AY225383*	B	1(a)	41(c)
		29	AY225384*	D	6	71(c)
		64b	AY225385*	A	1(a)	10(c)
Algarve	Praia do Alemão	PA-5	JN191655	D	0	0
		PA-6	JN191656)	D	0	6
	Portimão	PM-1	EU652175	D	2	51
		PM-14	EU652176	D	2	33
		PM-17	EU652120*	D	2	84
	Portimão II	PMI-1	EU652177	B	1	80
		PMI-6	EU652121*	A	1	81
Madeira	Serra d'Água	SA-4	EU652122*	A	0	63
		SA-9	EU652178	A	0	36
		SA-12	EU652179	A	0	56
		SA-13	JN191654	A	0	58
		SA-17	EU652180	A	3	16

(a) Laranjo *et al.* (2001); (b) Laranjo *et al.* (2002); (c) Laranjo *et al.* (2008); ND–not determined; *complete 16S rRNA gene sequence

Since 2009 several new species have been described, so an update of the 16S rRNA-based phylogenetic analysis of chickpea isolates using the new nucleotide sequences available was performed. In order to analyze the molecular diversity of all rhizobia isolates, phylogenetic analysis was performed using a parcial 16S rRNA gene sequence of rhizobia isolates. A dendrogram was generated by the neighbor-joining method from a 578 bp-long alignment. The 16S rRNA gene-based phylogenetic analysis of 121 isolates shows that all isolates assigned to the genus *Mesorhizobium* (*data not shown*). The 16S rRNA gene based phylogeny of 33 isolates among the 121 rhizobia isolates was performed (Fig.

2.1). Isolates form a large cluster together with the *Mesorhizobium* type strains, which received 99% bootstrap support. Four main clusters (A-D) can be identified, each cluster including isolates from at least two different provinces. The largest cluster (Cluster A) comprises the type strains *M. opportunistum*, *M. huakuii*, *M. amorphae, M. septentrionale* and *M. plurifarium* as well as 11 chickpea isolates distributed for five provinces. Although *M. septentrionale* and *M. plurifarium* type strains are included in this cluster, no isolate was found to group closely to these strains. In terms of 16S rRNA gene sequence, two isolates, LM-1-Lamego and STR-2-Santarém, are 100% identical to *M. amorphae.* On the other hand, five isolates in this cluster, such as C-14-Coimbra and 87-Évora, are 100% identical to *M. huakuii*. Three isolates share the same sequence similarity (99.6%) with both *M. opportunistum* and *M. huakuii,* namely C-1-Coimbra, CV-18Elvas and V-15b-Viseu, and may be new species. Cluster B includes 8 isolates, from five provinces, together with *M. loti*, *M. ciceri, M. alhagi, M. camelthorni, M. australicum* and *M. shangrilense* type strains. Six of the isolates share the same sequence similarity (100%) with both *M. loti* and *M. ciceri* type strains whereas the two remaining isolates showed a similarity of 99,6% with these type strains. Although *M. alhagi, M. camelthorni, M. australicum* and *M. shangrilense* type strains are also in this cluster, no isolate was found to group closely or to be similar to these strains. Cluster C includes 7 isolates together with *M. tianshanense, M. metallidurans*, *M. caraganae*, *M. gobiense* and *M. tarimense* type strains. All isolates in this cluster share high sequence similarity (100%) with *M. metallidurans*, *M. caraganae*, *M. gobiense* and *M. tarimense* type strains. No isolate was found to group closely to *M. tianshanense*. Within cluster D are 7 isolates distributed for four provinces and *M. mediterraneum* and *M. temperatum* type strains. Four isolates share identical sequence with both *M. mediterraneum* and *M. temperatum*.

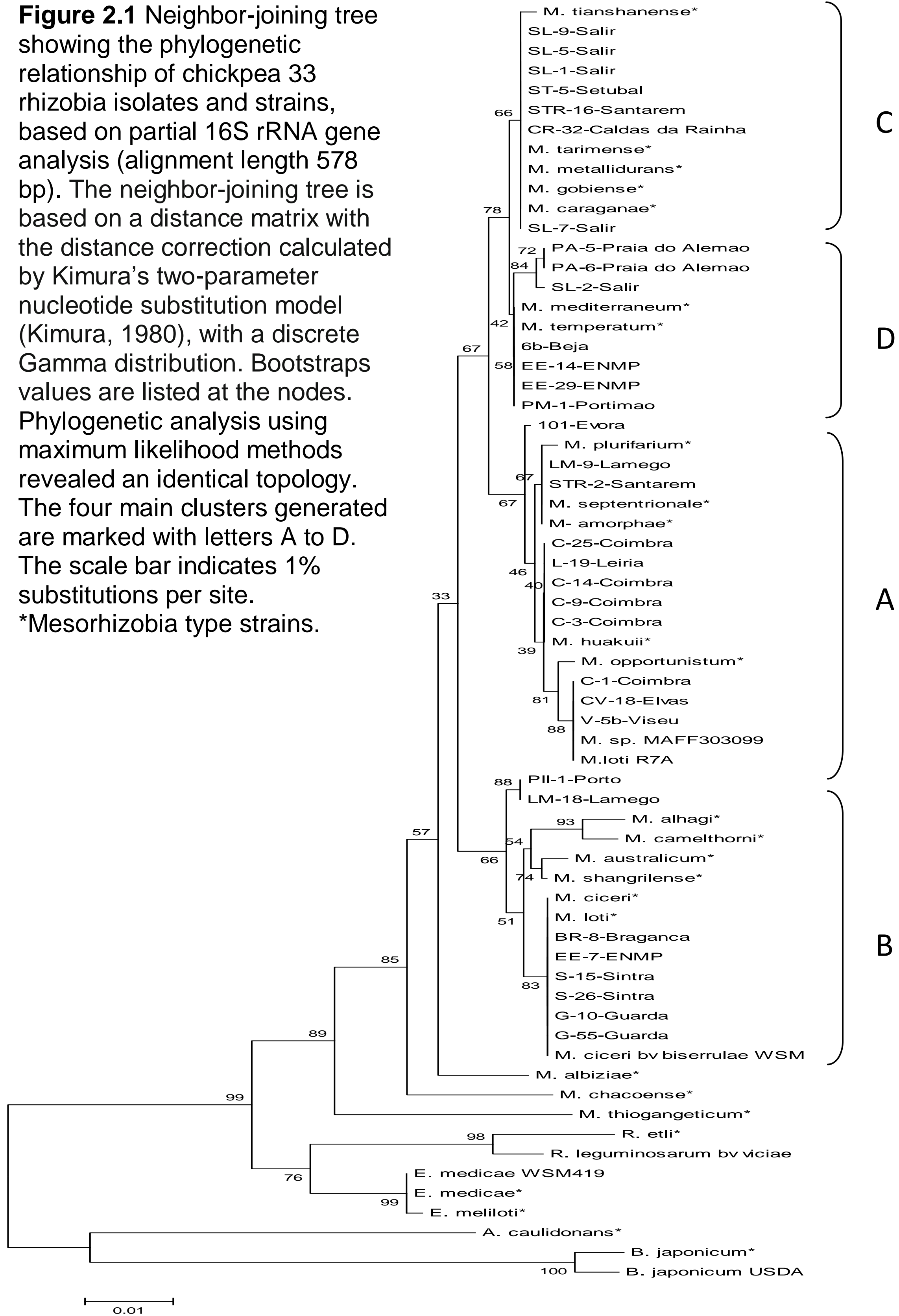

Figure 2.1 Neighbor-joining tree showing the phylogenetic relationship of chickpea 33 rhizobia isolates and strains, based on partial 16S rRNA gene analysis (alignment length 578 bp). The neighbor-joining tree is based on a distance matrix with the distance correction calculated by Kimura's two-parameter nucleotide substitution model (Kimura, 1980), with a discrete Gamma distribution. Bootstraps values are listed at the nodes. Phylogenetic analysis using maximum likelihood methods revealed an identical topology. The four main clusters generated are marked with letters A to D. The scale bar indicates 1% substitutions per site. *Mesorhizobia type strains.

Altogether, this analysis revealed that chickpea rhizobia isolates are highly diverse and group with several *Mesorhizobium* type strains. No isolate groups with *M. albiziae*, *M. chacoense* and *M. thiogangeticum* type strains.

Statistical analysis revealed an association between province of isolates and species clusters (χ^2= 130.667; *df*= 30; *P* < 0.01). In the north of Portugal (Trás-os-Montes e Alto Douro, Douro Litoral and Beira Baixa), isolates belonging to cluster B prevail; in the center (Beira Litoral, Ribatejo and Alto Alentejo), most isolates are from cluster A; and in the south (Baixo Alentejo and Algarve), isolates mainly belong to cluster D. All isolates from Madeira belong to cluster A. Moreover, Estremadura is the only province where isolates from cluster C predominate. Isolates from cluster C are found only in three provinces of the center of Portugal (Estremadura, Ribatejo, and Alto Alentejo) (Fig. 2.2).

The CA biplot (*data not shown*) indicated the existence of three classes of sites, consistent with the distribution of isolates. One class, which includes Estremadura, is associated with cluster C. A second class, Algarve and Baixo Alentejo, is mainly associated with cluster D. Finally, a class including Trás-os-Montes e Alto Douro, Alto Alentejo, Beira Baixa, Douro Litoral and Beira Alta, is associated with clusters A and B.

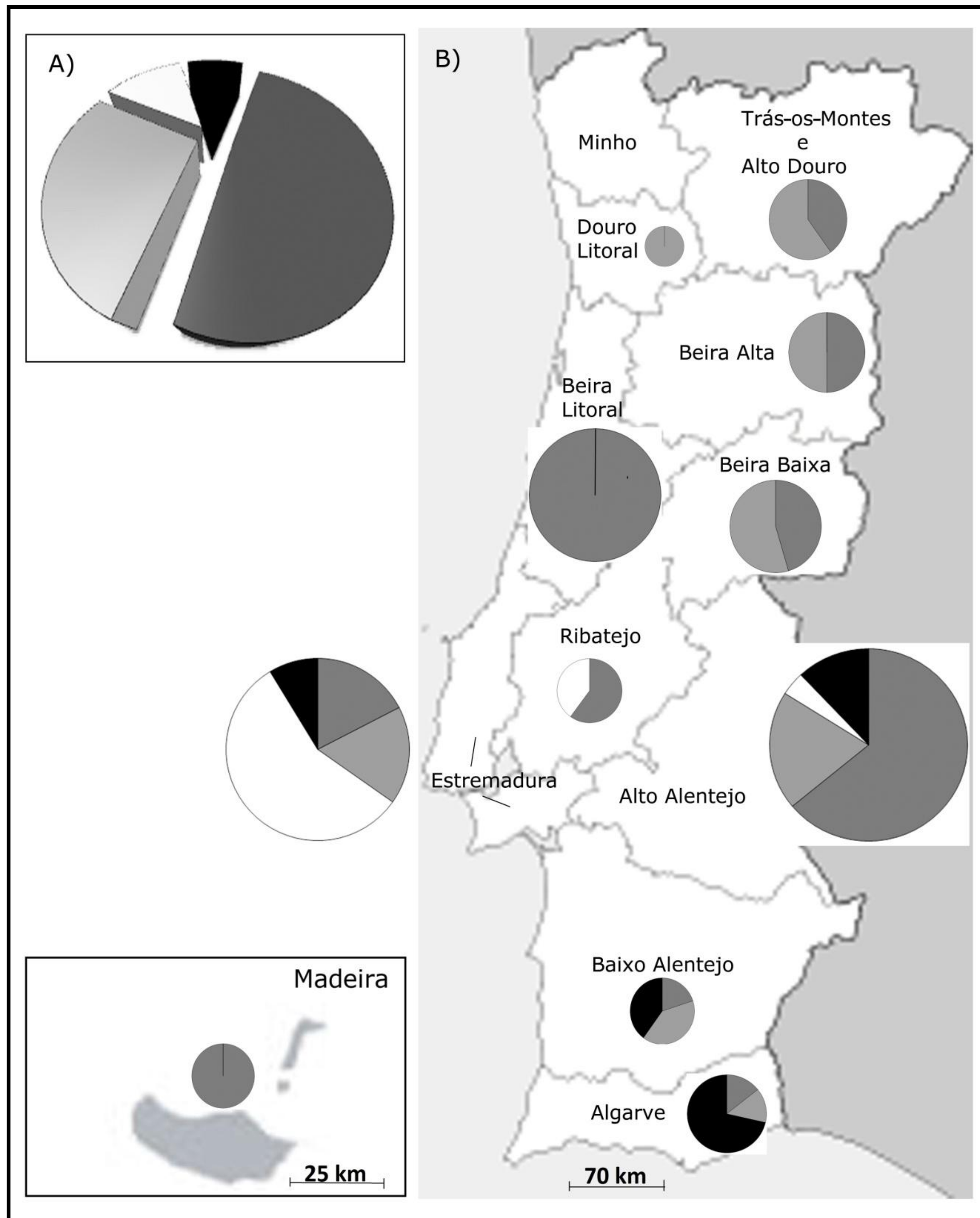

Figure 2.2 Distribution of the 121 chickpea rhizobia isolates by species clusters, as defined from de 16S rRNA gene-based phylogeny (A). Map of the provinces of Portugal and Madeira Island showing the distribution of isolates in each province, according to their cluster. Pie charts sizes are proportional to the number of isolates in each province (B). Isolates from: Cluster A (dark grey); Cluster B (light grey); Cluster C (white); Cluster D (black).

Plasmid profiles

Plasmid profiles were analyzed, and for most chickpea native rhizobia, at least one plasmid was detected (Table 2.2). Plasmid number ranges from zero to six, however, no isolate with five plasmids was found (Fig. 2.3).

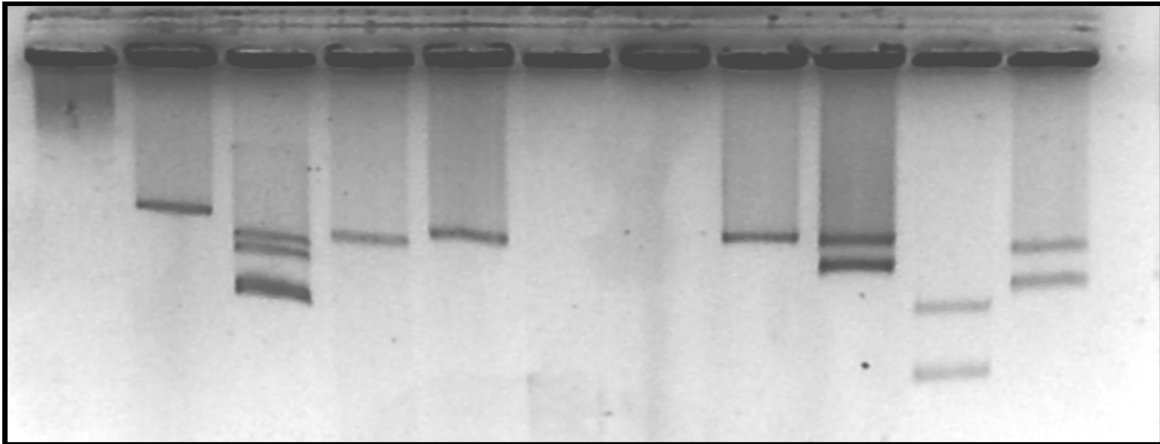

Figure 2.3 Example of an agarose gel showing plasmid profiles of chickpea rhizobia isolates. Lane 1 - L-19-Leiria; lane 2 - V-25b-Viseu; lane 3 - 29-Beja; lane 4 - BR-9-Bragança; lane 5- C-13-Coimbra; lane 6 - C-32-Caldas da Rainha; lane 7- G-55-Guarda; lane 8 – LM-1-Lamego; lane 9 - AII-5-Aveiro; lane 10 - PM-1-Portimão; lane 11- BR-16-Bragança.

For 36% of the isolates, one plasmid was detected. Only in about 27% of the isolates, two or more plasmids were detected. An association was found between plasmid number and province (χ^2=120,645; *df*=50; $P < 0.001$). Estremadura and Alto Alentejo are the provinces with isolates more variable in terms of plasmid number, harboring zero to four plasmids, while isolates from Douro Litoral, Beira Alta, Ribatejo, Algarve, and Madeira show the least variability in plasmid number. There is also an association between plasmid number and species clusters (χ^2=51.731, *df*=15, $P < 0.001$). The Fig. 2.4 shows the distribution of isolates in each cluster, according to their plasmid number. The CA biplot (*data not shown*) also shows an association between plasmid number and species clusters. Isolates from cluster A are associated to one plasmid, while in cluster C isolates

with no plasmids predominate. Cluster D is clearly associated to isolates with two or more plasmids. Isolates from cluster B are associated to plasmid number ranging from zero to two plasmids.

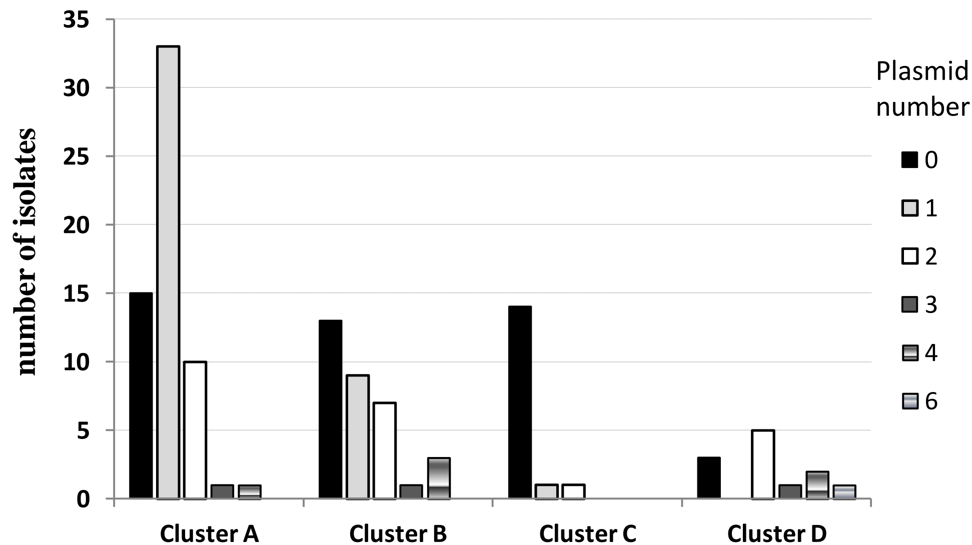

Figure 2.4 Number of isolates in each cluster according to their plasmid number.

Symbiotic effectiveness

Evaluation of SE was performed for all 121 isolates (Table 2.2). SE values range from 0% to 100%. SE trials revealed that 37% of the isolates show a SE above 50%. Twelve isolates, which represent about 10% of the total number of isolates, were found to be highly effective in fixing nitrogen in symbiosis with chickpea (SE values above 75%); most of these isolates belong to cluster B. The most effective isolates were S-24-Sintra and G-55-Guarda from cluster B (SE values of 100% and 88%, respectively). Although isolates closer to *M. ciceri / M. loti* (cluster B) have the highest mean SE (52%), no correlation was found between SE and species clusters. No correlation was found between SE and plasmid number, contrary to a previous study with a smaller set of isolates from Alentejo provinces (Laranjo *et al.,* 2002).

Table 2.3 Bacterial strains and gene accession numbers (GenBank) used in this work.

Strain	Origin	Host	*acdS#*	*nifH*	*nodC*	16S rRNA
A. caulinodans ORS571[I]	Senegal	*Sesbania rostrata*	AP009384	M16709	L18897	X67221
B. japonicum USDA110	USA	*Glycine max*	BA000040	blr1769	blr2027	rrn16S
B. japonicum USDA6 [I]	Japan	*Glycine max*	NA	NA	NA	U69638
R. leguminosarum bv viciae 3841	UK	*Pisum sativum*	AM236084	pRL100162	pRL100187	U29386
R. etli CFN42[I]	México	*Phaseolus vulgaris*	NA	RESP0005F	RESP0032F	U28916
E. meliloti USDA 1002[I]	USA	*Medicago sativa*	NA	M55229	EF209423	X67222
E. medicae A-321[I]	France	*Medicago spp.*	NA	DQ450936	DQ450944	L39882
E. medicae WSM419	Italy	*Medicago lupulina*	CP000740	CP000740	CP000740	CP000738
M. amorphae ACCC 19665[I]	China	*Amorpha fruticosa*	**ND**	EU267714	AF217261	AF041442
M. albiziae CCBAU 61158[I]	China	*Albizia kalkora, Albizia julibrissin, Glycine max, Leucaena leucocefala*	**JQ013380**	DQ311093	GQ167236	DQ100066
M. alhagi CCNWXJ12-2[I]	China	*Alhagi sparsifolia*	NA	NA	NA	EU169578
M. australicum LMG 24608[I]	Australia	*Biserrula pelecinus*	NA	AY601522	CP002447	AY601516
M. camelthorni ACCC14549 [I]	China	*Alhagi sparsifolia*	NA	NA	NA	EU169581
M. caraganae CCBAU 11299[I]	China	*Caragana spp.*	NA	NA	NA	EF149003
M. chacoense LMG 19008[I]	Argentina	*Prosopis alba*	**JQ013381**	DQ450927	DQ450937	AJ2778249
M. ciceri UPM-Ca7[I]	Spain	*Cicer arietinum*	**JQ013382**	DQ450928	DQ407292	DQ444456
M. ciceri bv. biserrulae WSM1271	Italy	*Biserrula pelecinus*	CP002447	CP002447	CP002447	CP002447
M. gobiense CCBAU 83330[I]	China	*Oxytropis glabra*	NA	NA	NA	EF035064
M. huakuii CCBAU 2609[I]	China	*Astragalus sinicus*	**ND**	NA	NA	FJ491264
M. sp. MAFF303099	Japan	*Lotus corniculatus*	BA000012	mlr5905	mlr6163	RRN16Sb
M. loti NZP 2213T[I]	New Zealand	*Lotus corniculatus*	**JQ013383**	DQ450929	DQ450939	X67229
M. loti R7A	New Zealand	*Lotus corniculatus*	AL672114	ML0303	ML0132	U50166
M. mediterraneum UPM-Ca36[I]	Spain	*Cicer arietinum*	**JQ013384**	DQ450930	DQ450940	AM181745
M. metallidurans STM 2683[I]	France	*Anthyllis vulneraria*	NA	NA	NA	AM930381
M. opportunistum WSM2075[I]	Australia	*Biserrula pelecinus*	CP002279	CP002279	CP002279	AY601515
M. plurifarium ORS 1032[I]	Senegal	*Acacia senegal, Prosopis juriflora*	**JQ013385**	DQ450931	FJ745283	Y14158
M. septentrionale HAMBI 2582[I]	China	*Astragalus adsurgens*	**JQ013386**	DQ450932	DQ450941	AF508207
M. shangrilense	China	*Caragana spp.*	NA	NA	NA	EU074203
M. tarimense CCBAU 83306[I]	China	*Glycyrrhiza uralensis, Lotus corniculatus, Lotus frondosus*	**JQ013387**	EU252607	EF050786	EF035058
M. temperatum HAMBI 2583[I]	China	*Astragalus adsurgens*	NA	DQ450933	DQ450942	AF508208
M. tianshanense A-1BS[I]	China	*Glycyrrhiza pallidiflora, Caragana spp*	**JQ013388**	DQ450934	DQ450943	AF041447
M. thiogangeticum SJT[I]	India	*Clitoria ternatea*	**JQ013389**	NA	NA	AJ864462

(Table 2.3 continued)

Isolate	**Origin**	**Host**	***acdS#***	***nifH***	***nodC***	**16S rRNA**
101-Évora	Portugal	*Cicer arietinum*	**JQ013399**	NA	NA	*
6b-Beja	Portugal	*Cicer arietinum*	**JQ013398**	DQ431732	DQ431753	*
BR-8-Bragança	Portugal	*Cicer arietinum*	**JQ013390**	JQ033936	JQ033958	*
C-1-Coimbra	Portugal	*Cicer arietinum*	**JQ013400**	NA	NA	*
C-14-Coimbra	Portugal	*Cicer arietinum*	**JQ013401**	NA	NA	*
CV-18-Elvas	Portugal	*Cicer arietinum*	**JQ013402**	DQ431741	DQ431762	*
EE-7-Elvas	Portugal	*Cicer arietinum*	**JQ013391**	DQ431743	DQ431764	*
EE-14-Elvas	Portugal	*Cicer arietinum*	**JQ013403**	DQ431745	DQ431766	*
EE-29-Elvas	Portugal	*Cicer arietinum*	**JQ013404**	DQ431746	DQ431767	*
G-10-Guarda	Portugal	*Cicer arietinum*	**JQ013392**	**JQ033940**	**JQ033946**	*
G-55-Guarda	Portugal	*Cicer arietinum*	**JQ013393**	**JQ033931**	**JQ033947**	*
L-19-Leiria	Portugal	*Cicer arietinum*	**JQ013394**	NA	NA	*
PII-1-Porto	Portugal	*Cicer arietinum*	**JQ013405**	NA	NA	*
PM-1-Portimão	Portugal	*Cicer arietinum*	**JQ013396**	NA	NA	*
S-15-Sintra	Portugal	*Cicer arietinum*	**JQ013397**	NA	NA	*
STR-16-Santarém	Portugal	*Cicer arietinum*	**JQ013406**	NA	NA	*
V5b-Viseu	Portugal	*Cicer arietinum*	**JQ013407**	NA	NA	*
C-3-Coimbra	Portugal	*Cicer arietinum*	NA	**JQ033937**	**JQ033942**	*
C-9-Coimbra	Portugal	*Cicer arietinum*	NA	**JQ033930**	**JQ033943**	*
C-25-Coimbra	Portugal	*Cicer arietinum*	NA	**JQ033938**	**JQ033944**	*
CR-32-Caldas da Rainha	Portugal	*Cicer arietinum*	NA	**JQ033939**	**JQ033945**	*
LM-9-Lamego	Portugal	*Cicer arietinum*	NA	**JQ033932**	**JQ033948**	*
PA-5-Praia do Alemão	Portugal	*Cicer arietinum*	NA	NA	**JQ033949**	*
PA-6-Praia do Alemão	Portugal	*Cicer arietinum*	NA	NA	**JQ033950**	*
S-26-Sintra	Portugal	*Cicer arietinum*	NA	**JQ033933**	**JQ033951**	*
SL-1-Salir	Portugal	*Cicer arietinum*	NA	NA	**JQ033952**	*
SL-2-Salir	Portugal	*Cicer arietinum*	NA	NA	**JQ033953**	*
SL-5-Salir	Portugal	*Cicer arietinum*	NA	NA	**JQ033954**	*
ST-5-Setubal	Portugal	*Cicer arietinum*	NA	NA	**JQ033955**	*
STR-2-Santarém	Portugal	*Cicer arietinum*	NA	**JQ033934**	**JQ033956**	*
SL-7-Salir	Portugal	*Cicer arietinum*	NA	NA	**JQ033959**	*
SL-9-Salir	Portugal	*Cicer arietinum*	NA	NA	**JQ033960**	*
LM-18-Lamego	Portugal	*Cicer arietinum*	NA	**JQ033941**	**JQ033961**	*

Accession numbers in bold were obtained in this study; #Results obtained in collaboration with F. Nascimento; NA- not available; ND- not detected. * Accession number indicated in Table 2.2. ^{T}- Type strains.

Phylogeny analysis of *nifH*, *nodC* and *acdS* genes

GenBank accession numbers for the *nifH*, *nodC* and *acdS* gene sequences obtained herein as well as the accession numbers from those available on GenBank are listed in Table 2.3. As expected, all chickpea rhizobia isolates tested showed similar *nifH* and *nodC* genes sequences similar to the ones carried by the typical chickpea microsymbionts. Regarding the phylogeny based on the *nifH* gene sequences (Fig 2.5), the chickpea symbionts form an independent branch (Cluster A), which includes the type strains *M. ciceri* and *M. mediterraneum.* All other species from the genus *Mesorhizobium* are found outside this cluster. The strains known to be able to nodulate *Biserrula pelecinus,* namely *M. ciceri* bv. *biserrulae*, *M. opportunistum and M. australicum*, are in the same cluster (B). The strains (*M. loti*, *M. loti* R7A, *M. sp.* MAFF303099 and *M. tarimense*) able to nodulate *Lotus corniculatus* grouped together and form a third cluster (C). In terms of phylogeny based on the *nodC* gene sequences (Fig 2.6), all chickpea symbionts are again in the same cluster (A), which includes the type strains *M. ciceri* and *M. mediterraneum*. Similarly, the two clusters (B and C) mentioned above are also found in the *nodC*-based phylogeny, which correspond to symbionts nodulating *Biserrula pelecinus* and *Lotus corniculatus*, respectively.

Interestingly, the phylogenetic tree based on *acdS* gene sequences (Fig. 2.7) shows the three main clusters mentioned above. Mesorhizobia type strains and the Portuguese mesorhizobia isolates that nodulate *Cicer arietinum* form one cluster (cluster A). The type strains nodulating *Biserrula pelecinus* form another cluster (cluster B). Strains able to nodulate *Lotus corniculatus* form cluster C. Phylogenetic analysis of the *acdS* gene from *Mesorhizobium* strains indicates that rhizobia able to nodulate the same plant host have a similar *acdS* gene. The *acdS* gene sequences here obtained share high identity (84 to 99%) to the previously described *acdS* gene of *M. sp.* MAFF303099.

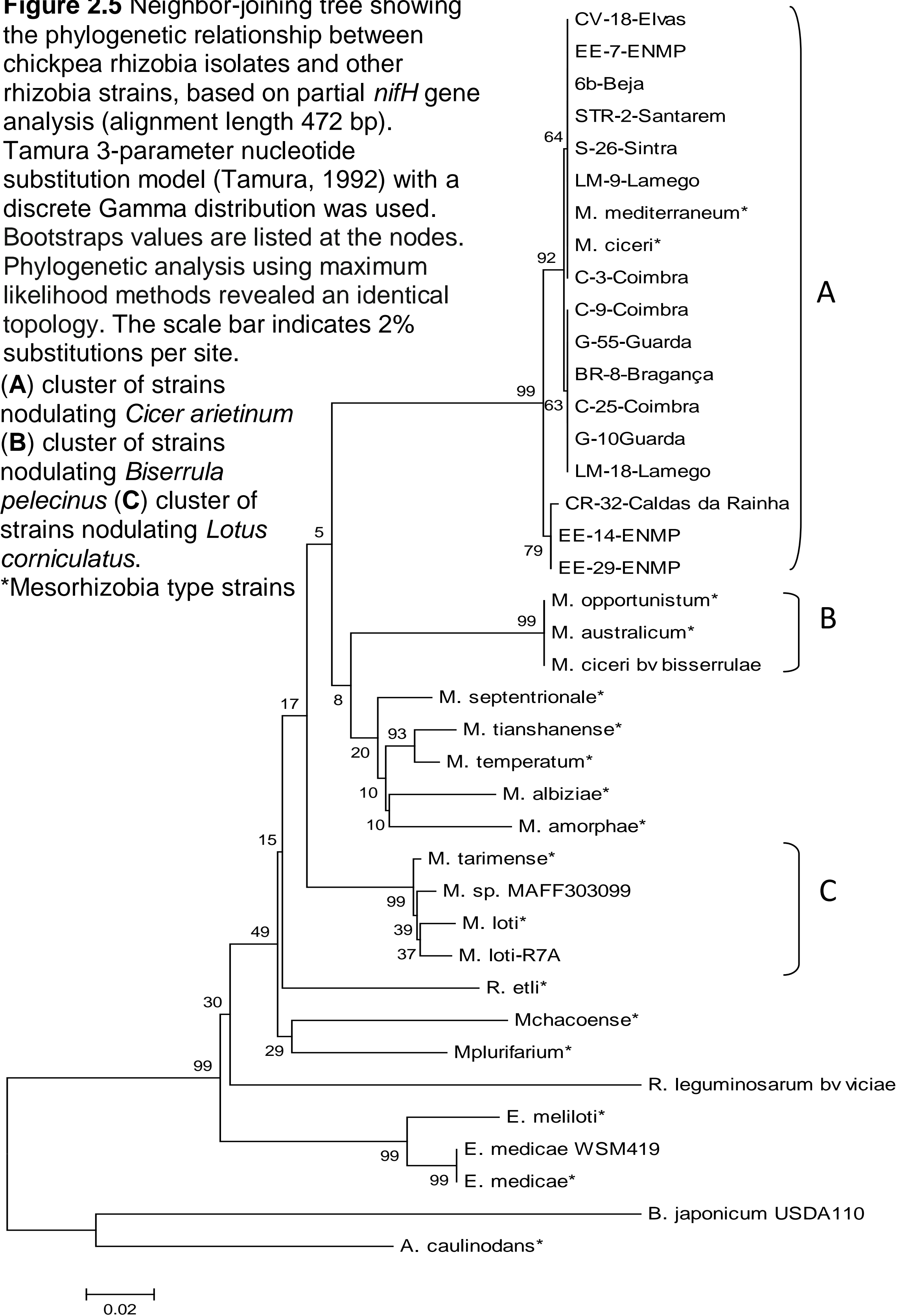

Figure 2.5 Neighbor-joining tree showing the phylogenetic relationship between chickpea rhizobia isolates and other rhizobia strains, based on partial *nifH* gene analysis (alignment length 472 bp). Tamura 3-parameter nucleotide substitution model (Tamura, 1992) with a discrete Gamma distribution was used. Bootstraps values are listed at the nodes. Phylogenetic analysis using maximum likelihood methods revealed an identical topology. The scale bar indicates 2% substitutions per site.
(**A**) cluster of strains nodulating *Cicer arietinum* (**B**) cluster of strains nodulating *Biserrula pelecinus* (**C**) cluster of strains nodulating *Lotus corniculatus*.
*Mesorhizobia type strains

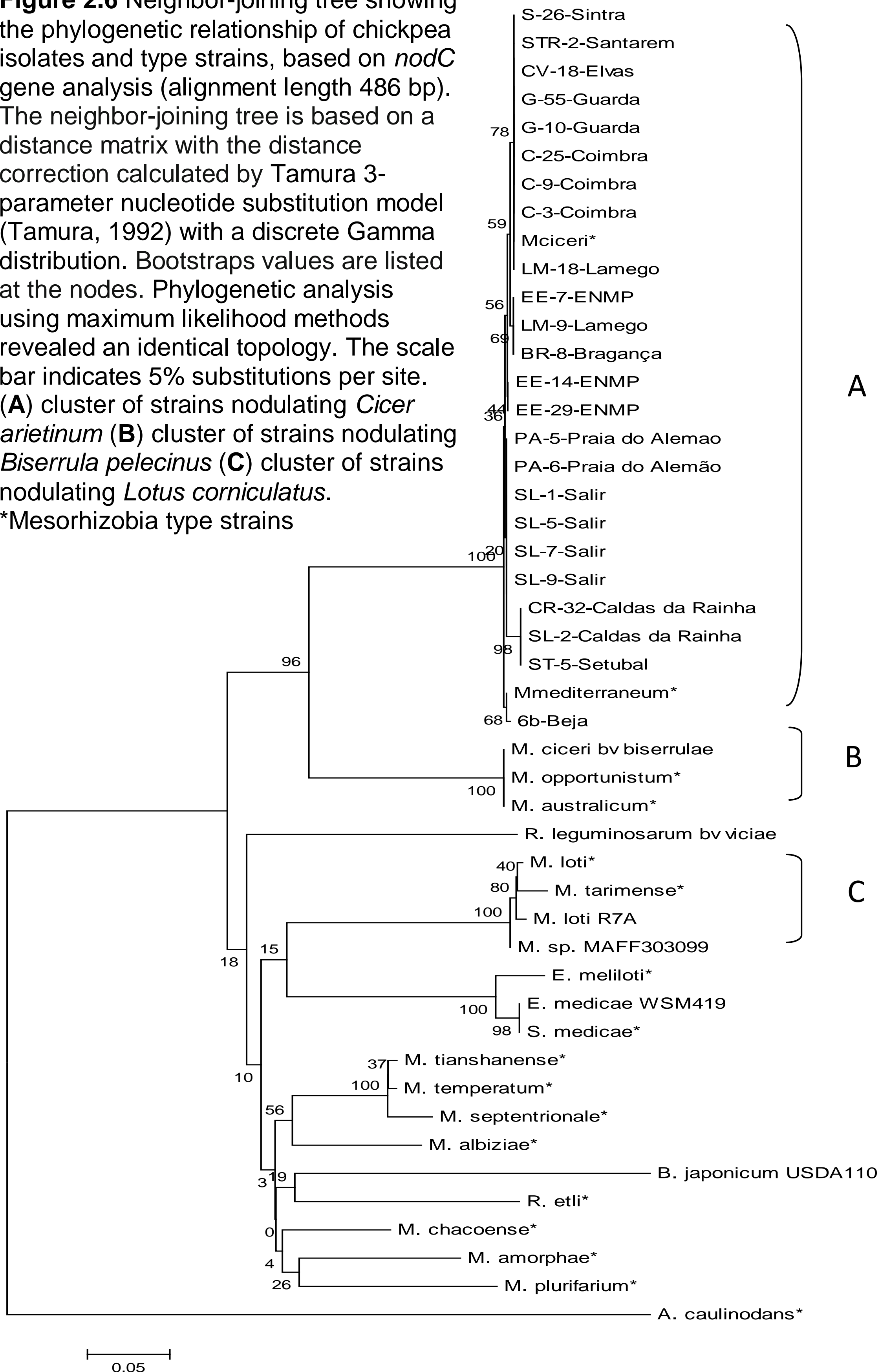

Figure 2.6 Neighbor-joining tree showing the phylogenetic relationship of chickpea isolates and type strains, based on *nodC* gene analysis (alignment length 486 bp). The neighbor-joining tree is based on a distance matrix with the distance correction calculated by Tamura 3-parameter nucleotide substitution model (Tamura, 1992) with a discrete Gamma distribution. Bootstraps values are listed at the nodes. Phylogenetic analysis using maximum likelihood methods revealed an identical topology. The scale bar indicates 5% substitutions per site. (**A**) cluster of strains nodulating *Cicer arietinum* (**B**) cluster of strains nodulating *Biserrula pelecinus* (**C**) cluster of strains nodulating *Lotus corniculatus*.
*Mesorhizobia type strains

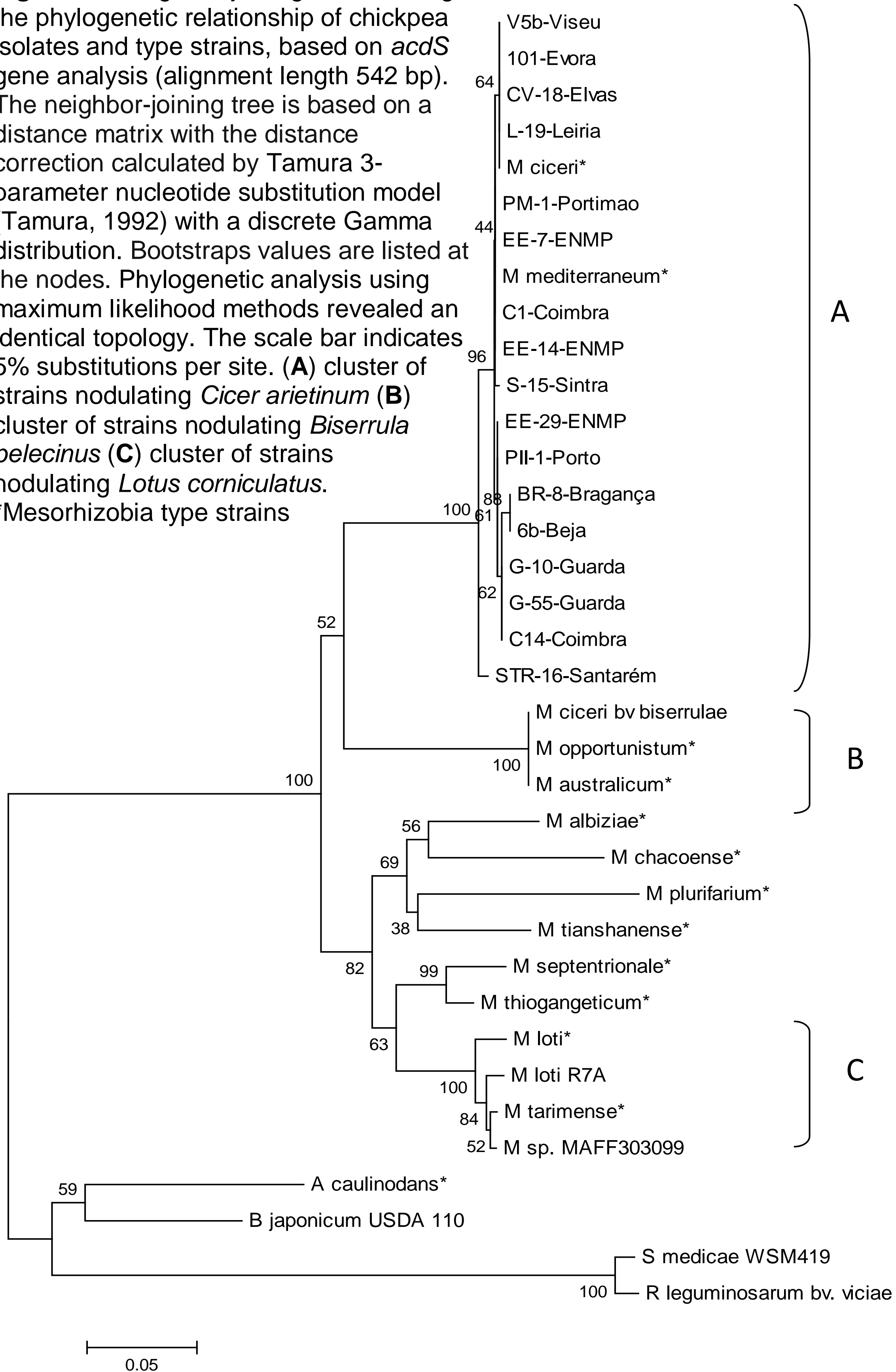

Figure 2.7 Neighbor-joining tree showing the phylogenetic relationship of chickpea isolates and type strains, based on *acdS* gene analysis (alignment length 542 bp). The neighbor-joining tree is based on a distance matrix with the distance correction calculated by Tamura 3-parameter nucleotide substitution model (Tamura, 1992) with a discrete Gamma distribution. Bootstraps values are listed at the nodes. Phylogenetic analysis using maximum likelihood methods revealed an identical topology. The scale bar indicates 5% substitutions per site. (**A**) cluster of strains nodulating *Cicer arietinum* (**B**) cluster of strains nodulating *Biserrula pelecinus* (**C**) cluster of strains nodulating *Lotus corniculatus*. *Mesorhizobia type strains

2.5 Discussion

The present study is the first survey of chickpea rhizobia native populations covering the Portuguese territory. One hundred and twenty one isolates were confirmed as chickpea symbionts and identified as *Mesorhizobium* sp., forming a monophyletic cluster with all *Mesorhizobium* type strains in the 16S rRNA gene phylogeny.

Despite the fact that a few isolates affiliation changed with the inclusion of the new species described after 2009, the four main clusters found before remain the same. The four clusters of the partial 16S rRNA gene-based phylogeny show that isolates positioning is scattered within the *Mesorhizobium* genus. Isolates from cluster A, which are more related to *M. huakuii, M. opportunistum* and *M. amorphae* than to any other type strain, are the most abundant chickpea microsymbionts found in Portuguese soils. This was unexpected since these type strains are unable to nodulate chickpea. *M. huakuii* was originally isolated from *Astragalus sinicus* (Chen *et al.*, 1991) that does not exist in Portugal. *M. amorphae* was originally isolated from *Amorpha fruticosa* (Chen *et al.*, 1991), a plant unrelated to *Cicer arietinum*, which is uncommon and considered invasive in Portugal. Nevertheless, a few isolates also affiliated to *M. opportunistum*, which was isolated from *Biserrula pelecinus* (Nandasena *et al.*, 2009). *Biserrula pelecinus* is a pasture leguminous plant adapted to acid soils and found in Iberian Peninsula, however, its microsymbionts are unable to nodulate *Cicer arietinum* (Nandasena *et al.,* 2001; Nandasena *et al.*, 2007a). *M. ciceri* and *M. mediterraneum* species groups (clusters B and D, respectively) could be expected to include the majority of native isolates, as these species were described as the specific chickpea microsymbionts (Nour *et al.*, 1994b; Nour *et al.*, 1995). However, only 37% of the isolates grouped with these two type strains. Isolates related to *M. amorphae* (cluster A), *M. loti* (cluster B) and *M. tianshanense* (cluster C) were found, as in previous studies on chickpea rhizobia isolated from Portugal and Spain (Rivas *et al.*, 2007, Laranjo *et al.*, 2004). However, the isolates in the cluster C, which formerly only included *M*

tianshanense, are closer to the new species *M. metallidurans*, *M. caraganae*, *M. gobiense* and *M. tarimense* than to *M. tianshanense*. The present work screened the entire Portuguese territory, confirmed the high diversity of native rhizobia and revealed an unexpected high proportion of isolates unrelated to *M. ciceri* and *M. mediterraneum*.

To our knowledge this is one of the few studies addressing the diversity of chickpea rhizobia covering an entire country, namely Morocco (Maâtallah *et al.*, 2002a) and Tunisia (L'Taief *et al.,* 2007). Using PCR-RFLP analysis of the 16S rRNA gene, Maâtallah *et al.* (2002a) found a lower diversity of chickpea rhizobia than the one revealed in the present study, since most isolates were described as close to *M. ciceri*, *M. loti* and *M. mediterraneum*. More recently, L'Taief *et al.* (2007) isolated chickpea native rhizobia from several regions of Tunisia and found isolates belonging only to either *M. ciceri* or *M. mediterraneum*. Probably, the low diversity found in Tunisia and Morocco is related to the history of chickpea cultivation on the sampled sites, as supported by several studies reporting a decrease in rhizobia diversity associated with the presence of the host plant (Coutinho *et al.*, 1999). Accordingly, the high diversity found in Portuguese soils could be explained by the absence of chickpea crop in Portugal (Duarte Maçãs, 2003) and the non-existence of chickpea wild relatives (Talavera *et al.*, 1999); furthermore, there are no records of the use of commercial inoculants that could reduce the natural chickpea rhizobia diversity. Interestingly, isolates from the single site where chickpea has been cultivated (Elvas-ENMP) group only with *M. ciceri* or *M. mediterraneum*.

A correlation between isolates species cluster and origin soil pH was found ($P < 0.001$). For example, all isolates assigned to the *M. mediterraneum* / *M. temperatum* species (cluster D) were obtained from the soils with higher pH values. This may indicate that genetic determinants, which allow rhizobia survival in alkaline soil conditions, are species specific.

Considering the correlation found between species cluster and soil pH, it is likely that pH is a key environmental parameter determining the species geographic distribution. This hypothesis is supported by wider studies addressing soil bacterial communities, suggesting that soil pH is the variable that best explains the population diversity and overall community composition (Fierer and Jackson, 2006). Several studies in rhizobia showed that the pH affects survival and competitiveness in soil, as well as the nodulation process (Zahran, 1999). The effect of pH in chickpea rhizobia growth has been addressed in previous studies (Rodrigues *et al.*, 2006). Altogether these studies suggest that pH is a key environmental factor for rhizobia population composition, acting on bacteria, both free-living and in symbiosis.

In each 16S rRNA gene-based cluster, isolates with high and low symbiotic effectiveness were found. A large set of isolates with very high SE values (above 75%) are good candidates for field inoculation. Many of these isolates are from the *M. ciceri* cluster (B). About 67% of the isolates from cluster B present a SE value above the corresponding type strain *M. ciceri*, which showed a SE of 41%, estimated in a previous study (Laranjo *et al.*, 2008). In the cluster D, which includes *M. mediterraneum* / *M. temperatum* type strains, 42% of the isolates showed a SE above 39%, which is the value described for the type strain of *M. mediterraneum* (Laranjo *et al.*, 2008).

The plasmid number of rhizobia isolates was found to be associated with species cluster, suggesting that this feature might be species constrained. In most isolates from cluster A, one plasmid was detected, similarly to *M. amorphae* (Wang *et al.*, 1999). In cluster D, both isolates and *M. mediterraneum* (Cadahía *et al.*, 1986) showed more than one plasmid. In the majority of isolates belonging to cluster C no plasmid were detected, similarly to *M. tianshanense* (Chen *et al.*, 1995). Isolates from cluster B seem to be more diverse in plasmid number, including isolates with zero, one and two plasmids. The type strain of *M. ciceri* (cluster B) harbours one plasmid (Cadahía *et al.*, 1986).

Contrary to previous studies, which suggested that most rhizobia nodulating chickpea are *M. ciceri* and *M. mediterraneum*, this wider survey shows a predominance of other species. The obtained isolates collection, highly diverse in terms of species, as well as SE, provides an important source of rhizobia strains to be used, namely as potential inoculants.

ACC deaminase genes are naturally present in many strains of *Rhizobium* spp. and are prevalent in isolates from different geographical locations (Ma *et al*., 2003; Duan *et al.,* 2009). Nevertheless, little was known in terms of prevalence and phylogeny of this gene in *Mesorhizobium* genus. Our results reveal that 10 of the 12 analyzed *Mesorhizobium* type strains, from different geographical locations and nodulating different leguminous plants, possess the *acdS* gene, suggesting that ACC deaminase is a common feature in *Mesorhizobium* spp. Furthermore, the *acdS* gene was detected in the 17 chickpea mesorhizobia isolates tested, indicative that many of the Portuguese chickpea mesorhizobia possess an *acdS* gene, and suggesting that ACC deaminase genes are prevalent in these chickpea-nodulating mesorhizobia.

The phylogenetic analysis based on symbiosis genes (*nifH* and *nodC*) is in agreement with other studies (Rivas *et al.,* 2007; Laranjo *et al.,* 2008) which suggest the lateral transfer of symbiosis genes across different species. This study confirms that both genes are good markers for rapid detection of chickpea mesorhizobia. The *acdS*-based phylogenetic tree shows a topology similar to the symbiosis (*nodC* and *nifH*) genes-based trees, grouping isolates that nodulate the same host, rather than by species as in the 16S rRNA gene-based phylogeny. Several studies show that many *Mesorhizobium* strains have acquired the ability to nodulate a specific host by acquiring the symbiosis island carrying specific symbiosis genes (Sullivan *et al*., 1995; Sullivan and Ronson, 1998; Nandasena *et al*., 2006; Nandasena *et al*., 2007b; Laranjo *et al.* 2008). Therefore, our results suggest that the *acdS* gene is likely to be horizontally transferred between *Mesorhizobium* species by exchange of the symbiosis island. Moreover, Uchiumi *et al.* (2004) verified high expression levels of

transposase genes during symbiosis, which could give rise to DNA rearrangement and thus may contribute to the rapid evolution of symbiosis islands in mesorhizobia.

Altogether, our results may indicate that lateral gene transfer plays a crucial role in the acquisition of genes involved in the symbiotic nitrogen fixation process by rhizobia, which is an advantage for competiveness among the native rhizobia strains. Moreover, the *acdS* genes found herein appear to be horizontally transferred between different *Mesorhizobium* species by exchange of the symbiosis island. Thus, it is possible that lateral transfer of genes may be a natural event that occurs with more frequency that it was thought.

The present study is the first systematic assessment of *Cicer arietinum* microsymbionts in Portugal and contributes to clarify the biogeography of chickpea rhizobia, providing a global picture of how species are distributed across the country. This study also brings new insights on the evolution mesorhizobia-leguminous plant associations.

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Chapter 3

Tolerance of chickpea mesorhizobia to salinity

One of the biggest concerns in agriculture is the increase of salt-affected land worldwide. Salt stress affects both soil bacteria and plants, and therefore the symbiotic legume-rhizobia relationship may be negatively affected in several processes, thus reducing plant growth and development.

In this chapter, chickpea mesorhizobia tolerance to salt stress was evaluated. Additionally, the molecular bases of salt tolerance in rhizobia were studied by comparing the expression levels of major chaperone genes in tolerant and sensitive isolates, within the same species, to find differences that could be related to the different susceptibility to salt stress.

This chapter is based on the manuscript:

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

3.1 Summary

Salinity is an important abiotic stress that limits rhizobia-legume symbiosis, affecting plant growth, thus reducing crop productivity. Our aims were to evaluate the tolerance to salinity of native chickpea rhizobia as well as to investigate the expression of chaperone genes *groEL, dnaKJ* and *clpB* in both tolerant and sensitive isolates. One hundred and six native chickpea mesorhizobia were screened for salinity tolerance by measuring their growth with 1.5% and 3% NaCl. Most isolates were salt-sensitive, showing a growth below 20% compared to control. Nevertheless, a few isolates presented a growth above 30% with 1.5% NaCl, namely BR-8-Bragança, CR-32-Caldas da Rainha, PA-6-Praia do Alemão and SL-2-Salir. An association between salt tolerance and province of origin of the isolates was found. The transcriptional analysis by northern hybridization of chaperone genes was performed using tolerant and sensitive isolates belonging to different *Mesorhizobium* species. Upon salt shock, most isolates revealed a slight increase in the expression of the *dnaK* gene, whereas the *groESL* and *clpB* expression was unchanged or slightly repressed. No clear relationship was found between the chaperone genes induction and the level of salt tolerance of the isolates. This is the first report on transcriptional analysis of the major chaperones genes in chickpea mesorhizobia under salinity, which may contribute to a better understanding of the mechanisms that influence rhizobia salt tolerance.

3.2 Introduction

Rhizobia are soil bacteria able to establish nitrogen fixing symbioses with legumes. The biological nitrogen fixation contributes to an ecological and sustainable agriculture, as it reduces the need for chemical nitrogen fertilizers and improves crop productivity. One of the major problems that agriculture is currently facing is the effect of abiotic environmental stresses, leading to yield reductions and subsequent economic losses (Ashraf *et al.,* 2008). Among the abiotic stresses, salinity seriously limits the productivity of agricultural crops (Ashraf *et al.,* 2008; Katerji *et al.* 2009; Grewal, 2010) and affects about 800 Mha of arable lands worldwide (Munns and Tester, 2008).

Legumes represent a very significant group of crops in agriculture and therefore their tolerance to salt stress is important worldwide. Chickpea (*Cicer arietinum* L.) is one of the most important grain legume crops because it is a relevant protein source in both human and animal diets. Like other legumes, chickpea is very sensitive to salinity, which affects its growth and development (Elsheikh and Wood, 1990a).

Salinity may negatively affect the rhizobia-plant symbioses in several processes: growth and survival of rhizobia in soil, root colonization, infection and nodule development and functioning (Kulkarni *et al.,* 2000). The rhizobia isolated from chickpea nodules and cultured *in vitro* are usually much more tolerant to salt than their host (Zahran, 1999). Nevertheless, chickpea rhizobia differ in NaCl tolerance, some strains are able to grow at salt concentrations as high as 500 mM NaCl (Kucuk and Kivanc, 2008), others cannot grow even when NaCl concentration is lowered to 100 mM NaCl (Elsheikh and Wood, 1990a,b; Zurayk *et al.*, 1998; Kucuk and Kivanc, 2008).

A major consequence of salt-stress is the loss of intracellular water, which imposes a water deficit because of osmotic effects on a wide variety of metabolic activities (Fatnassi *et al.,* 2011). Proteins are at permanent risk of unfolding,

especially when cells are exposed to environmental stress conditions, such as high salt concentration. When protein denaturation occurs, molecular chaperones enable denatured proteins to acquire their native folding faster and more reliably than they otherwise would (Hartl and Hayer-Hartl, 2009).

Several classes of chaperones are induced under stress conditions, such as salinity. The DnaK machinery comprises the co-chaperone DnaJ and the nucleotide exchange factor GrpE, whereas the GroEL system includes the co-chaperone GroES (Chaudhuri *et al.*, 2009). ClpB belongs to the Clp family, which is ubiquitous among prokaryotes and eukaryotes, acting as both protease and chaperone (Gottesman *et al.*, 1997). Under extreme conditions, ClpB interacts with the DnaK chaperone system catalyzing protein disaggregation and reactivation (Motohashi *et al.,* 1999; Zolkiewski, 1999). In *Ensifer meliloti* cells subjected to salt stress, Domínguez-Ferreras *et al.* (2006) reported the induction of several genes, including *clpB* as well as the repression of some *groESL* operon copies. Additionally, the co-chaperone *dnaJ* was described as being involved in *Rhizobium tropici* salt tolerance (Nogales *et al.,* 2002). However, in chickpea rhizobia, little is known about the expression of chaperone genes under saline conditions.

The present study describes the screening of a collection of Portuguese chickpea rhizobia for salinity tolerance and investigates the gene expression of the well characterized chaperone systems *dnaK*-*dnaJ*, *groEL*-*groES* and *clpB*. The transcription of these chaperone genes upon salt shock was analyzed, using sensitive and tolerant mesorhizobia isolates, belonging to several *Mesorhizobium* species.

3.3 Materials and Methods

Bacterial isolates

A total of 106 chickpea rhizobia isolates were used in the present study (Table 3.1). Overall, isolates were collected from 26 soil samples (Chapter 2, Table 2.1), covering almost all Portuguese territories with the exception of Azores Islands and Minho province. Four *Mesorhizobium* type strains were also used: *M. ciceri* UPM-Ca7, *M. loti* LMG 6125; *M. amorphae* ACCC 19665 and *M. mediterraneum* UPM-Ca36. All isolates were preserved in 30% (v/v) glycerol at -80ºC and cultured in yeast extract mannitol (YEM) broth (Vincent, 1970) for routine use.

Salt stress tolerance

The salt tolerance of bacterial isolates was screened by evaluation of their growth based on optical density (OD) readings at 540 nm. The YEM medium was supplemented with 1.5% and 3% NaCl for stress conditions. For control conditions, standard YEM was used. After overnight growth in YEM, bacterial cultures were standardized to an initial OD of 0.03 and grown for 48h at 28ºC. Three replicas per treatment were done.

Statistical analysis

In order to compare differences in isolate tolerance, optical density values were converted into percentage values, considering growth at control conditions as 100%. Average value and standard deviation of the three replicas were calculated. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA). The Krustal-Wallis test was used in order to explore the relationship between stress tolerance (continuous dependent variable) and categorical independent variable, as for instance species group or province of origin. To identify categories that differ significantly from others, three different

post hoc tests (Tamhane, Dunnett T3 and Games-Howell) were used. To detect structure in the relationships between categorical variables, the correspondence analysis (CA) was conducted as an exploratory data analysis technique (Benzécri, 1973). Isolates were divided into three classes: sensitive (growth < 10%), tolerant (growth between 10 and 20%) and highly tolerant (growth > 20%), in order to investigate the relationships between these classes and isolates province of origin. Spearman's correlations were performed in order to determine if any of the soil characteristics were related with salt-tolerance.

RNA extraction and northern hybridization

RNA extraction was performed using cell cultures in exponential growth phase, submitted to a salt shock in YEM supplemented with 5% NaCl, for one hour. Control RNA was extracted from cells grown in YEM. Total RNA extraction was performed according to the protocol for Rapid Isolation of RNA from Gram-negative Bacteria (Ausubel *et al.,* 1997).

The nonradioactive DIG system (Roche Applied Science) was used for northern experiments. RNA samples were denatured in a loading buffer (50% deionized formamide; 6.1% formaldehyde; 1 × MOPS) and separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde in 1× MOPS (20 mM MOPS buffer, 5 mM sodium acetate, 2 mM EDTA, pH 7.0). After electrophoresis, capillary transfer into a positively charged nylon membrane (Roche Applied Science) was carried out in 20 ×SSC (3 M NaCl; 300 mM sodium citrate, pH 7.0). RNA was fixed by baking the membrane at 120ºC for 30 min. The *groEL* and *dnaKJ* RNA probes were obtained as previously described (Alexandre and Oliveira 2011). The *clpB* RNA probe was obtained using a gene fragment of 1388 bp that was amplified using the primers clpB-F (5'-CGCCGAACCAAGAACAATCC-3') and clpB-R (5'-ACCCTCCTCATAGCCGACAT-3') (Stabvida). The PCR reaction was prepared with 2 U Taq DNA polymerase (Fermentas), 1 × reaction buffer, 1.5 mM $MgCl_2$,

0.2 mM of each dNTP (Invitrogen), 20 pmol of each primer and DNA of *Mesorhizobium* sp. MAFF 303099. The amplification program used was: 3 min at 95ºC for initial denaturation; 30 cycles of 1 min at 94ºC, 50 sec at 62ºC and 2 min at 72ºC and a final extension step at 72ºC during 5 min. The PCR product was purified using the GFX™ PCR DNA or Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The *clpB* gene fragment was cloned using pGEM-T Easy Vector System (Promega) following the manufacturer's instructions. All RNA probes were obtained by *in vitro* transcription labelling, using DIG Northern Starter Kit (Roche Applied Science). The DNA probe for 16S rRNA was labeled using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science). The 16S rRNA gene PCR amplification was performed using DNA of *M. mediterraneum* Ca36^T, as previously described (Alexandre and Oliveira, 2011).

Hybridizations were carried out overnight at 68ºC, after a pre-hybridization period of 30 min at the same temperature. For the 16S rRNA detection, the membranes were re-hybridized overnight at 50ºC with a DNA probe. After hybridization, stringency washes and immunological detection were performed according to the manufacturer's instructions.

Hybridization signals were analyzed using ImageQuantTL™ v7.01 (GE Healthcare). The 16S rRNA signal was used as internal control for the amount of total RNA loaded. To determine the expression levels, the ratio between transcript signals and the corresponding 16S rRNA signals was calculated and the fold difference was determined using the ratio of the previous value between control and the salt shock conditions.

Table 3.1 List of the isolates used in the present study (from Chapter 2).

Province	Origin	Isolate
Trás-os-Montes e Alto Douro	Bragança	BR-8
		BR-9
		BR-15
		BR-16
		BR-28
	Lamego	LM-1
		LM-9
		LM-13
		LM-18
		LM-21
Douro Litoral	Porto	PII-1
		PII-2
		PII-3
		PII-4
Beira Litoral	Aveiro	A-3
		A-8b
	Aveiro II	AII-5
		AII-7
	Coimbra	C-1
		C-3
		C-7
		C-9
		C-13
		C-14
		C-15
		C-23
		C-24
		C-25
		C-27b
Beira Alta	Guarda	G-1
		G-4
		G-10
		G-24
		G-55
	Viseu	V-5b
		V-15b
		V-18
		V-20
		V-25b
Beira Baixa	Castelo Branco	CB-10
		CB-19
		CB-23
		CB-30
		CB-38
		CB-75
	Telhado	T-3
		T-4
		T-5
		T-7
		T-8
Madeira	Serra d' Água	SA-9
		SA-12
		SA-13
		SA-17
Estremadura	Alenquer	AL-13
	Caldas da Rainha	CR-3
		CR-16
		CR-18
		CR-29
		CR-32
	Leiria	L-19
	Salir	SL-1
		SL-2
		SL-3
		SL-5
		SL-6
		SL-7
		SL-9
	Setúbal	ST-2
		ST-5
		ST-8
		ST-20
		ST-33
	Sintra	S-1
		S-8
		S-15
		S-24
		S-26
Ribatejo	Santarém	STR-2
		STR-4
		STR-10
		STR-14
		STR-16
Alto Alentejo	Elvas	75
		78
		85
		CV-18
	ENMP	EE-7
	Évora	90
		93
		94
		98
		102
	Portalegre	PT-35
Baixo Alentejo	Beja	6b
		7a
		27
		29
		64b
Algarve	Portimão	PM-1
		PM-14
		PM-17
	Portimão I	PMI-1
		PMI-6
	Praia do Alemão	PA-5
		PA-6

3.4 Results

Salt stress tolerance

In general, the chickpea mesorhizobia isolates showed low tolerance to salinity (Fig. 3.1). At 1.5% NaCl, the isolates growth ranged from 3% to 46% while at 3% NaCl, the growth range was between 2% and 30%. Actually, a positive correlation was found between growth at both NaCl concentrations (r = 0.354, P < 0.01). Only four isolates grew above 30% with 1.5% NaCl, namely BR-8-Bragança, CR-32-Caldas da Rainha, PA-6-Praia do Alemão and SL-2-Salir.

In order to investigate if rhizobial species clusters are significantly different in terms of their ability to tolerate salt stress, several statistical tests were performed. For both NaCl concentrations used, the Kruskal-Wallis test indicated that there were differences between species clusters (χ^2 = 11.949, df = 3, P < 0.05 for 1.5% NaCl and χ^2 = 77.062, df = 3, P < 0.01 for 3% NaCl). Despite the fact that at 1.5% NaCl, the *M. huakuii* / *M. amorphae* and the *M. ciceri* / *M. loti* isolates present the lowest growth average, no significant differences were found between these and the other two species clusters (with the highest growth average). On the other hand, at 3% NaCl significant differences were observed: *M. huakuii* / *M. amorphae* and the *M. ciceri* / *M. loti* clusters are the species groups with the lowest average tolerance and are significantly different from each other and from the remaining two species clusters.

Comparing the tolerance levels between the type strain and the isolates belonging to the same species cluster, differences in tolerance phenotype were detected. For instance, the *M. mediterraneum* type strain (cluster D) grew above 10% at both stress conditions, and while some isolates of the same species group show a similar phenotype, others show a high sensitivity to salt.

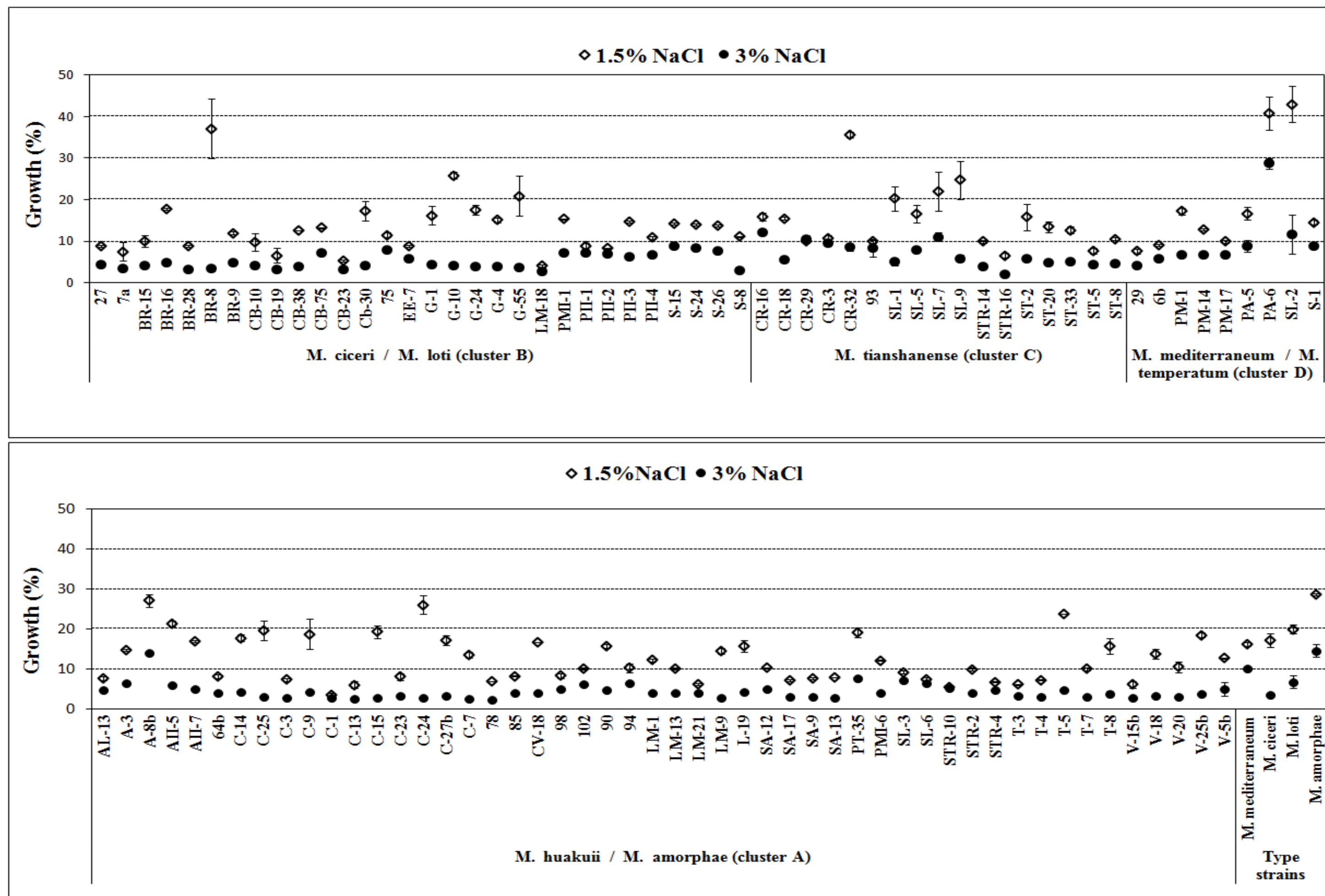

Figure 3.1 Growth of chickpea mesorhizobia under different salt stress conditions: 1.5% NaCl (open diamonds) and 3% NaCl (closed circles). Percentages were calculated considering the control condition (YEM with no extra NaCl) as 100% growth. Presented values are the average of three replicas (standard deviation bars are shown).

In order to investigate if salt tolerance is related to the provinces of origin of the isolates, statistical analyses were performed. For both NaCl concentrations tested, the Kruskal-Wallis test indicated that there are differences between provinces of origin of the isolates ($\chi^2 = 77.528$, $df = 10$, $P < 0.01$ for 1.5% NaCl and $\chi^2 = 141.368$, $df = 10$, $P < 0.01$ for 3% NaCl). Regarding the ability to grow with 1.5% NaCl, the isolates from the three provinces with the lowest growth averages (Ribatejo, Baixo Alentejo and Madeira) were found to be significantly different from the isolates from the provinces with highest growth average (Algarve, Estremadura, Beira Litoral and Beira Alta). These differences are also evident in the correspondence analysis (CA) biplot, which revealed an association between some provinces of origin and isolates' ability to tolerate 1.5% NaCl (Fig. 3.2). Madeira, Beira Baixa, Ribatejo and Baixo Alentejo isolates are associated with low tolerance to salt stress, while Algarve, Beira Alta and Beira Litoral isolates are associated with high tolerance to this stress. Isolates from Algarve, Estremadura and Douro Litoral provinces show the highest growth averages at 3% NaCl. The *post hoc* tests indicated that Estremadura and Douro Litoral provinces are significantly different from the provinces with low growth averages.

Since significant differences between provinces of origin of the isolates with regard to salt tolerance were obtained, statistical analyses were performed in order to investigate if soil characteristics (Chapter 2, Table 2.1) were related with salt tolerance. For both salt concentrations, a positive correlation between salt tolerance and electrical conductivity of the origin soil of the isolates was found ($r = 0.228$, $P < 0.01$ for 3% NaCl and $r = 0.207$, $P < 0.01$ for 1.5% NaCl). A negative correlation between growth with 1.5% NaCl and organic matter levels was found ($r = -0.142$, $P < 0.01$). On the other hand, the pH value of the origin soil and salt-tolerance at 3% NaCl were found to be positively correlated ($r = 0.298$, $P < 0.01$).

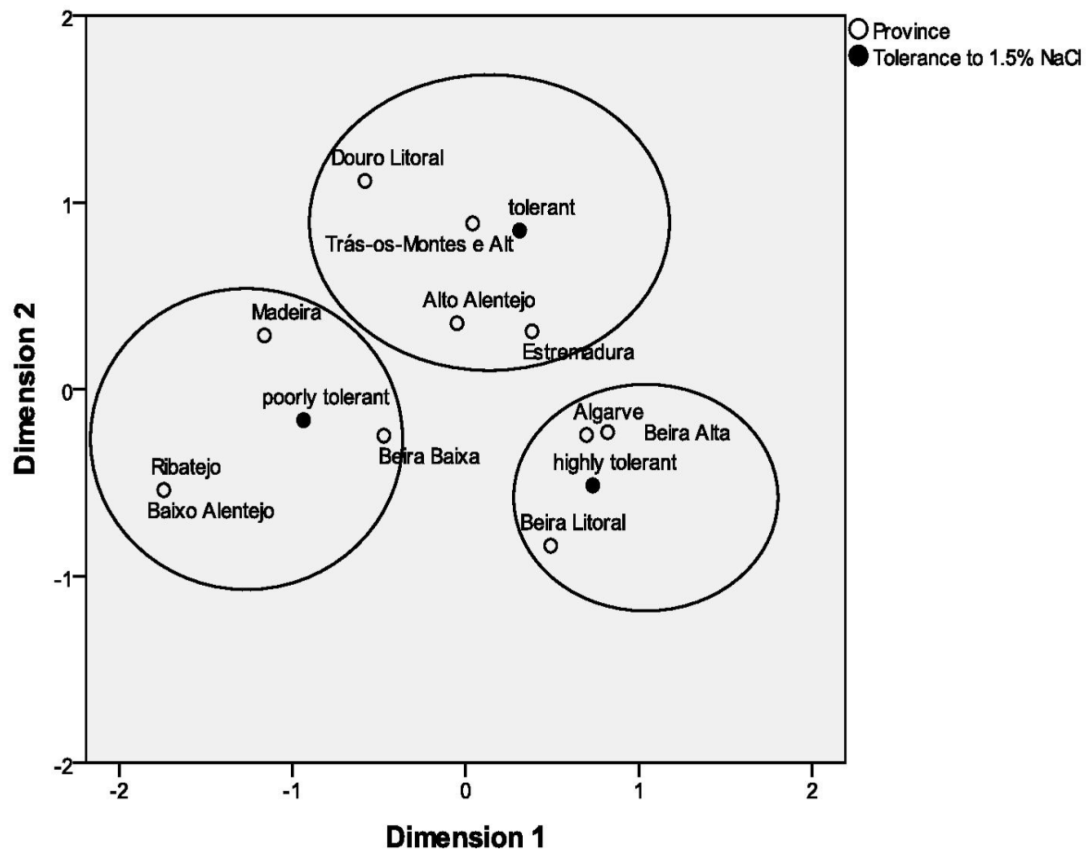

Figure 3.2 CA biplot of the relationship between provinces of origin of the isolates and tolerance to 1.5% NaCl. (Note: the dots corresponding to provinces are occasionally overlaid).

Transcriptional analysis of the major chaperone genes upon salt shock

The transcription of *groEL*, *dnaKJ* and *clpB* genes was analyzed in isolates showing different phenotypes upon salt stress, in order to investigate the involvement of these genes in tolerance to salinity. The transcriptional levels of the major chaperone genes were evaluated by northern hybridization after a salt shock and compared to those of control conditions. Based on the salt tolerance screening at 1.5% NaCl (Fig. 3.1), 12 chickpea rhizobia isolates, comprising the four species clusters and including both tolerant (growth > 20%) and sensitive (growth < 10%) isolates, were selected. From cluster A, isolates C-3-Coimbra

and C-23-Coimbra as salt-sensitive and isolates C-25-Coimbra and AII-5-Aveiro as salt-tolerant were chosen. Isolates BR-8-Bragança and G-10-Guarda as salt-tolerant and LM-18-Lamego and EE-7-ENMP as salt-sensitive, belonging to cluster B, were selected. From clusters C and D, one isolate of each category of tolerance was chosen, namely ST-5-Setúbal and 29-Beja as salt-sensitive and CR-32-Caldas da Rainha and PA-6-Praia do Alemão as salt-tolerant, respectively.

The northern blot analysis with the *dnaKJ* RNA probe allows the detection of three different transcripts, namely the mRNAs of *dnaK* and of *dnaJ* and also the bicistronic *dnaKJ* mRNA (Alexandre and Oliveira, 2011). A transcript with approximately 2 kb, which is consistent with the predicted size of the *dnaK* gene transcript, was detected in all analyzed isolates (Fig. 3.3a).

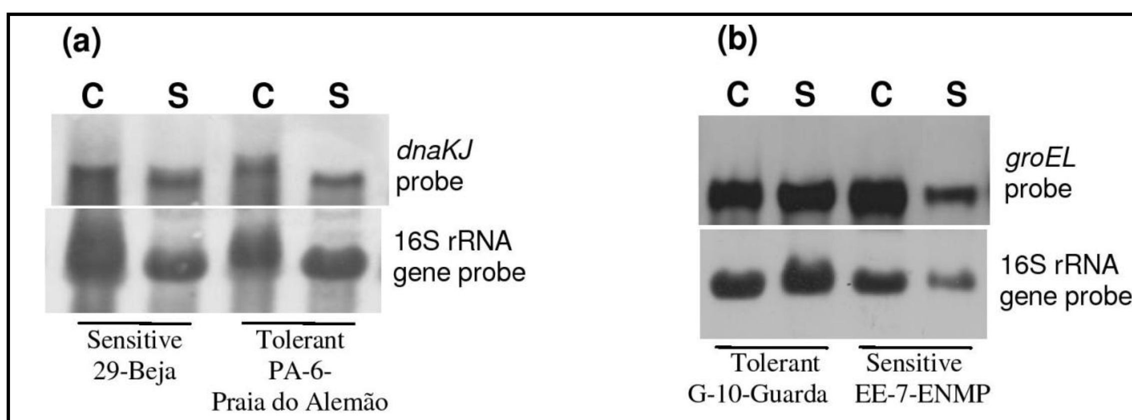

Figure 3.3 Comparison of transcriptional analysis of the *dnaK* gene (a) and the *groESL* operon (b) between a salt-tolerant and a salt-sensitive isolate submitted to salt shock. Northern blot hybridization of total RNA with probes specific for *dnaKJ*, *groEL* and 16S rRNA under control conditions (C) and upon salt shock (S).

Most isolates showed a slight increase in the *dnaK* mRNA levels upon salt shock (Fig. 3.4), however, the sensitive isolate C-23-Coimbra showed a significant increase in *dnaK* transcript levels (approximately 3 fold) after salt shock. The level of *dnaK* gene transcription was not directly related with the sensitivity or tolerance of the isolates for all species cluster. For instance, the sensitive isolates

from clusters C and D, revealed a higher induction of *dnaK* than the tolerant isolates from the same species. On the other hand, in the tolerant isolates from cluster B, low induction of *dnaK* gene was detected, while in the sensitive isolates no induction or a slight repression of *dnaK* gene occurred, after salt shock.

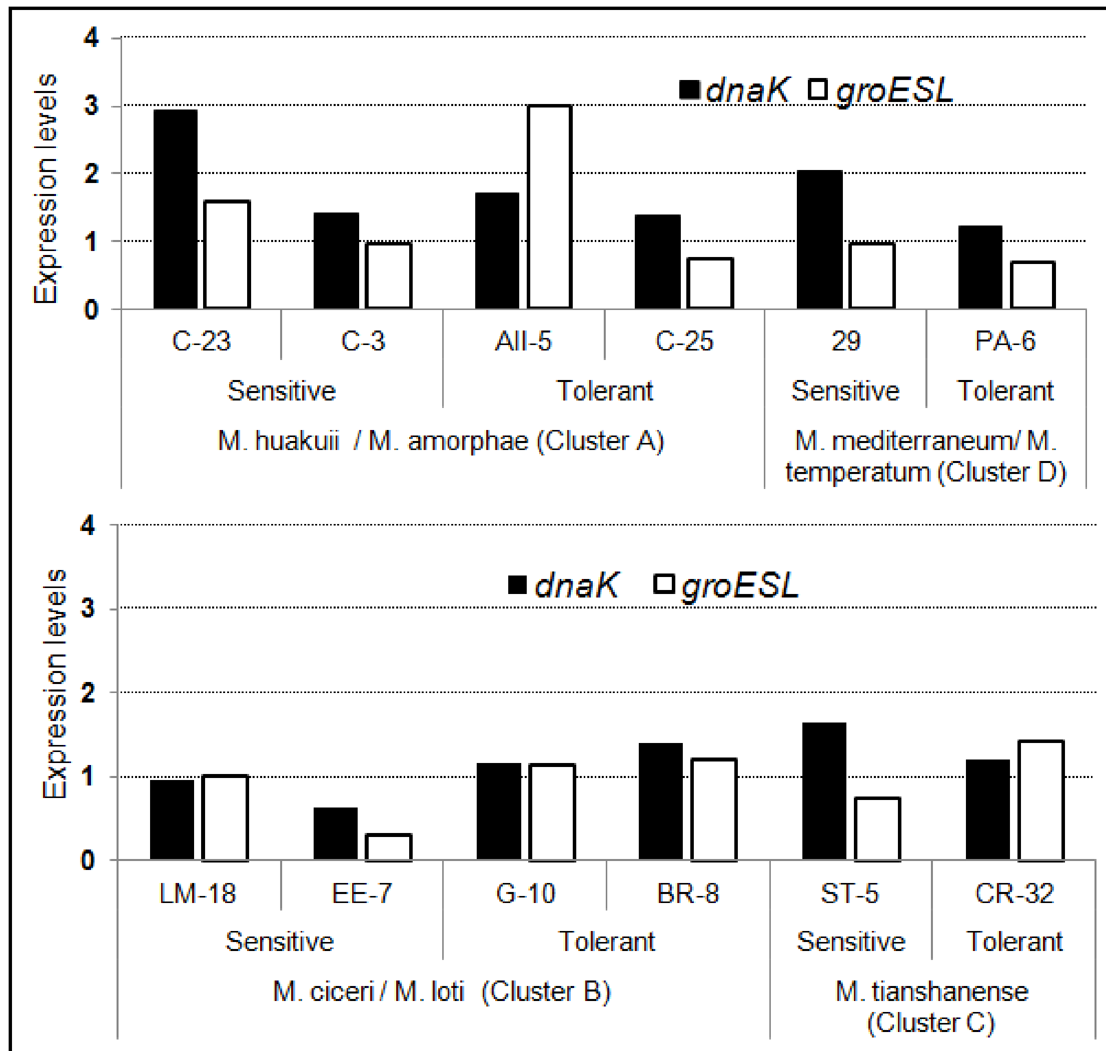

Figure 3.4 Expression levels of *dnaK* and *groESL* genes after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the four species clusters (A-D).

Concerning the analysis of the *groESL* chaperone system, the *groEL* RNA probe could detect two putative transcripts: the bicistronic *groESL* transcript and the *groEL* transcript (Alexandre and Oliveira, 2011). In this study, the signal detected was approximately 2 kb long, which corresponds to the bicistronic *groESL* mRNA (Fig. 3.3b). The majority of the isolates displayed unchanged or slightly lower *groESL* mRNA levels upon salt shock (Fig. 3.4). However, three isolates belonging to different species groups, revealed induction of *groESL* operon after salt shock, namely isolates C-23-Coimbra (sensitive), AII-5-Aveiro (tolerant) and CR-32-Caldas da Rainha (tolerant). Both sensitive and tolerant isolates, from the *M. ciceri* / *M. loti* and *M. mediterraneum* / *M. temperatum* clusters, showed no significant differences on *groESL* expression level after salt shock, with exception of the sensitive isolate EE-7-ENMP, which showed a decrease in the *groESL* mRNA levels. Tolerant isolate AII-5-Aveiro showed the highest induction of *groESL*, nevertheless within the same cluster another tolerant isolate (C-25-Coimbra) revealed repression of the same operon.

Regarding the transcriptional analysis of the ClpB chaperone gene, the *clpB* RNA probe detects a single transcript, according to the genome of *M. sp.* MAFF303099 and *M. ciceri* bv. *biserrulae* WSM1271. In order to analyse the expression of the *clpB* gene upon salt shock, isolates from cluster B, namely BR-8-Bragança (tolerant), G-10-Guarda (tolerant) and EE-7-ENMP (sensitive), were analyzed. In tolerant isolates, after salt shock the *clpB* transcript levels were similar to those obtained under control conditions (Fig. 3.5).However, for the sensitive isolate EE-7-ENMP, a repression of *clpB* gene expression was observed.

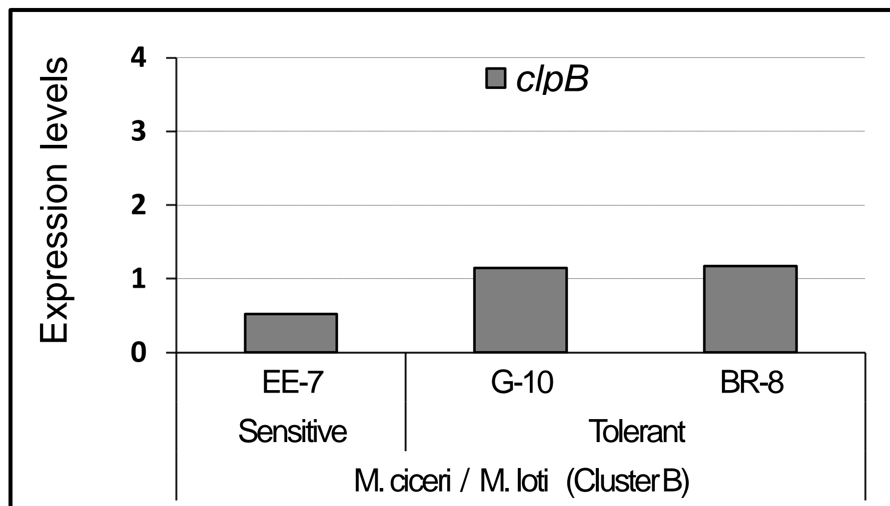

Figure 3.5 Expression levels of *clpB* gene after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the Cluster B.

3.5 Discussion

In the present study, a large collection of native chickpea rhizobia were screened for salt stress tolerance. In all tested mesorhizobia, the growth was reduced upon salt stress. Bacterial growth was severely affected with 3% NaCl. Nevertheless, some strains such as LM-18-Lamego showed similar growth in both stress conditions. Only a few isolates showed a significant growth with 1.5% NaCl. These results are in agreement with several studies, which showed that growth of most rhizobial strains is inhibited by 100 mM (approximately 0.6%) NaCl, while a few strains can tolerate more than 300 mM (approximately 1.8%) NaCl (Elsheikh and Wood, 1990a, b; Zurayk *et al.,* 1998; Maâtallah *et al*., 2002a, b). Several studies indicated that fast-growing strains are generally more tolerant to high salt concentrations than slow-growing strains (Odee *et al.,* 1997; Zerhari *et al.,* 2000; Maâtallah *et al.,* 2002b). Jarvis *et al.* (1997) described mesorhizobia growth rate as lower than the fast-growing members of the genus *Rhizobium* and higher than the slow-growing members of the genus *Bradyrhizobium*. In general, our isolates are more sensitive than *Rhizobium* sp. strains and more tolerant than

Bradyrhizobium sp. strains (Elsheikh and Wood, 1990b) which is in agreement with what was mentioned above.

Despite the fact that salt tolerance seems to be diverse among chickpea rhizobia, an association between province of origin of isolates and salt tolerance was obtained, which is in agreement with the observations by Kulkarni *et al.* (2000). Correlations between the soil organic matter levels and the size or strain composition of population of rhizobia have been reported (Bezdicek, 1972). Zahran *et al.* (1992) observed that the majority of *Rhizobium* strains isolated from saline soils were salt-tolerant. Similarly, herein correlations between origin soil characteristics, such as soil pH, electrical conductivity and organic matter levels and salt tolerance of the isolates was found, suggesting that strains phenotype is closely related with soil characteristics, which act as a selective pressure.

Shamseldin (2008) found that all salt-tolerant strains of common bean rhizobia contained a 250 kb plasmid with the exception of one strain, suggesting that this plasmid may play a role in the salt tolerance mechanism. However, no relationship between salt tolerance and the presence of plasmids was found in our study.

Although several reports show that many genes may be involved in the response to salt stress in rhizobia (Talibart *et al*., 1994; Nogales *et al*., 2002; Rüberg *et al.,* 2003; Wei *et al.,* 2004; Domínguez-Ferreras *et al.,* 2006), the mechanisms for osmotic adaptation in rhizobia exposed to salt stress are still unclear. Mutational studies allowed the identification of multiple genes involved in salt tolerance, as for example genes encoding cation efflux proteins (*pha* genes), methyltransferase (*metH*), trigger factor (*tig*) and genes involved in exopolysaccharides synthesis (EPS) (Jiang *et al*., 2004; Miller-Williams *et al*., 2006). Taking into account previous studies suggesting the direct or indirect involvement of DnaKJ, GroESL and ClpB in salt stress tolerance (Nogales *et al.* 2002; Wei *et al*., 2004; Domínguez-Ferreras *et al*., 2006), the transcriptional analysis of these chaperone genes was performed using sensitive and tolerant

isolates within four species cluster. In the majority of the tested isolates, the *dnaK* transcripts increased after salt shock, while no change or a slight repression was observed with the *groESL* operon. However, the detected levels of induction of these genes are very low when compared with the levels obtained by Alexandre and Oliveira (2011) in chickpea mesorhizobia submitted to heat shock.

The increase of the *dnaK* mRNA levels found in the present study is in agreement with several former reports in others organisms, such as *Enterococcus faecalis* (Laport *et al.,* 2004), *Lactobacillus sanfranciscensis* (Hörmann *et al.,* 2006) and *Bifidobacterium* (Ventura *et al*., 2005) under salinity conditions. Nevertheless, Susin *et al.* (2006) verified that *Caulobacter crescentus* cells with DnaKJ depleted are not affected by the presence of high concentrations of NaCl. On the other hand, Nogales *et al.* (2002) showed that a mutant with a disrupted *dnaJ* gene presented less salt tolerance when compared to the wild type.

The *groESL* operon was slightly repressed or unchanged in the majority of the isolates when submitted to salt shock, which is in agreement with other studies in *Mesorhizobium* and *Ensifer* (Domínguez-Ferreras *et al.,* 2006; Laranjo and Oliveira, 2011). However, in other microorganisms, such as *Enterococcus faecalis* and *Caulobacter crescentus*, the *groESL* operon was induced when the cells were submitted to salt shock (Laport *et al.,* 2004; Susin *et al.,* 2006).

ClpB was described to be involved in salt tolerance (Nag *et al.,* 2005; Ventura *et al.,* 2005), high temperature tolerance (Eriksson and Clarke, 2000; Nag *et al.,* 2005; Acébron *et al.,* 2009), and virulence (Chastanet *et al*., 2004). Our preliminary data on transcriptional analysis of the *clpB* gene showed no induction in the salt-tolerant isolates, while in the salt-sensitive isolate a significant repression was observed. These results are different from the ones reported by Domínguez-Ferreras *et al.* (2006) who described an induction (2.13 fold) of the *clpB* gene in *Ensifer meliloti*, upon exposure to 400 mmol l^{-1} (± 2.5%) NaCl for one hour.

Our results suggest that chaperone genes are not significantly induced in chickpea mesorhizobia under salt shock. Nevertheless, according to Domínguez-Ferreras *et al.* (2006) the induction levels greatly depend on the salt type and concentration, as well as the duration of the imposed salt stress. Alexandre and Oliveira (2011) reported a higher increase in the amount of *dnaK* and *groESL* transcripts in heat-tolerant isolates in comparison with sensitive ones, within the same species group, under heat stress, suggesting that increased levels of these chaperones may contribute to a higher tolerance to heat in rhizobia. However, in the present study, using salt-tolerant and -sensitive isolates, no clear relationship was observed between isolate phenotypes and chaperone gene induction. Similar results were observed by Laranjo and Oliveira (2011), who compared the induction levels of *groESL* of two *Mesorhizobium* species, with different salt tolerance phenotypes.

In conclusion, our results show that most chickpea mesorhizobia are sensitive to salt stress. Nevertheless, some isolates were able to grow 30 to 46% compared to control conditions. No relationship was found between the level of salt tolerance and the chaperone genes induction, among the tested isolates. Taking this into account, a higher salt tolerance does not appear to be due to higher transcriptional levels of these chaperone genes. To our knowledge, this is the first report in chickpea mesorhizobia focusing on the transcriptional analysis of the main chaperone genes under salt shock. Response and adaptation to environmental stresses are complex phenomena involving many biochemical processes that likely reflect changes in gene expression and in the activity and transport of proteins. Further studies are required to understand the molecular basis of salt tolerance in rhizobia.

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Chapter 4

Evaluation of the symbiotic performance of *acdS*-transformed mesorhizobia under salt stress

Symbiotic performance of rhizobia in saline soils depends on the salt-tolerance of both the host and the microsymbiont. The selection of native rhizobia highly efficient and simultaneously highly tolerant to environmental stresses is not always easy to accomplish. Thus, alternative strategies, such as the genetic transformation of the microsymbiont partner, in order to improve its symbiotic performance under stressful conditions, may be useful for agriculture applications. In an attempt to improve the symbiotic performance under salinity conditions of chickpea mesorhizobia, genetic transformation of two isolates with an exogenous ACC deaminase gene (*acdS*) was performed. The ACC deaminase is associated to the lowering of ethylene levels in plant tissues by the cleavage of its immediate precursor (ACC) into ammonia and α-ketobutyrate.

This chapter is based on the manuscript:

Brígido, C., Nascimento, F.X., Duan, J., Glick, B.R., Oliveira, S. “Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Mesorhizobium* spp. reduces the negative effects of salt stress in chickpea” *Submitted*

4.1 Summary

Salinity stress is one of the most important abiotic stresses due to its impact in reducing crop yield worldwide. Our goal was to study the symbiotic performance of two *Mesorhizobium ciceri* strains, transformed with an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene, in chickpea plants under salinity stress. The *M. ciceri* EE-7 (salt-sensitive) and *M. ciceri* G-55 (salt-tolerant) strains were transformed with an *acdS* gene present on plasmid pRKACC, by triparental conjugation. A plant growth assay was conducted using chickpea plants inoculated with either *acdS*-transformed or wild-type strains, under control and salt-stress conditions.

Salinity significantly reduced the overall growth of chickpea plants inoculated with either wild-type strains. Although the growth of plants inoculated with either salt-sensitive or salt-tolerant strain was reduced, the native salt-tolerant mesorhizobia strain showed a higher ability to nodulate chickpea under salt stress compared to the salt-sensitive strain. Both *acdS*-transformed strains showed an improvement in chickpea growth compared to the wild-type strains, under salinity. All of the plant growth parameters were higher in plants inoculated with the *acdS*-transformed strains compared to the plants inoculated with the native strains. The negative effects of salt stress on nodulation were reduced when using *acdS*-transformed strains in comparison to the wild-type strains. Interestingly, by expressing the exogenous ACC deaminase gene, the salt-sensitive strain was able to induce nodules in the same extent as the salt-tolerant strain, contributing for a significant increase in the shoot dry weight of plants.

As far as we know, this is the first report on the genetic modification of a *Mesorhizobium* strain that improved its nodulation abilities under salt stress, indicating that ACC deaminase can play an important role in facilitating plant-*rhizobium* interaction under salinity conditions.

4.2 Introduction

Salinity stress is one of the most important abiotic stresses due to its impact in reducing crop yield worldwide. More than 800 million hectares of land are salt-affected (Essah *et al*., 2003), accounting for more than 6% of the world's total land area. Moreover, about half of the land devoted to irrigated crops is adversely affected by salt (Gamalero *et al*., 2009).

Salt tolerance in plants is very diverse, ranging from halophytes to extremely salt-sensitive plants showing a wide range of adaptations (Gamalero *et al*., 2010). Legumes represent a very significant group of crops in agriculture and therefore their tolerance to salt stress is important to worldwide agricultural practice. Chickpea (*Cicer arietinum* L.) is one of the most important grain legume crops because it is a protein source in both human and animal diets. Furthermore, it plays a significant role in the maintenance of soil fertility, through its symbiotic association with rhizobia (Saxena and Singh, 1987). Like other legumes, chickpea is very sensitive to salinity, which affects its growth and development. On the other hand, rhizobial tolerance to salinity is important for the symbiosis, particularly if salt concentrations could have a detrimental effect on rhizobial populations as a result of direct toxicity and/or as through osmotic stress (Tate, 1995).

Despite the fact that legume plants are more sensitive to salinity than their rhizobial partners, the establishment of the symbiosis between them is highly sensitive to salt stress (Zahran, 1999). Ethylene is produced by plants in response to several environmental stresses (Bari and Jones, 2009) and is also known for its negative role in nodulation (Ma *et al*., 2002; Middleton *et al*., 2007), as it inhibits the formation and functioning of nodules (Nandwal *et al*., 2007; Ding and Oldroyd, 2009).

Symbiotic performance in saline soils depends on the salt-tolerance of both the host and the microsymbiont (Saxena and Rewari, 1992; Zahran, 1999). The cultivated chickpea has a narrow genetic variation (Udupa *et al*., 1993), which

makes it difficult for breeders to produce elite cultivars with durable resistance to the many major abiotic stresses. However, the use of genomic analysis of potential salt-tolerant chickpea cultivars may contribute significantly to the selection of salt-tolerant cultivars (Mantri *et al.*, 2007; Varshney *et al.*, 2009). Meanwhile, several strategies have been followed in an attempt to improve plant growth under stressful conditions, such as selection of high-salt tolerant rhizobia strains and reduction of deleterious ethylene concentrations in the plant, that seem to be promising approaches.

Despite some reports showing that high salt-tolerant rhizobia strains are symbiotically more efficient than salt-sensitive ones under saline conditions (Elsheikh and Wood, 1995; Saxena and Rewari, 1992), some researchers are critical of these reports. Under salinity, the ethylene levels increase in plant tissues (Abeles *et al.*, 1992). Nandwal *et al.* (2007) reported a positive correlation between the levels of salinity and the amount of 1-aminocyclopropane-1-carboxylate (ACC) content (the immediate ethylene precursor) and ethylene production in chickpea nodules. From this perspective, the approach of lowering the ethylene levels in plants through the activity of ACC deaminase (encoded by the *acdS* gene), that converts ACC into α-ketobutyrate and ammonia, may be an alternative to help chickpea plants to overcome the negative effects caused by salt stress. Several reports showed the beneficial effects of the utilization of plant-growth promoting bacteria expressing *acdS* in different plants subjected to salinity conditions (Sergeeva *et al.*, 2006; Cheng *et al.*, 2007; Jalili *et al.*, 2008; Gamalero *et al.*, 2010; Siddikee *et al.*, 2010, 2011).

Rhizobia that express the enzyme ACC deaminase typically exhibit only a low level of enzyme activity compared with free-living plant growth-promoting bacteria (i.e., 10- to 30-fold less than free-living bacteria). This suggests that there may be at least two types of ACC deaminase-producing bacteria (Glick *et al.*, 2007a). There are free-living bacteria that bind relatively non-specifically to plants and have a high level of ACC deaminase activity, protecting plants from different stresses by lowering ethylene levels throughout the plant. Alternatively, rhizobia

bind tightly to the roots of specific plants and have a low level of enzyme activity which facilitates nodulation by locally lowering ethylene levels.

Taking into account the previous results on the use of *acdS* genes from free-living bacteria to lower stress ethylene levels (Glick, 2003) it should be possible to transform *Mesorhizobium* spp. with *acdS* genes from free-living bacteria and thereby enhance nodulation and growth of legumes under stressful conditions. Thus, the main objectives of the work reported here were: (i) to evaluate the symbiotic performance of native salt-tolerant and salt-sensitive rhizobia strains in chickpea plants under salt stress; and (ii) to improve the symbiotic performance of *Mesorhizobium ciceri* strains in chickpea plants under salinity stress by transformation with an exogenous *acdS* gene.

4.3 Materials and Methods

Bacterial Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 4.1. Two chickpea mesorhizobia were selected based on their salt tolerance in liquid media (Chapter 3), and their similar symbiotic effectiveness under control conditions (Chapter 2), namely *Mesorhizobium ciceri* EE-7 (salt-sensitive) and *Mesorhizobium ciceri* G-55 (salt-tolerant). The mesorhizobia strains were grown at 28ºC in tryptone-yeast (TY) medium (Beringer, 1974) or in minimal medium (Robertsen *et al*., 1981), containing sucrose as the only carbon source. The growth medium for transformed mesorhizobia strains was supplemented with tetracycline (20 $\mu g.ml^{-1}$). The *Escherichia coli* DH5α and MT616 strains were grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001) at 37ºC. Appropriate antibiotics were added to the medium when necessary. For the *E. coli* strain containing pRKACC, 15 $\mu g.ml^{-1}$ of tetracycline was used, while for the strain with pRK600, the medium was supplemented with 25 $\mu g.ml^{-1}$ of chloramphenicol.

Table 4.1 Bacterial strains and plasmids used in this study.

Plasmid or Bacteria	Relevant features	Reference or source
Plasmids		
pRKACC	The broad-host-range plasmid pRK415 containing the *acdS* gene from *Pseudomonas putida* UW4, Tc^r	Shah *et al.*, 1998
pRK600	pRK2013, *npt::*Tn9, Cm^r	Finan *et al.*, 1986
Bacteria		
M. ciceri EE-7	*M. ciceri* strain, salt-sensitive	Chapter 3
M. ciceri G-55	*M. ciceri* strain, salt-tolerant	Chapter 3
M. ciceri EE-7 (pRKACC)	*M. ciceri* EE-7 strain containing pRKACC	This study
M. ciceri G-55 (pRKACC)	*M. ciceri* G-55 strain containing pRKACC	This study
E. coli DH5α	SupE44 *Δ lacU169 (φ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Sambrook and Russell 2001
E. coli MT616	Strain containing helper plasmid pRK600	Finan *et al.*, 1986

Tc^r- Tetracycline resistance; Cm^r- Chloramphenicol resistance

Triparental Mating

In this study, plasmid pRKACC, containing the *acdS* gene from *Pseudomonas putida* UW4 (Shah *et al.*, 1998), was used to transform the two selected chickpea mesorhizobia strains, by triparental conjugation. *E. coli* DH5α cells containing plasmid pRKACC were used as the donor, mesorhizobia isolates were the recipient and *E. coli* MT616 cells, with pRK600 acted as a helper, in triparental mating (Nascimento *et al.,* 2012). The *E. coli* cells were grown overnight at 37ºC on LB plates, containing the appropriate antibiotics. The mesorhizobia isolates were grown on TY plates at 28ºC for 48-72 hours. After growth, a small amount of each cell culture of the *E. coli* strains was transferred to each mesorhizobia strain on TY plates and mixed. These mixtures were grown overnight at 28ºC to allow plasmid transfer to occur. The mixtures were picked and resuspended in liquid minimal medium with no antibiotics. The cells were diluted and then

transferred to minimal medium plates containing 20 μg.ml^{-1} tetracycline and allowed to grow at 28ºC. To avoid *E. coli* cell contamination, the selected isolates were purified by at least four single colony passages on minimal medium supplemented with 20 μg.ml^{-1} tetracycline.

In order to confirm the transfer of plasmid pRKACC to mesorhizobia cells, the tetracycline resistant mesorhizobia colonies were picked and plasmids were extracted following the manufacturer's procedures (DNA-Spin^{TM} Plasmid DNA Purification Kit, Intron) and visualized in a 1.0% agarose gel stained with ethidium bromide.

ACC deaminase activity assay

The native mesorhizobia cells and the respective transconjugants were tested for ACC deaminase activity. ACC deaminase induction was performed according to Duan *et al.* (2009). After induction, ACC deaminase activity was measured based on the determination of α-ketobutyrate resulting from ACC cleavage by ACC deaminase, as described by Penrose and Glick (2003). Total protein content of cells was quantified by the method of Bradford (1976). The final ACC deaminase activity was expressed in μmol α-ketobutyrate/mg protein/h.

Chickpea seed sterilization

Chickpea seeds (cultivar Chk 3226) were surface sterilized with 5% sodium hypochlorite diluted with sterile distilled water (1/1: v/v) for 30 minutes. After sterilization, seeds were rinsed six times in sterilized distilled water and incubated for 2 hours at 28ºC. Seeds were placed in sterilized vermiculite and then incubated in the dark for 48 hours at 28ºC.

Evaluation of the chickpea plants tolerance to salt

After germination, the seeds were transferred to plastic pots (±11.5 cm diameter; ±10.5 cm high) filled with sterile vermiculite and grown in a growth chamber, under a 16/8h light/dark cycle and 24/18ºC day/night temperature and at a relative humidity of 65%.

In order to determine the salt concentration to use in the plant growth trial, the chickpea plants were tested with three different NaCl concentrations: 0.075%, 0.15% and 0.3% supplemented in the nitrogen-free nutrient solution (Broughton and Dilworth, 1971) applied in alternate watering. Chickpea plants watered with nutrient-free solution without supplemented NaCl were considered as control plants. Three plants per treatment were used. The nutrient-free solution was applied three times a week. Two months after sowing, the chickpea plants were harvested and the nonlethal salt concentration was determined.

Plant growth assay under control and salt stress

In order to evaluate the symbiotic performance of the *acdS*-transformed and wild-type mesorhizobia strains in chickpea plants under control and salinity conditions, a plant growth assay was conducted in a growth chamber. The *Mesorhizobium* strains were grown in TY liquid medium (supplemented with 20 $\mu g.ml^{-1}$ tetracycline for *acdS*-transformed mesorhizobia strains) at 28ºC for 72 hours. After incubation, the cell suspension was centrifuged at 10,000×g and washed twice with TY liquid medium (without antibiotic). The bacterial cultures were standardized to an OD_{540nm} of 0.8 and 1 ml of the bacterial suspension was used to inoculate each seed.

For control conditions, a nitrogen-free nutrient solution was used (Broughton and Dilworth, 1971). For salinity conditions, 15 days after inoculation, the plants were subjected to salt stress using the nutrient solution supplemented with 0.15% NaCl and applied in alternate watering. The nutrient solutions were applied three

times a week. Uninoculated nitrogen-free plants were used as negative controls. After eight weeks, the plants were harvested and several parameters were measured, such as shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN) and nodule dry weight (NDW).

Statistical analysis

Six replicates per treatment were used for statistical analysis. These data were examined by an analysis of variance and multiple comparisons among treatment means was made by Student-Newman-Keuls test. Statistical analyses were performed using MSTAC software, version 2.1.

4.4 Results

The two selected chickpea mesorhizobia were transformed with the plasmid pRKACC, containing the ACC deaminase gene from the plant growth promoting bacterium, *Pseudomonas putida* UW4. The successful transformation of the two *Mesorhizobium* strains was confirmed by plasmid extraction and visualization in an agarose gel (*data not shown*).

The ACC deaminase activity was measured in all mesorhizobia strains, both native and *acdS*-transformed strains. No ACC deaminase activity was detected in any of the native mesorhizobia strains in free-living conditions. On the other hand, the *acdS*-transformed mesorhizobia strains displayed ACC deaminase activity in free-living conditions. The *acdS*-transformed mesorhizobia strains showed different levels of ACC deaminase activity; the ACC deaminase activity of *Mesorhizobium ciceri* EE-7 (pRKACC) and *Mesorhizobium ciceri* G-55 (pRKACC) was 0.768±0.036 and 0.398±0.068 μmol of α-ketobutyrate/mg of protein/h, respectively.

The chickpea cultivar CHK3226 was tested for salt tolerance. Of the three NaCl concentration tested, only 0.3% NaCl was found to be lethal to the chickpea

plants (*data not shown*). The other two NaCl concentrations applied to chickpea plants also showed negative effects on plants growth, however, in a lower extent. For this reason, 0.15% NaCl concentration was chosen for the evaluation of symbiotic performance of the mesorhizobia strains under salt stress.

A chickpea plant growth assay was performed in order to assess the symbiotic performance of the native salt-tolerant and salt-sensitive rhizobia strains as well the transformed strains, expressing the exogenous ACC deaminase gene, under control and salt stress conditions. Under control conditions the symbiotic performance of the two native mesorhizobia EE-7 and G-55 strains was similar. Shoot dry weight (SDW), root dry weight (RDW), the number of nodules (NN) and the nodule dry weight (NDW) of plants inoculated with either strain showed no significant differences under control conditions (Fig. 4.1 and 4.2).However, in the presence of salt, the growth of chickpea plants inoculated with each native strain was significantly inhibited compared to plants grown in control conditions (Fig. 4.1). The salt stress imposed on the chickpea plants led to a reduction in shoot dry weight, root dry weight, nodule dry weight, number of nodules and chickpea total biomass when compared to the plants grown under control conditions (Fig. 4.1 and 4.2). Although the symbiotic performance of both wild-type strains was negatively affected by salt, the symbiotic performance of the salt-sensitive strain EE-7 was affected in a greater extent when compared to the salt-tolerant strain G-55. Indeed, the chickpea plants inoculated with the salt-sensitive strain were affected to a greater extent by salinity, in all parameters analyzed compared to the plants under control conditions. Under saline conditions, the plants inoculated with the salt-sensitive *Mesorhizobium,* strain EE-7, showed a reduction of 54.5% in the shoot dry weight and 48.7% in the total biomass while the plants inoculated with the salt-tolerant *Mesorhizobium,* strain G-55, showed a decrease of 35.9% in the shoot dry weight and 33.6% in the total biomass, when compared to plants grown under control conditions (Fig. 4.1 A, C).

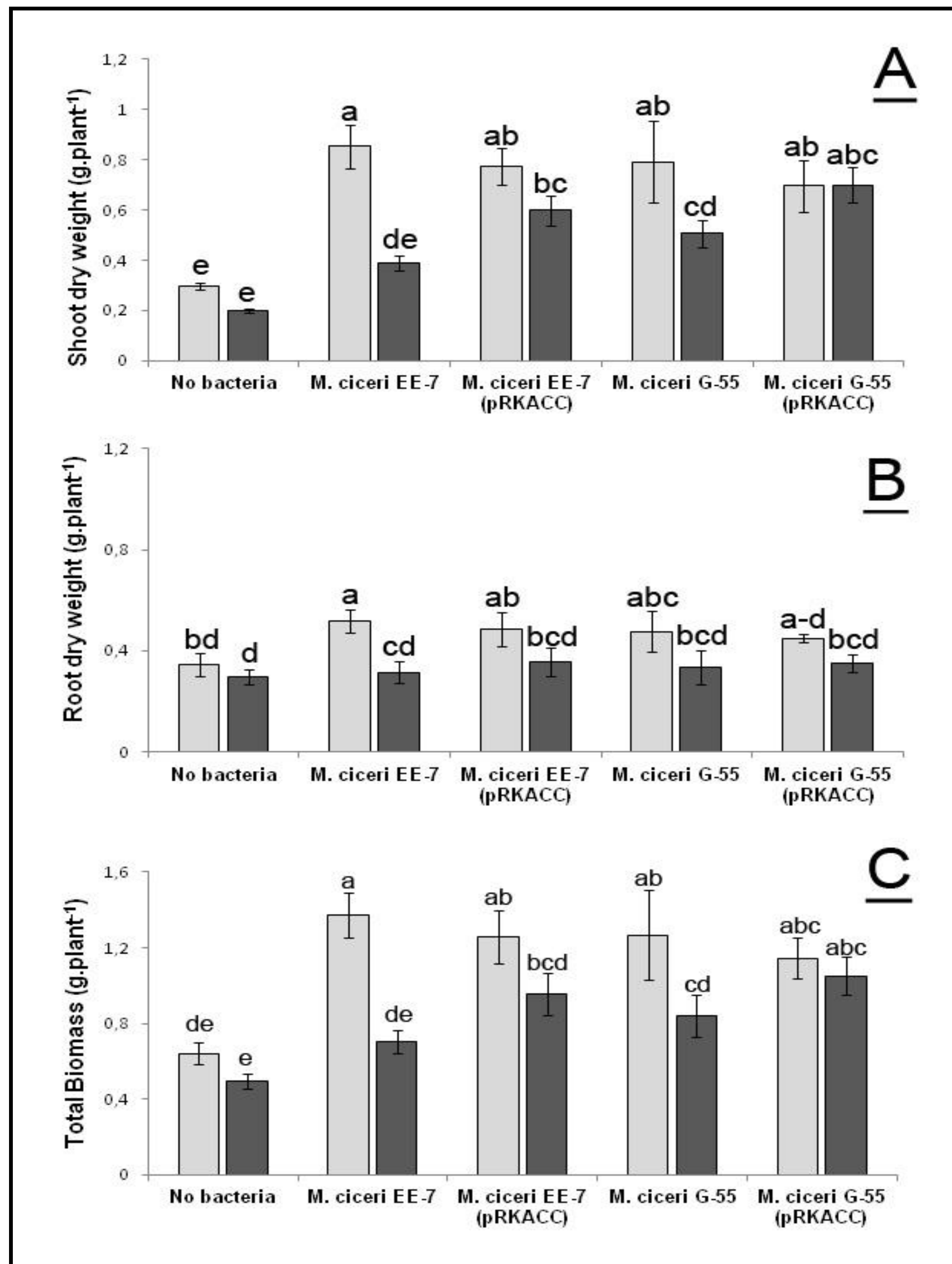

Figure 4.1 Results obtained from plant growth assay using chickpea plants inoculated with transformed and native mesorhizobia salt-tolerant and salt-sensitive strains under control and salinity conditions. Data correspond to the mean and standard error values of six plants after eight weeks of inoculation. Light grey bars plant under control conditions and grey bars correspond to plants subjected to salinity conditions. **A)** Shoot dry weight (g.plants^{-1}) **B)** Root dry weight (g.plants^{-1}) and **C)** Chickpea total biomass (g.plants^{-1}). Means sharing the same letters are not significantly different at $p \leq 0.05$ ($n = 6$)

Concerning the symbiotic performance of the *acdS*-transformed mesorhizobia strains, both transformed strains promoted the growth of chickpea plants in the presence of salt (Fig 4.1). Under salinity and with both strains, all parameters analyzed (SDW, RDW, NN, NDW, total biomass) from plants inoculated with the transformed mesorhizobia strain are higher compared to the plants inoculated with the native strain (Fig. 4.1 and 4.2).

A significant difference between shoot dry weight of plants inoculated with *M. ciceri* EE-7 (pRKACC) and *M. ciceri* EE-7 wild-type was observed under salinity. Moreover, the salt-sensitive strain EE-7 (pRKACC) expressing *acdS* revealed higher symbiotic performance under salt-stress when compared to the corresponding native strain (Fig. 4.2). Strain EE-7 (pRKACC) promoted an increase of shoot dry weight and chickpea total biomass of 54% and 35%, respectively, in plants under salinity stress when compared to those inoculated with the wild-type strain under the same conditions (Fig. 4.1 A, C). An increase in the nodulation abilities of the *Mesorhizobium ciceri* EE-7 (pRKACC) was also observed, especially regarding the number of nodules formed in chickpea plants under salinity. *M. ciceri* EE-7 (pRKACC) strain showed an increase of 120% in the number of nodules when compared to the number of nodules formed by the wild-type strain EE-7, under salinity conditions (Fig. 4.2 A). Interestingly, the number of nodules formed by the *acdS*-transformed mesorhizobia strains in chickpea plants under salt stress conditions was similar to the number of nodules formed in plants inoculated with the same strains, under control conditions (Fig. 4.2 A).

Although in a lesser extent, the plants inoculated with the *Mesorhizobium ciceri* G-55 (pRKACC) strain also showed an improvement in tolerance to salinity conditions, compared to the plants inoculated with the native strain. The use of the *M. ciceri* G-55 (pRKACC) strain as inocula of plants submitted to salinity showed an increase of the symbiotic performance compared to the plants inoculated with its corresponding native strain under the same conditions. The *M. ciceri* G-55 (pRKACC) strain resulted in a 38% and 25% increase of shoot dry

weight and chickpea total biomass, respectively, when compared to the plants inoculated with the native *M. ciceri* G-55, under salinity conditions.

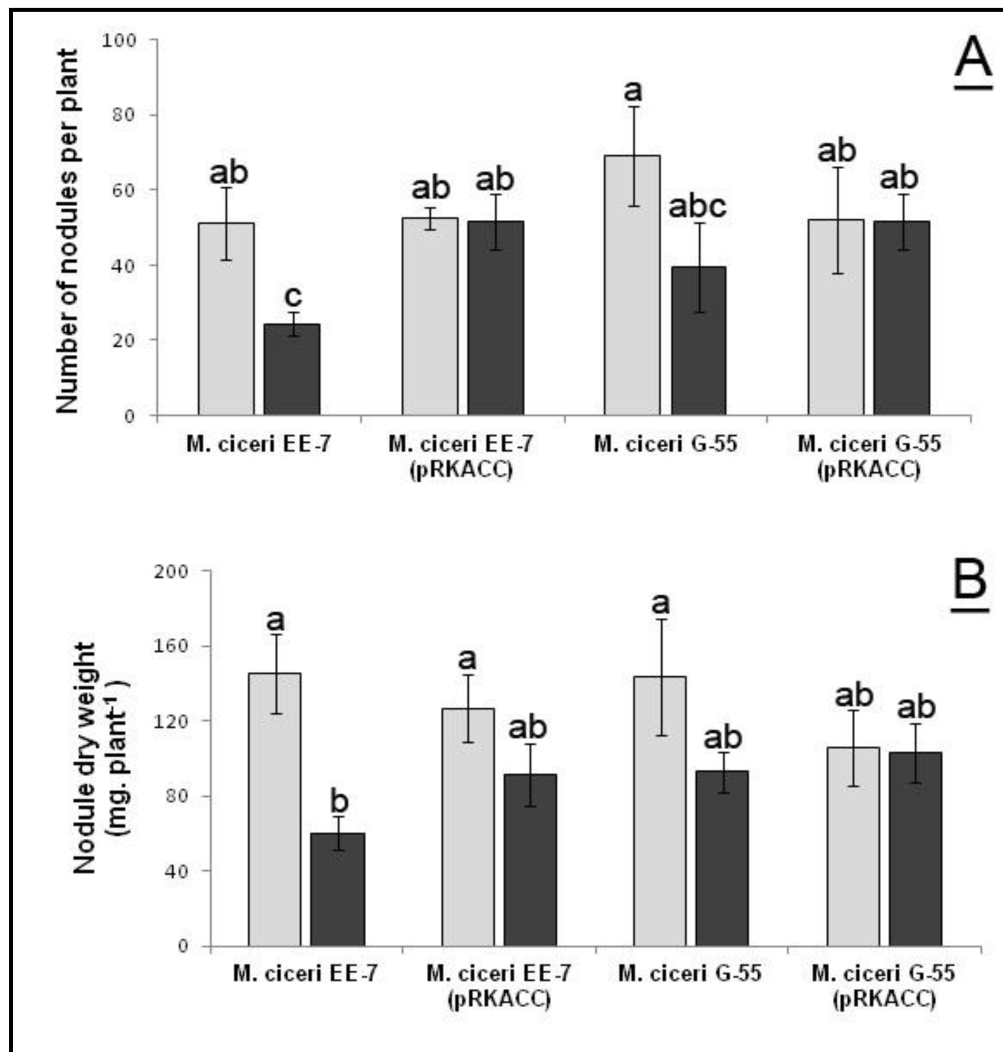

Figure 4.2 Results obtained from plant growth experiments using chickpea plants inoculated with *acdS*-transformed and no-transformed mesorhizobia salt-tolerant and salt-sensitive strains under control and salinity conditions. No nodules were obtained in uninoculated plants. Data correspond to the mean and standard error values of six plants after eight weeks of inoculation. Light grey bars indicate plants under control conditions and grey bars correspond to plants subjected to salinity conditions. **A)** Number of nodules per plant; and **B)** Nodule dry weight (mg). Means sharing the same letters are not significantly different at $p \leq 0.05$ ($n = 6$)

4.5 Discussion

Selection of rhizobia strains and host cultivars is required for effective symbiosis in order to maximize plant production under stressful conditions, such as salinity. In the present work, the effect of NaCl on chickpea plants inoculated with two specific *Mesorhizobium ciceri* strains was evaluated as well as the impact of the expression of an *acdS* gene in the improvement of the symbiotic performance of the strains.

Salinity significantly reduced the overall growth of chickpea plants when inoculated with either of the native strains. This is evident from the reduced shoot and root dry weights of plants subjected to salinity stress, even when the plants were inoculated with either salt-tolerant or salt-sensitive mesorhizobia strains. Similar depressive effects of NaCl in chickpea growth have been reported in other studies (Soussi *et al*., 1998; Garg and Singla, 2004; Eyidogan and Öz, 2007).

Soussi *et al*. (1998) have shown that salt applied at the vegetative growth stage contributes to a strong inhibition of nodulation even with the lowest NaCl concentration tested (i.e. 50mM). Considerable inhibition of nodulation was also detected in the present study. A reduction in the nodule dry weight and number of nodules was obtained in plants inoculated with both wild-type salt-tolerant and salt-sensitive mesorhizobia strains under salinity. This notwithstanding, the native salt-tolerant mesorhizobia strain showed an increased ability to nodulate chickpea under salt stress compared to the salt-sensitive strain. Although the symbiotic performance of the two mesorhizobia strains used in this study was affected by salinity, the nodulation process was less affected when a salt-tolerant mesorhizobia was used. These results are in agreement with the observations in other reports (Elsheikh and Wood, 1995; Saxena and Rewari, 1992; Mhadhbi *et al*., 2004), suggesting that the salt-tolerance of the microsymbiont partner influence the symbiosis performance. However, the differences observed between plants inoculated with the salt-tolerant and the salt-sensitive

Mesorhizobium strains are insufficient to preferentially use salt-tolerant rhizobia as a major approach to overcome the negative effects of salinity in plants.

It is well established that high amounts of ethylene have inhibitory effects on plant growth and the reduction in endogenous ethylene levels in plant tissues, by bacterial ACC deaminase activity can promote plant growth. Ethylene is also known for its negative role inhibiting nodulation of legumes by rhizobia (Ma *et al.*, 2002; Ding and Oldroyd, 2009).

In the present study, the *acdS* gene from *Pseudomonas putida* UW4 was introduced into chickpea mesorhizobia, and their symbiotic performance was evaluated under control and salinity conditions. No ACC deaminase activity was detected in the native mesorhizobia strains in free-living conditions. Similarly, in other studies with mesorhizobia strains possessing the *acdS* gene, no ACC deaminase activity is detected in free-living conditions (Ma *et al*., 2003; Glick *et al*., 2007b).

Herein, the two transformed strains displayed different levels of ACC deaminase activities in spite of the fact the same exogenous gene was introduced in the same plasmid. This difference might reflect differences in promoter recognition between the two strains. Alternatively, since the *P. putida* UW4 *acdS* gene is regulated in a rather complex manner (Saleh and Glick, 2001; Cheng *et al*., 2008) by a number of different factors, it more likely is a reflection of metabolic differences between the two host mesorhizobia strains.

While the detected ACC deaminase activities of the two *acdS*-transformed mesorhizobia strains are low compared to the ACC deaminase activity of *Pseudomonas putida* UW4 (Ma *et al*., 2003), the ACC deaminase activities detected in this study are within the expected range of activities detected previously (Shah *et al*., 1998; Nascimento *et al*., 2012).

Regardless of the precise level of exogenous ACC deaminase activity displayed by *acdS*-transformed rhizobacteria strains, beneficial effects on plant growth

were obtained with *Mesorhizobium* strains, as described by Nascimento *et al*. (2012) with *Mesorhizobium ciceri* LMS-1 (pRKACC) in chickpea plants, under control conditions. Furthermore, transconjugants of *Rhizobium* sp. strains TAL1145 containing the native- and *Sinorhizobium* sp. strain BL3-*acdS* genes increased root mass and produced more nodules on *Leucaena leucocephala* than the native strain (Tittabutr *et al*., 2008).

Both *acdS*-transformed mesorhizobia strains improved the chickpea plant growth under salt conditions when compared to plants inoculated with native mesorhizobia strains under the same stressful conditions. However, the observed growth promotion was not equal with the two *acdS*-transformed mesorhizobia strains. With the salt-sensitive *M. ciceri* EE-7 (pRKACC), expressing the exogenous ACC deaminase gene, an increased ability to nodulate chickpea plants under salinity was observed. Moreover, the salt-sensitive transformed strain is able to form nodules in the same extent of the salt-tolerant *M. ciceri* G-55. However, the expression of an exogenous ACC deaminase in the salt-tolerant strain *M. ciceri* G-55 did not significantly influenced the nodulation abilities of this strain. It is possible that the different improvement obtained in the symbiotic performance of the strains upon transformation with the *acdS* gene is due to the different levels of ACC deaminase activity in the two strains, with a higher ACC deaminase activity contributing to a higher alleviation of the negative effects of ethylene. The reduction of the negative effects of salt stress was observed in all of the parameters analyzed. Similar results were previously obtained with other plants (canola, tomato, cucumber and red pepper) inoculated with rhizobacteria expressing ACC deaminase and subjected to salt stress (Cheng *et al*., 2007; Gamalero *et al*., 2010; Siddikee *et al*., 2010, 2011).

On the other hand, the *acdS*-transformed strains did not promote plant development under optimal conditions, suggesting that the beneficial effect on plant growth was related to plant stress and to the modulation of ethylene levels through ACC deaminase. Similar results were observed by Gamalero *et al*. (2008), with cucumber plants. In addition, the increased symbiotic performance of

acdS-transformed mesorhizobia strains under salinity is achieved by reducing the ethylene levels in plant tissues, which in turns, lead to an increased number of nodules.

This study shows that expression of an exogenous *acdS* gene in mesorhizobia improved the symbiotic performance of the bacteria when they were used as inoculants of chickpea plants grown under saline conditions, thus almost completely alleviating the negative effects caused by salinity. This suggests that the expression of an exogenous *acdS* by *Mesorhizobium* strains may be a useful tool in the development of inocula for sustainable agricultural purposes, particularly in salt-damaged lands.

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Chapter 5

Tolerance of chickpea mesorhizobia to acidity

Due to the agricultural practices and environmental changes, the amount of land affected by acidity is increasing, limiting crop productivity worldwide. The demand for rhizobia tolerant to low pH is imperative, in order to develop legume-rhizobia associations able to tolerate soils acidity. In contrast to the temperature and salt response, the acidity stress response has not been widely studied in rhizobia.

The present study comprised the evaluation of acid stress tolerance of chickpea rhizobia, in order to identify isolates tolerant to acidity and to investigate a possible relationship between stress tolerance and the species or the site origin of the isolates. Additionally, to study the molecular bases of acid stress response in rhizobia, we analysed the expression of chaperone genes *dnaKJ* and *groESL* upon stress, using acid tolerant and sensitive chickpea rhizobia isolates, belonging to different *Mesorhizobium* species.

This chapter is based on the following manuscripts:

Brígido, C., Alexandre, A., Laranjo, M., Oliveira, S. (2007) "Moderately acidophilic mesorhizobia isolated from chickpea" Letters in Applied Microbiology, 44:168-174.

Brígido, C., Oliveira, S. (2012) "Most acid-tolerant chickpea mesorhizobia show induction of major chaperone genes upon acid shock" Accepted for publication in *Microbial Ecology*, DOI 10.1007/s00248-012-0098-7

5.1 Summary

The ability of rhizobia to endure stress is important in order to achieve high symbiotic performance in suboptimal conditions. The rhizobia-legume symbiosis is affected by environmental conditions such as acidity. Our goals were to evaluate the tolerance of mesorhizobia to acid and alkaline conditions as well as to investigate whether acid tolerance is related to the species or the origin site of the isolates. In addition, to investigate the molecular basis of acid tolerance, the expression of chaperone genes *groEL* and *dnaKJ* was analyzed, using acid-tolerant and sensitive mesorhizobia.

Tolerance to pH 5 and 9 was evaluated in liquid medium for 98 Portuguese chickpea mesorhizobia belonging to four species clusters. All isolates showed high sensitivity to pH 9. In contrast, mesorhizobia revealed high diversity in terms of tolerance to acid stress: 35% of the isolates were acid-sensitive and 45% were highly tolerant to pH 5 or moderately acidophilic. An association between mesorhizobia tolerance to acid conditions and the origin soil pH was found. Furthermore, significant differences between species clusters regarding tolerance to acidity were obtained. Ten isolates were used to investigate the expression levels of the chaperone genes by northern hybridization. Interestingly, most acid-tolerant isolates, assigned to *M. ciceri* / *M. loti* and *M. huakuii / M. amorphae* clusters, displayed induction of the *dnaK* and *groESL* genes upon acid shock while the sensitive ones showed repression. This study suggests that acid tolerance in mesorhizobia is related to the pH of the origin soil and to the species cluster of the isolates. Additionally, the transcriptional analysis suggests a relationship between induction of major chaperone genes and higher tolerance to acid pH in mesorhizobia.

This is the first report on transcriptional analysis of the major chaperones genes in mesorhizobia under acidity, contributing to a better understanding of the molecular mechanisms of rhizobia acidity tolerance.

5.2 Introduction

Agricultural practices and environmental changes increase the amount of land affected by acidity, limiting crop productivity worldwide. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic nitrogen fixation. Soil acidity affects the nodulation and nitrogen fixation processes undertaken by rhizobia, since it reduces rhizobial persistence and survival in the soil as well as nodulation efficiency (Graham *et al*., 1982; Ibekwe *et al*., 1997).

In Portugal, most of the soils are acid mainly due to the agricultural practices and the mild and dry climate, which favors a fast mineralization of the organic matter (Torrent *et al.,* 2007). This results in an important constraint to most agricultural crops, such as chickpea production. This legume has a great economic importance due to its use worldwide as food for both humans and animals. Although chickpea (*Cicer arietinum* L.) is a successful legume on alkaline soils (Rao *et al*., 2002), its symbiotic relationship with mesorhizobia is better adapted to acidity (Siddique *et al.,* 1999; Howieson *et al*., 2000).

To avoid losses in the productivity of leguminous crops in acidic soil conditions, the development of legume-rhizobia associations able to tolerate such stress (Dilworth *et al*., 2001) or the selection of rhizobia tolerant to low pH (Ruiz-Díez *et al*., 2009) are possible strategies. Chen *et al*. (2005) described the pH range for mesorhizobia growth between 4 and 10, although, the optimal pH range was between 6 and 8. Some exceptions have been identified as is the case of strains of *Mesorhizobium loti* that showed a high tolerance to acidity (pH 4) (Correa and Barneix, 1997; Jarvis *et al*., 1997).

Stress response in bacteria is essential for effective adaptation to changes in the environment. Bacteria have the ability to sense protein folding and other signals, leading to the activation of proteins such as molecular chaperones, proteases and regulatory factors, which play an important role in promoting homeostasis under stress conditions, such as acidity (Jakob *et al*., 1993; Hartl, 1996;

Frydman, 2001). Molecular chaperones recognize non-native states of other proteins and assist their folding and/or prevent their aggregation. The DnaK-DnaJ-GrpE and GroEL-GroES complexes are the best characterized molecular chaperone systems, especially in *Escherichia coli* (Sabate *et al*., 2010).

Although several studies evaluated the tolerance to acid and alkaline pH of strains belonging to the *Mesorhizobium* genus (Laranjo and Oliveira, 2011), little is known about the factors that determine acid tolerance in rhizobia. Furthermore, the molecular mechanisms enabling tolerant strains to endure low pH, and thus the molecular basis of rhizobia tolerance to acidity, remain mostly unknown.

The present study evaluates the tolerance to acid and alkaline conditions of a Portuguese chickpea rhizobia collection and investigates whether acid tolerance is related to the species cluster, origin soil pH or geographical origin of the isolates. Additionally, it investigates changes in the expression levels of the major chaperone systems *groESL* and *dnaKJ* upon acidic shock, using tolerant and sensitive mesorhizobia isolates, belonging to several *Mesorhizobium* species.

5.3 Materials and Methods

Bacterial isolates

A total of 98 isolates from a chickpea rhizobia collection (Chapter 2), which covers almost all Portuguese territory including Madeira Island, were used in the present study (Table 5.1). All isolates were preserved in 30% (v/v) glycerol at -80ºC and cultured in yeast extract mannitol (YEM) broth (Vincent, 1970) at 28ºC for routine use.

Table 5.1 List of the isolates used in the present study (from chapter 2).

Province	Origin (soil pH)	Isolate	Province	Origin (soil pH)	Isolate
Trás-os-Montes e Alto Douro	Bragança	BR-8	**Estremadura**	Alenquer	AL-13
		BR-9		Caldas da Rainha	CR-3
		BR-15			CR-18
		BR-16			CR-32
		BR-28		Salir	SL-1
	Lamego	LM-1			SL-2
		LM-9			SL-3
		LM-13			SL-5
		LM-18			SL-6
		LM-21			SL-7
Douro Litoral	Porto	PII-1			SL-9
		PII-2		Setúbal	ST-2
		PII-3			ST-5
		PII-4			ST-8
Beira Litoral	Aveiro	A-3			ST-20
	Aveiro II	AII-5			ST-33
		AII-7		Sintra	S-1
	Coimbra	C-3			S-8
		C-7			S-15
		C-9			S-24
		C-13			S-26
		C-14	**Ribatejo**	Santarém	STR-2
		C-15			STR-4
		C-23			STR-10
		C-24			STR-14
		C-25			STR-16
		C-27b	**Alto Alentejo**	Elvas	75
	Leiria	L-19			78
Beira Baixa	Castelo Branco	CB-10			85
		CB-19		ENMP	EE-7
		CB-23		Évora	90
		CB-30			93
		CB-38			94
		CB-75			98
	Telhado	T-3			102
		T-4		Beja	6b
		T-5			7a
		T-7			27
		T-7			29
Algarve	Portimão	PM-1	**Beira Alta**	Guarda	G-1
		PM-14			G-4
		PM-17			G-10
	Portimão I	PMI-1			G-24
		PMI-6			G-55
	Praia do Alemão	PA-5		Viseu	V-15b
		PA-6			V-18
Madeira	Serra d'Água	SA-9			V-20
		SA-12			V-25b
		SA-13			
		SA-17			

pH stress tolerance

The pH stress tolerance of the bacterial isolates was screened by evaluating their growth based on optical density (OD) readings at 540 nm. The pH stresses and control conditions were performed according to Laranjo and Oliveira (2011). Briefly, the YEM medium was buffered with 25 mM homopiperazine-N, N'-bis-2-(ethanesulfonic acid) (Homopipes) for pH 5 and with 26 mM 2-amino-2-methyl-1,3-propanediol (AMPD) for pH 9. For control conditions, YEM was buffered with 20 mM 2-morpholinoethanesulfonic acid (MES) at pH 7. After overnight growth, bacterial cultures were standardized to an initial OD of 0.03 and grown for 48 h at 28 ºC. Three replicas per isolate under each condition were used.

Statistical analysis

In order to compare isolates tolerance, optical density values were converted into percentage values, considering growth at control conditions (pH 7) as 100%. Average value and standard deviations of the three replicas were calculated. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA). Both Krustal-Wallis and the Welch tests were used when there is no homogeneity of variances, in order to explore the relationship between stress tolerance (continuous dependent variable) and categorical independent variable, as for instance species group or province of origin. To identify categories that differ significantly from others, three different *post hoc tests* (Tamhane, Dunnett T3 and Games-Howell) were used. To detect structure in the relationships between categorical variables, the correspondence analysis (CA) was conducted as an exploratory data analysis technique (Benzécri, 1973). In order to investigate whether distinct acid tolerance phenotypes were related to the pH value of the origin soil of the isolates, these were divided into four classes according to their growth at pH 5: sensitive (growth < 30%), tolerant (growth between 30% and 70%), highly tolerant (growth between 70% and 100%) and moderately acidophilic (growth > 100%).

Nonparametric correlations between percentages of growth at acid pH and symbiotic effectiveness as well as the soil pH value from the origin site of isolates were determined using Spearman's rank order correlation coefficient.

RNA extraction and northern hybridization

The transcription of *groEL* and *dnaKJ* genes was analyzed in ten isolates showing different phenotypes upon acid conditions, in order to investigate the involvement of these genes in tolerance to acidity. The transcriptional levels of the major chaperone genes were evaluated by northern hybridization after an acid shock and compared to those of control conditions. Total RNA extraction was performed using cell cultures in exponential growth phase, submitted to an acidic shock in YEM (pH 3) for one hour. Control RNA was extracted from cells grown in YEM (pH 7). Total RNA extraction was performed according to the protocol for Rapid Isolation of RNA from Gram-negative Bacteria (Ausubel *et al.*, 1997).

All procedures for northern hybridization and stringency washes were followed as described previously in Chapter 3. The *groEL* and *dnaKJ* RNA probes were obtained as previously described (Alexandre and Oliveira 2011). The DNA probe for 16S rRNA was labeled using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science). The 16S rRNA gene PCR amplification was performed using DNA of *M. mediterraneum* Ca36^T, as previously described (Alexandre and Oliveira, 2011).

Hybridization signals were analyzed using ImageQuantTL™ v7.01 (GE Healthcare). The 16S rRNA signal was used as internal control for the amount of total RNA loaded.To determine the expression levels, the ratio between the transcript signals and the corresponding 16S rRNA signals was calculated and the number of folds was determined using the ratio of the previous value between control and the pH shock conditions minus 1.

5.4 Results

Evaluation of mesorhizobia tolerance to acidic and alkaline stresses

Isolates from the chickpea mesorhizobia collection were tested for tolerance to pH 5 and 9 (Fig. 5.1). The screening revealed that chickpea mesorhizobia varies in terms of tolerance to acid conditions (pH 5). In contrast, all chickpea mesorhizobia were sensitive to alkaline conditions (pH 9). Interestingly, isolates from *M. mediterraneum* / *M. temperatum* and *M. tianshanense* clusters are highly sensitive to both tested pH stress conditions, whereas isolates belonging to the other two species clusters (*M. ciceri* / *M. loti* and *M. huakuii* / *M. amorphae*) showed high diversity in tolerance to pH 5. In total, only 35% of the isolates tested were sensitive to acidic conditions. On the other hand, 45% of the isolates were highly tolerant or prefer acidic pH. Moreover, isolates C-25-Coimbra, T-5-Telhado and Al-13-Alenquer belonging to *M. huakuii* / *M. amorphae* cluster as well as isolates 27-Beja and S-8-Sintra from the *M. ciceri* / *M. loti* cluster can be considered as moderately acidophilic (growth above 100% in pH 5).

Statistical analysis indicated that there are significant differences between species clusters regarding their tolerance to acidic conditions ($\chi^2 = 125.822$; $df = 3$; $P < 0.01$). For instance, *M. ciceri* / *M. loti* isolates showed the highest growth average at pH 5 while the *M. tianshanense* isolates showed the lowest growth average, and are significantly different from each other and from the other two species clusters. Actually, these results are reinforced by the *post hoc tests,* which indicate that the growth averages at pH 5 are significantly different among species clusters.

Similarly, the statistical analysis showed that provinces of origin are significantly different in terms of the isolates tolerance to acid pH ($\chi^2 = 102.260$; $df = 10$; $P < 0.01$). The three provinces with the highest growth averages at pH 5 (Beira Alta, Trás-os-Montes e Alto Douro, Beira Litoral) were found to be significantly different from the provinces with the lowest growth average (Ribatejo, Algarve, Estremadura).

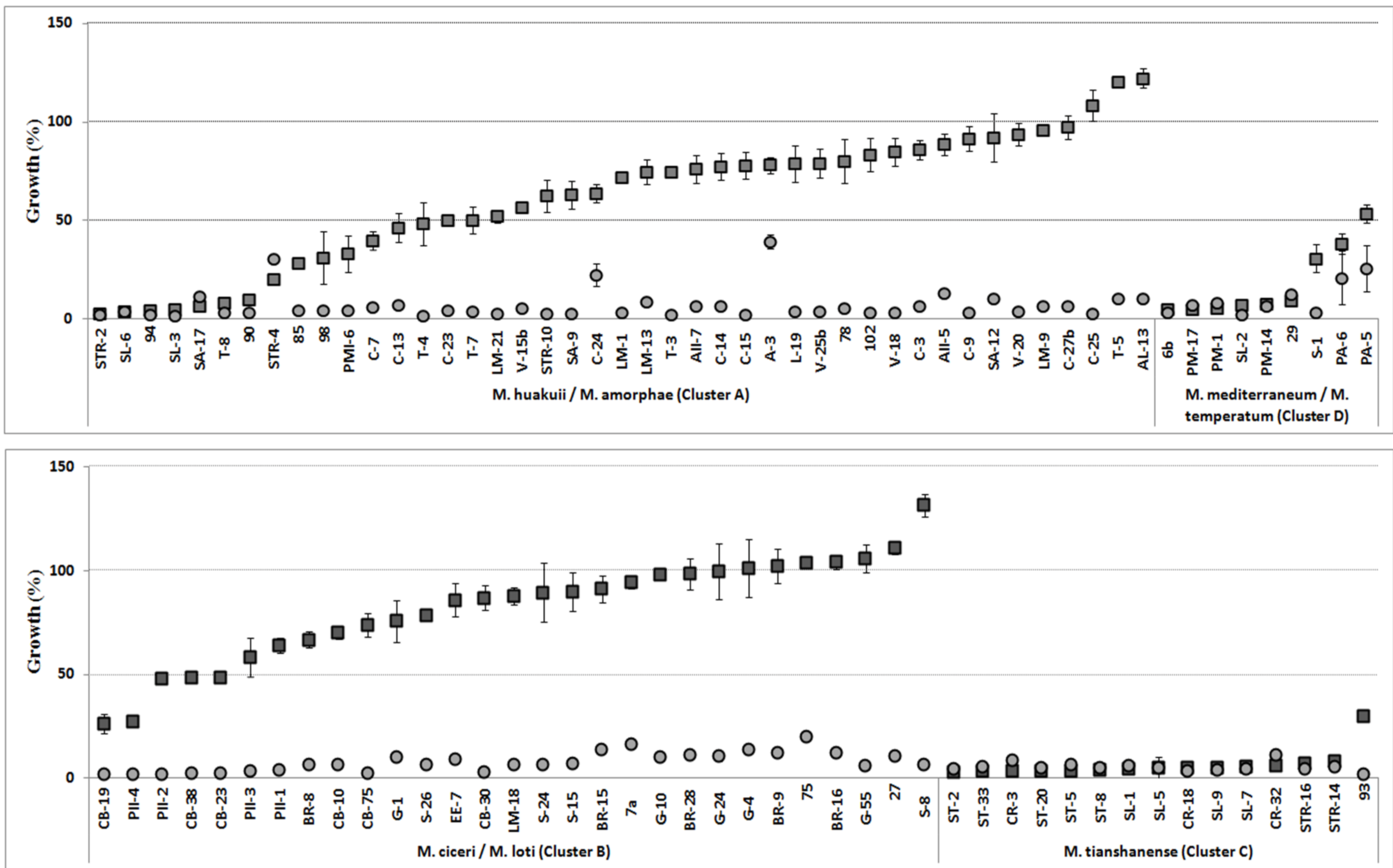

Figure 5.1 Growth of chickpea mesorhizobia under acid and alkaline stress conditions: pH 5 (squares) and pH 9 (circles). Percentages were calculated considering the control condition (pH 7) as 100% growth. Presented values are the average of three replicas (standard deviation bars are shown).

In order to investigate whether the pH value of the sampling site is one of the explanations for the significant differences found between provinces of origin, the sampling soils were classified into three classes based on their pH’s values. Sampling soils with pH’s values below 6.5 were considered acid soils, whereas the neutral soils include soils with pH’s values ranging from 6.5 to 7.4 and the alkaline soils represented the soils with pH’s values above 7.4.

The correspondence analysis biplot (Fig. 5.2) shows an association between the pH class of the origin soil and the isolate’s ability to tolerate pH 5. Additionally, a negative correlation was found between the isolates growth at acid pH and the pH value of the sampling soil (r = -0.358; P < 0.01). For instance, isolates from alkaline soils were more sensitive to pH 5 than the isolates from neutral or acidic soils. Sensitivity to acid conditions is clearly associated to alkaline soils whereas the acid tolerance is associated to neutral or acidic soils.

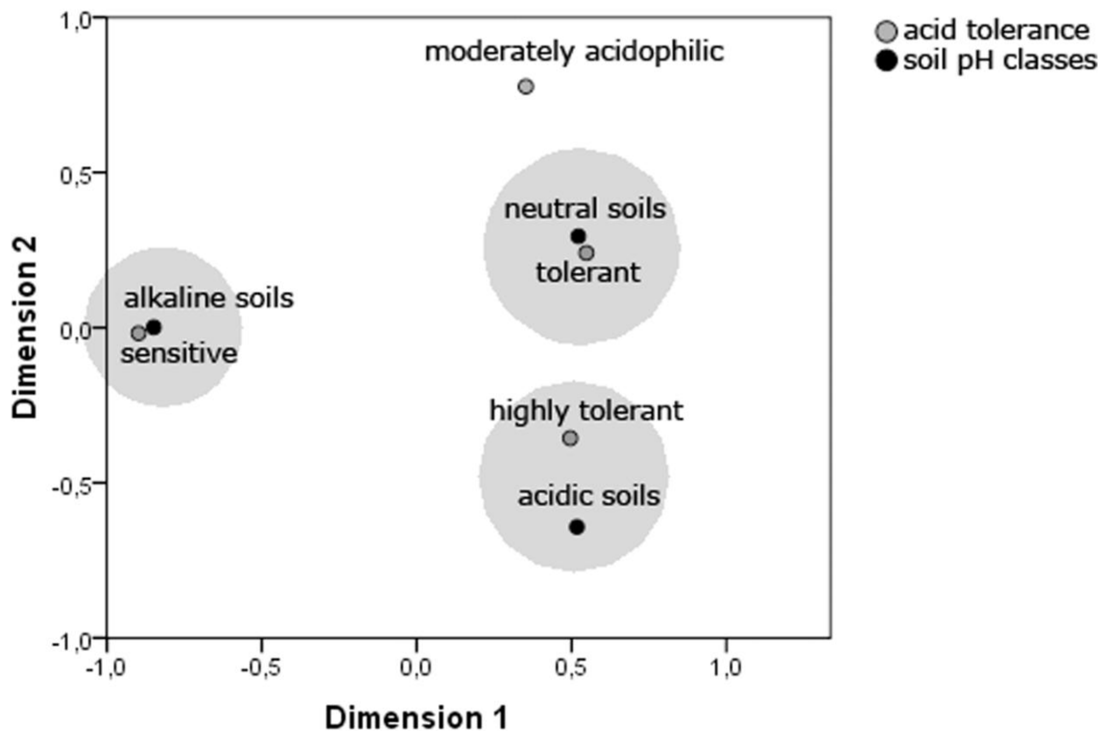

Figure 5.2 CA biplot of the relationship between origin soil pH and tolerance to acid pH of the isolates.

Our results indicate an association (χ^2 = 156.863; *df* = 6; *P* < 0.01) between the origin soil pH of the isolates and species clusters. Moreover, a CA biplot also detects an association between species clusters and soil pH classes, where the *M. ciceri* / *M. loti* and *M. huakuii* / *M. amorphae* species clusters are associated to neutral soils and acidic soils, respectively, and the remaining species clusters are associated to the alkaline soils (Fig. 5.3).

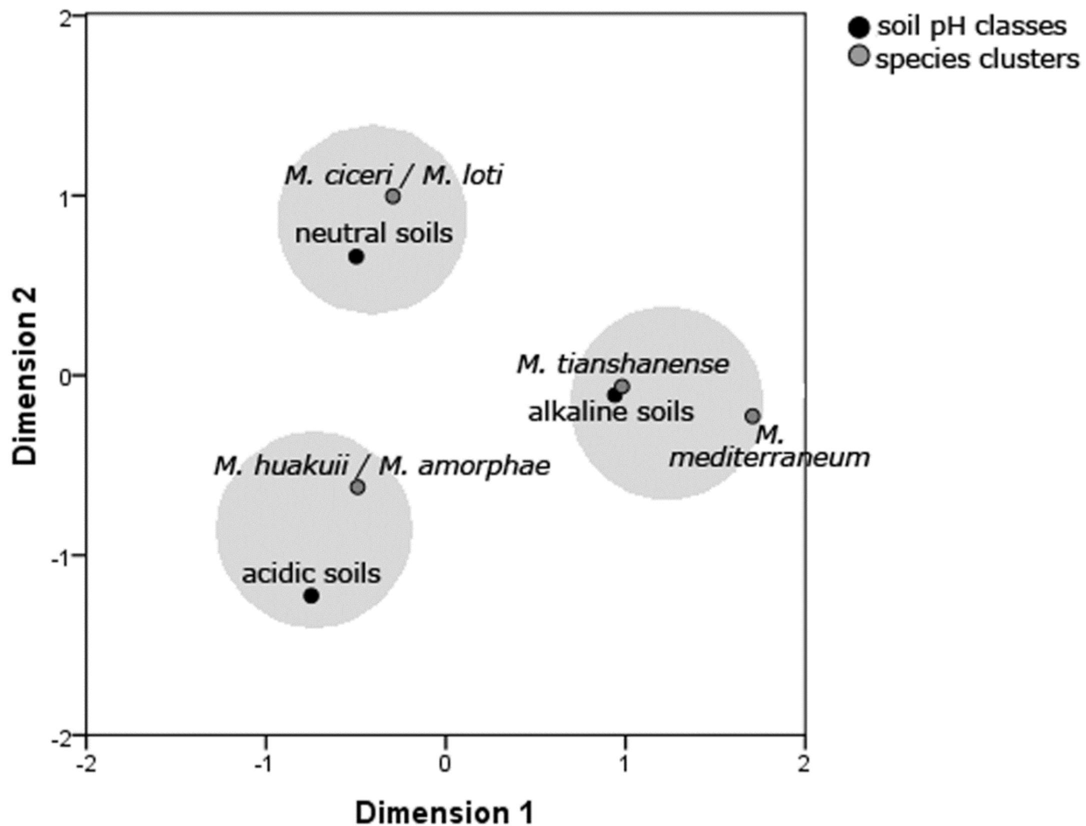

Figure 5.3 CA biplot of the relationship between origin soil pH and the isolates species clusters.

Curiously, a positive correlation between tolerance to acidic conditions and symbiotic effectiveness was found (*r* = 0.139; *P* < 0.05). For example, the isolate G-55-Guarda showed a growth of 106% at pH 5 and it is also highly efficient with a symbiotic effectiveness of 88%.

Transcriptional analysis of the major chaperones genes upon acidic shock

In order to investigate the involvement of the major chaperone genes in tolerance to acidic conditions, the transcription of *groEL* and *dnaKJ* genes was analyzed. Using northern hybridization, the transcriptional levels of these chaperone genes upon an acidic shock were compared to those under control conditions. Based on the pH stress tolerance screening (Fig. 5.1), ten chickpea mesorhizobia isolates, from the four species clusters, with different phenotypes under acidic conditions, were selected. From the *M. huakuii* / *M. amorphae* species cluster the following isolates were chosen: isolate PMI-6-Portimão as acid-sensitive, V-15b-Viseu and C-9-Coimbra as acid-tolerant and AL-13-Alenquer as moderately acidophilic. From the *M. ciceri* / *M. loti* cluster, the isolate PII-4-Porto as acid-sensitive, G-55-Guarda and G-10-Guarda as acid-tolerant and S-8-Sintra as moderate acidophilic were selected. The clusters *M. tianshanense* and *M. mediterraneum* / *M. temperatum* only include isolates sensitive to acidity, so only one isolate from each of these two clusters was chosen, namely ST-33-Setúbal from the *M. tianshanense* and PM-14-Portimão from *M. mediterraneum* / *M. temperatum*.

The northern blot analysis using the *dnaKJ* mRNA probe allows the detection of three different transcripts, with 1917, 1131 and 3048 nucleotides in size, corresponding to the predicted mRNAs of *dnaK*, *dnaJ* and also the bicistronic *dnaKJ* respectively, according to the *Mesorhizobium sp.* MAFF303099 genome. A transcript with approximately 2 kb, which is consistent with the size of the *dnaK* gene transcript, was detected in all isolates under control conditions and upon acidic shock (Fig. 5.4a). The majority of the isolates showed increase in the *dnaK* transcript levels (Fig. 5.5) after acidic shock.

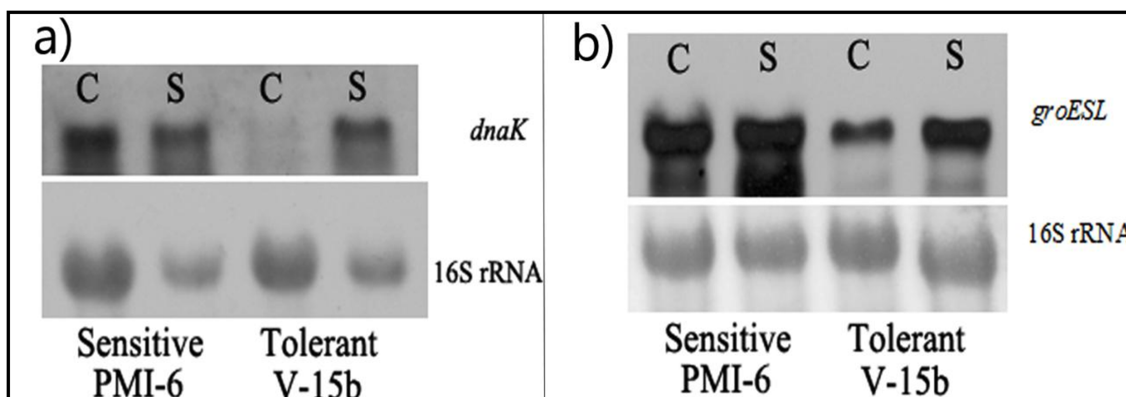

Figure 5.4 Comparison of transcriptional analysis of the *dnaK* gene (a) and the *groESL* operon (b) between acid-tolerant and acid-sensitive isolates submitted to acidic shock. Northern blot hybridization of total RNA with probes specific for *dnaKJ*, *groEL* and 16S rRNA under control conditions (C) and upon acidic shock (S).

The tolerant isolates belonging to *M. ciceri* / *M. loti* cluster showed an increase in the *dnaK* mRNA level, after acidic shock when compared with the control. However, the sensitive isolate PII-4-Porto showed a decrease in the expression of the *dnaK* gene after acidic shock. Similarly, in isolates from the *M. huakuii* / *M. amorphae* cluster, the level of *dnaK* gene transcription was positively related to the acid tolerance of the isolates, since a slight repression of the *dnaK* gene was observed in the sensitive isolate (PMI-6-Portimão) while induction was observed in the acid-tolerant ones (V-15b-Viseu, C-9-Coimbra and AL-13-Alenquer) (Fig. 5.5).

No significant changes in the transcriptional levels of *dnaK* gene were observed in the sensitive isolates neither from the *M. tianshanense* nor *M. mediterraneum* / *M. temperatum* clusters upon acidic shock.

Regarding the analysis of the *groESL* chaperone system, the *groEL* RNA probe allows the detection of two putative transcripts with 1947 and 1632 nucleotides, corresponding to the bicistronic *groESL* transcript and to the *groEL* transcript respectively, according to the *Mesorhizobium sp.* MAFF303099 genome. In this study, the signal detected was approximately 2 kb, which corresponds to the

bicistronic *groESL* mRNA (Fig. 5.4b). Most isolates showed an increase of the *groESL* mRNA transcript levels after the acidic shock. Interestingly, isolates belonging to the *M. ciceri* / *M. loti* cluster presented the same pattern in the *groESL* chaperone gene transcription as obtained for the *dnaK* chaperone gene (Fig. 5.5): tolerant isolates (G-10-Guarda, G-55-Guarda and S-8-Sintra) showed an increase of the *groESL* mRNA levels while the sensitive isolate (PII-4-Porto) revealed a repression of this chaperone gene, after acidic shock.

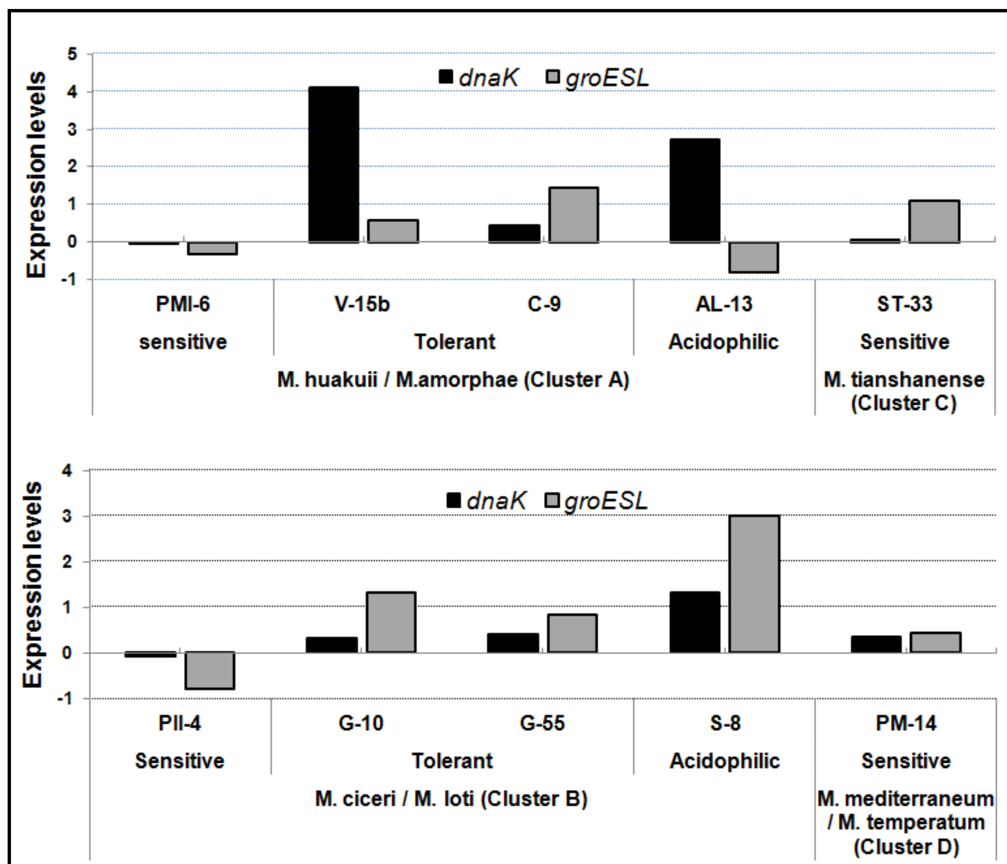

Figure 5.5 Expression levels of *dnaK* and *groESL* genes after salt shock evaluated by northern analysis using tolerant and sensitive isolates from the four species clusters.

Concerning the cluster *M. huakuii* / *M. amorphae,* the tolerant isolates (V-15b-Viseu and C-9-Coimbra) showed an increase in the *groESL* mRNA levels after acidic shock, whereas the sensitive isolate (PMI-6-Portimão) showed repression of the *groESL* gene. Although the moderately acidophilic isolate (AL-13-

Alenquer) showed a repression of the *groESL* gene, it shows one of the highest levels of *dnaK* gene induction, upon acidic shock.

The acid-sensitive isolates belonging to the *M. tianshanense* and *M. mediterraneum* / *M. temperatum* showed a slight induction of the *groESL* genes compared to the control.

For both *dnaK* and *groESL* transcription levels, the isolates from the *M. ciceri* / *M. loti* and *M. huakuii* / *M. amorphae* seem to exhibit a similar relationship between transcription levels and tolerance to acid pH, with the exception of the moderately acidophilic isolate AL-13-Alenquer for the *groESL* analysis.

5.5 Discussion

In this study, evaluation of growth in acid and alkaline conditions was performed for 98 isolates from a collection of native Portuguese chickpea mesorhizobia previously characterized (Chapter 2). All mesorhizobia showed sensitivity to alkaline stress, which is in agreement with previous studies (Laranjo and Oliveira, 2011). In contrast, the mesorhizobia isolates tested herein showed high diversity regarding tolerance to acid pH. Interestingly, almost half of the tested isolates were highly tolerant (growth > 70%), including 11 isolates with a higher growth at pH 5 than at pH 7, indicative of moderately acidophilic isolates. High tolerance to low pH has been previously reported in *Mesorhizobium* species, namely *M. huakuii* (Chen *et al*., 1991), *M. ciceri* (Nour *et al*., 1994), *M. loti* (Jarvis *et al*., 1982) and *M. amorphae* (Wang *et al*., 1999), which are all able to grow at pH 5. On the other hand, *M. mediterraneum* and *M. tianshanense* cannot grow at pH 5 (Chen *et al*., 1995; Nour *et al*., 1995). More recently, a high diversity in tolerance to acidic conditions within the *Mesorhizobium* genus was reported by Laranjo and Oliveira (2011).

Amarger *et al.* (1997) reported that tolerance to salinity, acidity and alkalinity is more strain-specific than species-specific. However, other studies suggest that

the tolerance to pH stress in rhizobia is species-related (Reeve *et al*., 2006). Herein, the evaluation of tolerance to acidity of a large set of chickpea mesorhizobia suggests that acid tolerance phenotype is related to the species clusters. Significant differences between species clusters regarding tolerance to acidity were obtained. For instance, the majority of isolates from *M. ciceri / M. loti* cluster are acid-tolerant whereas isolates belonging to *M. tianshanense* cluster are acid-sensitive. Moreover, several studies in rhizobia have reported that stress tolerance seems to be species related namely temperature stress tolerance (Alexandre and Oliveira, 2011) and tolerance to copper (Laguerre *et al*., 2006) as well as antibiotic resistance (Alexandre *et al.,* 2006).

An association between species clusters and origin soil pH of the isolates was already found in Chinese soybean rhizobia (Li *et al*., 2011). Our results suggest that soil pH contributes to determine the species that prevail in the rhizobia population. This type of association was found in other studies addressing soil bacterial communities, indicating soil pH as the variable that better explains the population diversity and mainly the community composition (Fierer and Jackson, 2006). On the other hand, in this study, the tolerance to acid pH of the chickpea mesorhizobia isolates was found to be associated to the origin soil pH, agreeing with some previous reports (Kulkarni and Nautiyal, 1999; Rodrigues *et al*., 2006). These results suggest that isolates collected from acidic or neutral soils may be more resistant to acidic environmental conditions than the ones from alkaline soils.

Interestingly, our results also reveal a positive correlation between the tolerance to acid pH and the symbiotic effectiveness of isolates, suggesting that the establishment of an effective symbiosis is related to tolerance to acidity. Bacterial persistence in soil is significantly dependent on soil pH, which may affect the symbiotic performance by reduction of the nodulation efficiency (Graham *et al*., 1982; Ibekwe *et al*., 1997; Brockwell *et al*., 1991). In addition, it is known that the pH in the rhizosphere of the leguminous host plant is lower due to the protons and organic acids excreted by the plants (Marschner, 2006), suggesting that the

rhizobial partner has to deal with this stressful condition to achieve effective symbiosis. Previous studies in our lab (*data not shown*) indicated that the maximum symbiotic performance achieved by a specific strain is related to its optimal growth pH. This suggests that reaching a higher symbiotic performance of rhizobia inoculants in chickpea crops should require the selection of a strain whose preferred pH is similar to the pH of the soil to be cultivated. Similar results were found in peanut rhizobia by Angelini *et al.* (2005).

Despite the fact that several studies have attempted to characterize the genes involved in tolerance to acidity, the molecular mechanisms to respond to acidity are still unknown in rhizobia. Molecular chaperones form a multi-protein network that prevent protein denaturation, and also help in the proper protein folding and refolding, transport, degradation and regulation. The functioning of this network is particularly important under stress conditions, such as acidic shock (Hartl and Hayer-Hartl, 2002). Our results from the transcriptional analysis of eight mesorhizobia isolates show a relationship between higher levels of transcriptional induction of both *dnaK* and *groESL* genes upon acidic shock and a higher ability of mesorhizobia to tolerate acid pH. Upon acid shock, both *dnaK* and *groESL* genes were induced only in acid-tolerant isolates and not in sensitive isolates, with the exception of the moderately acidophilic isolate (AL-13-Alenquer), which showed a repression of the *groESL* gene but one of the highest levels of *dnaK* gene induction. These results suggest that increased expression of chaperone genes may contribute to a higher tolerance to acid stress in rhizobia. However, these results only refer to the isolates belonging to *M. ciceri* / *M. loti* and *M. huakuii* / *M. amorphae* species clusters, since no comparison between tolerant and sensitive isolates was available for the remaining group species. Foster (1991) suggested a model to explain the acid response in *Salmonella* cells involving DnaK and GroEL, due to their ability to refold acid denatured proteins. Studies in *Streptococcus mutants* revealed that *dnaK* and *groEL* are part of the general stress response being both induced during the acid shock response (Lemos *et al.*, 2001, 2007; Matsui and Cvitkovitch, 2010). Other studies in *Escherichia coli* showed that the expression of the chaperones DnaK, DnaJ and

GrpE was inducible under acid shock (Zmijewski *et al*., 2004). However, contrary to these results, *dnaK* and *dnaJ* chaperone genes were found to be down-regulated upon acidic stress in *Streptococcus suis* S2 (Wei *et al*., 2011).

In rhizobia little is known about acid response. Recently, transcriptional analysis using *Ensifer meliloti* 1021 cells following an acidic upshift showed increased *groEL5* transcript levels (Hellweg *et al*., 2009; de Lucena *et al*., 2010). Hellweg *et al.* (2009) verified that the *groEL5* gene was not immediately up-regulated after the pH shift, but slowly increased its expression level during the time course (1 hour, pH 5.75).

Our previous studies in chickpea mesorhizobia suggest the existence of a relationship between higher levels of transcriptional induction of the *dnaK* and *groESL* chaperones genes and a higher ability of isolates to endure heat stress (Alexandre and Oliveira, 2011). In contrast, no correlation between salt tolerance and expression levels of these chaperones genes in mesorhizobia was found (Chapter 3). Altogether, it seems that *dnaK* and *groESL* genes may be involved in acid and heat tolerance in chickpea mesorhizobia.

Here we evaluated the diversity of tolerance to acid and alkaline stress conditions of a collection of chickpea mesorhizobia isolates belonging to four species clusters and investigated possible relationships between acid tolerance phenotype of isolates and their species cluster, geographical origin, origin soil pH and symbiotic effectiveness. Our findings suggest that tolerance to acid stress is related to the species clusters and to the origin soil pH. A relationship between induction of *dnaK* and *groESL* genes upon acidic shock and tolerance to acidic conditions was found, suggesting that induction of these chaperone genes is involved in the chickpea mesorhizobia tolerance to acid pH. However, further studies are required to clarify the role of these chaperone genes in acid stress tolerance of rhizobia.

5.6 References

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Chapter 6

Evaluation of the role of chaperone ClpB in chickpea mesorhizobia stress tolerance and symbiosis

Molecular chaperones play an important role in cells homeostasis, especially under stress conditions, due to their ability to prevent protein aggregation, assist refolding and mediate degradation of misfolded proteins. Chaperone systems are referred as heat shock proteins, since they are activated in response to high temperatures. Nevertheless, they are also involved in the response to other environmental stress conditions, such as acidity and salinity.

Chaperone systems from rhizobia are less studied in comparison to those in *E. coli.* In order to better understand the molecular basis of stress response that affects the legume-rhizobium interaction, the present work focused the role of the chaperone ClpB of mesorhizobia in the tolerance to several environmental stresses and in the symbiosis with chickpea plants.

This chapter is based on the following manuscript:

Brígido, C., Robledo, M., Menéndez, E., Mateos, P.F., Oliveira, S. "A ClpB chaperone knockout mutant of *Mesorhizobium ciceri* shows a delay in the root nodulation of chickpea plants" Accepted for publication in *Molecular Plant-Microbe Interactions*

6.1 Summary

Several molecular chaperones are known to be involved in bacteria stress response. To investigate the role of chaperone ClpB in rhizobia stress tolerance as well as in the rhizobia-plant symbiosis process, the *clpB* gene from a chickpea microsymbiont, strain *Mesorhizobium ciceri* LMS-1, was identified and a knockout mutant was obtained. The ClpB knockout mutant was tested to several abiotic stresses showing that it was unable to grow after a heat shock and it was more sensitive to acid shock than the wild-type strain. A plant growth assay performed to evaluate the symbiotic performance of the *clpB* mutant showed a higher proportion of ineffective root nodules obtained with the mutant than with the wild-type strain. Nodulation kinetics analysis showed a 6-8 day delay in nodule appearance in plants inoculated with Δ*clpB* mutant. The mutant strain showed lower levels of *nodC* expression, particularly in stress conditions. Analysis of histological sections of nodules formed by the *clpB* mutant showed that most of the nodules presented low amount of bacteroids. No differences in the root infection abilities of GFP-tagged *clpB* mutant and wild-type strains were detected. To our knowledge, this is the first study that evidences the involvement of the chaperone ClpB from rhizobia in the symbiotic nodulation process.

6.2 Introduction

Severe stresses result in extensive protein denaturation and aggregation. However, bacteria have developed molecular mechanisms, including chaperones and potentially destructive proteases, to deal with the stress effects ensuring cell survival. Molecular chaperones are protective systems within the cell to prevent protein aggregation, assist refolding and mediate degradation of misfolded proteins (Hart, 1996). Molecular chaperones like ClpB, DnaK, GroEL, and small heat shock proteins, such as IbpA and IbpB, are known to participate in bacterial stress response and tolerance to various stress conditions (Hartl *et al.*, 2011).

The *Escherichia coli* ClpB and its homologue in yeast, Hsp104, are essential for cell survival during extreme heat stress (Sanchez and Lindquist, 1990; Squires *et al.*, 1991), due to its ability to solubilize and reactivate protein aggregates that were previously viewed as dead-ended products in the life of proteins (Parsell *et al.*, 1994; Weibezahn *et al.*, 2005). Moreover, ClpB seems to be involved in virulence in *Listeria monocytogenes* (Chastanet *et al.*, 2004) and in other stresses, such as ethanol and acid stresses in *Brucella suis* (Ekaza *et al.*, 2001) and osmotic stress in *Bifidobacterium breve* UCC (Ventura *et al.*, 2005).

Hsp104/ClpB belongs to the Hsp100/Clp superfamily of AAA+ (ATPase associated with various cellular activities) (Neuwald *et al.*, 1999; Dougan *et al.*, 2002), which form large hexameric ring structures in an ATP-dependent manner (Zolkiewski *et al.*, 1999; Wang *et al.*, 2001; Lee *et al.*, 2003). Hsp100/Clp superfamily contains central components of the protein quality control system that degrade or disaggregate unfolded and aggregated proteins (Maurizi and Xia, 2004; Sauer *et al.*, 2004; Mogk *et al.*, 2008).

The bacterial ClpB monomer contains an N-terminal domain and two AAA+ nucleotide-binding domains (NBDs) separated by a coiled-coil middle-domain (M-domain) (Barnett *et al.*, 2000; Doyle and Wickner, 2009; DeSantis and Shorter, 2012). Protein disaggregation by ClpB *in vitro* requires the collaboration of a second ATP-dependent molecular chaperone, DnaK, to promote the

solubilisation and reactivation of proteins that misfold and aggregate following heat shock (Motohashi *et al.*, 1999; Goloubinoff *et al.*, 1999; Zolkiewski 1999). This cooperation is essential for the ability of cells to survive transient extreme stress conditions (Squires *et al.*, 1991).

Biological nitrogen fixation is of extreme importance in both agronomic and environmental terms, as it is an alternative to the use of chemical nitrogen fertilizers. Rhizobia are soil bacteria able to establish symbiotic relationships with legumes and can convert nitrogen into ammonia within the plants root nodules. In general, two main developmental processes are required for the formation of symbiotic N_2-fixing nodules: bacterial infection and nodule organogenesis (Gage, 2004; Oldroyd and Downie, 2008). These processes must be coordinated in both spatial and temporal manner to ensure nodule formation at the site of bacterial infection (for review see Oldroyd and Downie, 2008).

Early events in the symbiosis process such as molecular signalling, rhizobial attachment, root hair curling, infection thread formation, and nodule initiation, are particularly sensitive to high temperatures, salinity, acidity and other environmental stresses (Zhang and Smith, 1996; Hungria and Stacey, 1997; Hungria and Vargas, 2000). Furthermore, during the infection process rhizobia also have to deal with adverse conditions within the host cells and with the plant innate immunity that induce physiological stress responses and may interfere with the symbiosis (Soto *et al.*, 2009). These stresses may negatively affect the microsymbiont in free-living conditions as well as during the symbiotic relationship, thus leading to an ineffective nitrogen fixation (Zahran 1999).

Transcriptomic and proteomic analyses of rhizobia in symbiosis with host plants suggest the involvement of chaperones in this process (Pessi *et al.*, 2007; Djordjevic *et al.*, 2003; Karunakaran *et al.*, 2009; Tsukada *et al.*, 2009). These approaches provide a global view on putative genes involved in symbiosis but further strategies, such as gene knockout, are required to confirm the involvement of a specific gene. In order to investigate the potential role of the chaperone ClpB in the stress tolerance of mesorhizobia in free-living and in

symbiosis with chickpea plants, in this work the *clpB* gene of the strain *Mesorhizobium ciceri* LMS-1 was deleted.

6.3 Materials and Methods

Plasmids, bacterial strains and growth conditions

Bacterial strains and plasmids used are described in Table 6.1. *Mesorhizobium ciceri* strain LMS-1 and its derivatives LMS-1Δ*clpB* and complemented were grown in tryptone yeast (TY) or yeast extract mannitol (YEM) broth at 28ºC for routine use and preserved in 30% (v/v) glycerol at -80ºC. The *E. coli* strains were grown at 37ºC in Luria-Bertani (LB) broth supplemented with appropriate antibiotics. The final concentrations of antibiotics were: kanamycin at 50 µg ml^{-1}, ampicillin at 100 µg ml^{-1} and chloramphenicol at 25 µg ml^{-1}.

Table 6.1 Plasmids and strains used in the present study.

Strains and Plasmids	Description	Source or reference
Mesorhizobium ciceri		
LMS-1	Wild-type Nod^+ FiX^+	Nascimento *et al.*, 2012
LMS-1ΔclpB	Derivative from LMS-1 *clpB* minus	This Study
LMS-1*clpB*+	Derivative of LMS-1 clpB reintroduced	This Study
Escherichia coli		
DH5α	Host for cloning and triparental conjugation	Sambrook and Russel 2001
JM109	Host for cloning	Promega Corp.
MT616	Host containing the helper plasmid used for triparental conjugation	Finan *et al.*, 1986
Plasmids		
pGEM-T easy	Amp^r, M13*ori* pBR322*ori*, linear T overhangs vector	Promega Corp
pRK2013	Helper plasmid for mobilization with replicon ColE1; Km^r *tra*	Ditta *et al.*, 1980
pK18*mobsacB*	plasmid suicide; Km^r; *sacB*^{s}	Schafer *et al.*, 1994
pMRGFP	pBBR1MCS-2 derivative containing the *gfp* gene constitutively expressed; Km^r	Garcia-Fraile *et al.*, 2012
pRK600	Helper plasmid; pRK2013 npt::Tn9, Cm^r	Finan *et al.* 1986

Km^r, Amp^r and Cm^r indicate resistance to kanamycine, ampicilin and chloramphenicol, respectively.

DNA methods and construction of LMS-1 derivative strains

Total DNA from rhizobial strains was extracted using the E.Z.N.A. bacterial DNA kit (Omega bio-tek, USA) according to the manufacturer's suggested protocol. Plasmid DNA was obtained by using DNA-Spin^{TM} Plasmid DNA Purification Kit (Intron) following the manufacturer's instructions. Unless specified otherwise, molecular techniques were performed using standards protocols (Sambrook and Russel, 2001).

The *M. ciceri* LMS-1 *clpB* gene (2607 nucleotides) was isolated by PCR amplification. First, an internal fragment of 1388 bp was obtained using heterologous primers (Chapter 3) based on the *clpB* sequence from *Mesorhizobium* sp. MAFF303099. The stop region sequence was obtained by inverse PCR. The complete gene sequence was obtained by PCR amplification using a forward primer based on the gene sequence *Mesorhizobium* sp. MAFF303099. To generate the Δ*clpB* deletion mutant strain, a *clpB* gene fragment of 2558bp was amplified using the primers clpB-F1 (5'-TGAGAAGTACTCCGAGCGCGT-3') and clpB-R1 (5'-GGAAGTTCAGCCGGTCGGAA-3'). The PCR product was purified using the Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The *clpB* gene fragment was cloned using pGEM-T Easy Vector System (Promega) following the manufacturer's instructions. An internal 675 bp Eco47III-BamHI fragment from the cloned *clpB* gene fragment was removed. The truncated *clpB* gene was cloned as an EcoRI fragment into plasmid pK18mobsacB and transferred to the *Mesorhizobium ciceri* LMS-1 by triparental mating using pRK2013 as a helper. Double recombinants were selected as previously described (Schafer *et al*., 1994).

To complement the disruption of the *clpB* gene, replacement of the disrupted *clpB* gene by the complete *clpB* gene in its original genomic location was performed. The complete *clpB* gene (2607 bp) was amplified using the primers clpB-XbaI-F (5'-CTAGAGATGAACCTTGAGAAGTAC-3') and clpB-XbaI-R (5'-CTAGACAGGATCAGGCTGCCGCTT-3') and cloned using pGEM-T Easy Vector

System (Promega) and introduced as an XbaI fragment into plasmid pK18mobsacB. Complemented strain was generated by replacement of the *clpB* gene disrupted for the complete one by following the same approach to obtain the Δ*clpB* mutant strain as mentioned above.

To confirm the successful allele replacement in *M. ciceri* LMS-1 derivatives, PCR analysis and Southern hybridization were conducted. PCR analysis was performed as previously described (Chapter 3). Briefly, for Southern hybridization, about 2 µg of total DNA from *Mesorhizobium ciceri* LMS-1 and its derivatives were digested with the restriction enzyme HindIII. A 1388bp fragment of the *clpB* gene of *M. ciceri* LMS-1 was amplified as described before (Chapter 3) and used as a DNA probe, which was labeled using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Germany) according to the manufacture's protocol. The hybridization process was carried using Dig Easy Hyb hybridization buffer (Roche Applied Science, Germany) at 42ºC, followed by stringency washes at room temperature and 68ºC. The immunological detection was performed according to the manufacturer's instructions.

Mesorhizobium ciceri LMS-1 and Δ*clpB* deletion mutant strains were transformed with the *gfp* gene. For that, the plasmid pMRGFP (Garcia-Fraile *et al*., *submitted*), containing the *gfp* gene constitutively expressed, was used to transform both mesorhizobia strains, by triparental conjugation as previously described (Chapter 4).

Analysis of stress tolerance

To evaluate the stress tolerance of the *Mesorhizobium ciceri* LMS-1 Δ*clpB* strain and compared it to the wild-type strain, bacterial growth was evaluated by measuring optical density at 540 nm, every 6h during 54h of growth in liquid medium. *Mesorhizobium ciceri* LMS-1 and its derivatives were grown in YEM medium at 28ºC for overnight until late log phase (16-18h). After inocula growth,

the optical density was adjusted at 0.03 and submitted to different stress conditions. Three replicas per treatment were done.

For continuous cold and heat stress, mesorhizobia cells were grown at 15 ºC or 37ºC, respectively for 54h. For heat shock, cells were incubated at 45ºC for 1h, 48ºC for 15 min or 50ºC for 5 min, and then grown at 28ºC for 54h. For cold shock, cells were subjected to 4ºC for 1h followed by 28ºC for 54h. Growth at 28ºC for 54h was considered the control condition.

To study the effect of acid stress, mesorhizobia strains were grown in YEM medium buffered with 25 mM homopiperazine-N,N'-bis-2-(ethanesulfonic acid) (Homopipes) for pH 5. For control conditions, YEM was buffered with 20 mM 2-morpholinoethanesulfonic acid (MES) at pH 7. To evaluate the effect of an acidic upshift, the cells were subjected for one hour in YEM (pH 3), and then grown in YEM (pH 7) and YEM (pH 5) at 28º for 54h.

For salt stress, mesorhizobia cells were grown in YEM supplemented with 1.5% NaCl at 28ºC for 54h. For control conditions, YEM with no extra addition of NaCl was used. Bacterial cells were also submitted to a salt shock in YEM supplemented with 5% NaCl, at 28ºC for 1h, and then grown in YEM or in YEM supplemented with 1.5% NaCl, for 54h at 28ºC.

Immunoblotting

ClpB was detected in cells by immunoblotting using a rabbit polyclonal antibody against *E. coli* ClpB (kindly supplied by Professor Michal Zolkiewski). After growth under control and heat shock conditions, cells were harvested by centrifugation at 16.000×g for 3 min, washed in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) and resuspended in 500µL of the sterile water. Cells were kept on ice during sonication and then centrifuged at 13.500×g for 12 min at 4ºC and resuspended in 500µL of sterile water. Protein extracts containing similar concentration (±15µg) were then separated on a 12% linear polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad)

according to the manufacturer's instructions. Following transfer, the membrane was blocked with 1% (w/v) low fat milk in TBS buffer for 1 h, and incubated overnight with rabbit polyclonal antibodies against *E. coli* ClpB protein diluted 1:500. The membrane was washed 3 times with TBS-Tween (0.05% Tween20), and incubated for 1 h with 1:500 dilution of anti-rabbit IgG-peroxidase antibody (Sigma). The membrane was washed with TBS and visualized with peroxidase substrates (4-Chloro-1-naphthol, Sigma) in a color development buffer.

Analysis of the Symbiotic Phenotype

In order to evaluate the symbiotic performance of the Δ*clpB* and wild-type mesorhizobia strains in chickpea plants, a plant growth assay was conducted in a growth chamber. Chickpea seeds (cultivar Chk 3226) were surface sterilized and pre-germinated as described before (Chapter 4). After germination, the seeds were transferred to plastic pots filled with sterile vermiculite and grown in a growth chamber, under a 16/8h light/dark cycle and 24/18ºC day/night temperature and at a relative humidity of 65% for 8 weeks.

The rhizobia strains were grown in TY liquid medium at 28ºC for 72 hours. After incubation, the cell suspension was centrifuged at 10.000×g and resuspended in fresh TY liquid medium. Finally, the bacterial cultures were standardized to an OD_{540nm} of 1.0 and 1 ml of the bacterial suspension was used to inoculate each seed. Four 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. After eight weeks, the plants were harvested and several parameters were measured, such as shoot dry weight (SDW), root dry weight (RDW), number of nodules (NN) and nodule dry weight (NDW).

In order to evaluate the nodule kinetics and development, a hydroponic plant growth assay was conducted using chickpea plants inoculated with either *M. ciceri* LMS-1 or Δ*clpB* mutant strain. The bacterial cultures were grown in TY for

72 hours days. The bacterial suspension was centrifuged at 10,000×g and resuspended in nitrogen-free nutrient solution (Harper and Gipson, 1984) diluted in sterile water (1:4). The bacterial suspension was adjusted at an optical density of 0.6. Finally, the pre-germinated roots of the seeds were put into contact with the bacterial suspension overnight at 28ºC, in dark. The chickpea seeds were washed in nutrient solution and put in a container with 5L of nutrient solution in a growth chamber under controlled conditions. For negative controls, no bacterial culture was used. Five seeds per treatment were used. The nutrient solution was replaced every week and the nodule formation and development were recorded every 3 days for 35 days. The nutrient solution was aerated using an aquarium pump.

Analysis of *nodC* gene expression by semi-quantitative RT-PCR

To evaluate the *nod* genes expression in the Δ*clpB* strain, analysis of the *nodC* expression in the wild-type and mutant strains was performed by semi-quantitative RT-PCR (Moscatiello *et al*., 2009). Induction of the *nod* genes was performed using chickpea root exudates, which were obtained as described by Srivastava *et al.* (1999). The root exudates were added to exponential phase cells and the cultures were incubated for 6h at 28ºC (control condition). Cells were then submitted to control or heat shock (42ºC, for 30 min). conditions.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. After extraction, about 2μg of total RNA were treated with DNase I (Roche). Conversion of total RNA to cDNA was conducted using the RevertAid First Strand cDNA synthesis kit (Fermentas) according to the manufacture's suggestions. Amplification of 16S rRNA and *nodC* genes was performed as previously described (Laranjo *et al.* 2004; Rivas *et al*. 2007). Densitometric analysis of ethidium bromide-stained agarose gels was performed using Kodak Digital Science 1D version 2.0.3 (Eastman Kodak Company, New Haven, USA). The 16S rRNA gene was used to normalize relative *nodC*

transcript abundance. Three independent biological replicas were used to assess the *nodC* transcript levels. The viability of the mutant strain, subjected to this heat shock, was previously confirmed.

Statistical analysis

The data obtained from the chickpea plant growth assay was characterized by analysis of variance, and means were compared by One-way ANOVA. Statistical analysis was carried out using SPSS V.17 software (SPSS Inc., Chicago, USA***).***

Microscopy

For analysis of the infection performance of the rhizobia strains, 4 days-old germinated chickpea seeds were inoculated with GFP-tagged mesorhizobia strains as previously described (Robledo *et al*., 2011). After 4 days of inoculation, root hairs were examined by confocal spectral microscopy with a Leica confocal microscope equipped with krypton-argon laser using a blue excitation filter (excitation maximum 488 nm; 530 nm long-pass filter), allowing simultaneous visualization of GFP and propidium iodide fluorescence. Roots and root hairs were stained with 10 µM propidium iodide (Sigma-Aldrich). Projections were made from adjusted individual channels in the image stacks using Leica software.

Roots and nodules were excised from 25-day-old chickpea plants and processed for light microscopy. The internal morphological features of chickpea nodules were examined by microscopy after Toluidine blue staining. Pink nodules were fixed in 4% formaldehyde in 50 mM phosphate buffer (pH8), dehydrated in an increasing ethanol series, and embedded in paraffin. Toluidine blue-staining sections (2 µm) of embedded nodules were examined by light microscopy.

6.4 Results

Analysis of the *clpB* gene sequence from *Mesorhizobium ciceri* LMS-1

The complete nucleotide sequence (2607 bp) corresponding to the *clpB* gene of *M. ciceri* LMS-1 was determined by PCR amplification and compared to those from other bacteria (Fig. 6.1). The predicted amino acid sequence corresponds to a protein with approximately 96 kDa. The amino acid alignment with characterized prokaryotic ClpBs showed that the *M. ciceri* LMS-1 ClpB possesses an N-terminal domain, two nucleotide-biding regions (NBD1 and NBD2), and a coiled-coil middle (M) domain inserted into NBD1 (Fig. 6.1), corresponding to the typical domains of the bacterial ClpB subfamily.

The predicted amino acid sequence displays a high degree of similarity to predicted and experimentally proven ClpB proteins from a wide variety of organisms. As expected, the comparative analysis showed that the most similar protein (100% identity) to the predicted *M. ciceri* LMS-1 ClpB was the putative ClpB protein from *M. ciceri* bv. *biserrulae* WSM1271 (accession number E8TAM5). Nevertheless, identity levels > 50% were observed between the predicted *M. ciceri* LMS-1 ClpB amino acid sequence and the ClpB amino acid sequences from unrelated bacteria such as *E. coli* (58.1%) and *Thermus thermophilus* (57.2%). Moreover, the predicted ClpB sequence from *M. ciceri* LMS-1 shared high similarity (>67%) with the predicted ClpB among the bacteria belonging to the α-Proteobacteria class, especially in the regions corresponding to the nucleotide biding domains.

Figure 6.1 Alignment of ClpB proteins amino acid sequences from bacteria belonging to *Mesorhizobium* genus, *E. coli* and *Thermus thermophilus*. The conserved residues between all ClpB proteins are indicated in grey columns.LMS-1- M. ciceri LMS-1 (predicted ClpB); WSM1271- *M. ciceri* bv. biserrulae WSM1271 (E8TAM5); WSM2073- *M. australicum* WSM2073 (G4JW53); CCNWGS0123- *M. amorphae* CCNWGS0123 (G6YIV4); MAFF303099- *M. sp.* MAFF303099 (Q98G96); WSM2075- *M. opportunistum* WSM2075 (F7Y492); E.coliK12- *E. coli* strain K12 (P63284); T.termo.HB8- *Thermus thermophilus* strain HB8 (Q9RA63). The regions containing the two ATP-binding domains (ATP-1 and ATP-2) are boxed. Amino acids underlined in red correspond to the BamHI-Eco47III deletion fragment in *M. ciceri* LMS-1.

Confirmation of the *ΔclpB* knockout mutant construction

Deletion of an internal fragment of 675 nucleotides that encodes the M-domain and the initial sequence of NBD2 within the *clpB* gene from *M. ciceri* strain LMS-1 was performed (Table 6.1; Fig 6.1). The truncated *clpB* gene fragment was cloned in the mobilizable suicidal plasmid pK18mobsacB (Schafer *et al.*, 1994) and transferred to *M. ciceri* LMS-1. To confirm the allele replacement after plasmid integration, at the desired site of *M. ciceri* LMS-1 genome, total DNA HindIII-digested samples from wild-type and mutant strains were analyzed by Southern hybridization. A single band of about 2800bp was detected in the wild-type strain, indicating *clpB* as a single copy gene in *M. ciceri* LMS-1 genome (Fig. 6.2). In contrast, a shorter band with approximately 2100bp was detected in the DNA from the *clpB* knockout mutant strain, confirming the disruption of the *clpB* gene. Furthermore, the absence of ClpB protein using total proteins from the Δ*clpB* mutant strain was confirmed by immunoblotting assay. On the other hand, Southern hybridization of DNA from the complemented strain showed a band similar to the one obtained with the wild-type strain (Fig. 6.2).

In order to evaluate the effect of the loss of function of the ClpB chaperone in stress tolerance and symbiosis, wild-type, mutant and complemented strains were further tested under stress conditions and during symbiotic interaction with chickpea plants.

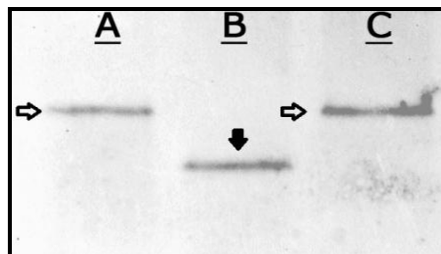


Figure 6.2 Southern hybridization of total DNA from *M. ciceri* LMS-1 (A), *ΔclpB* mutant (B) and complemented strain (C) digested with HindIII restriction enzyme. Open arrows indicate a band with approximately 2800 bp; closed arrow indicates a band of about 2100 bp.

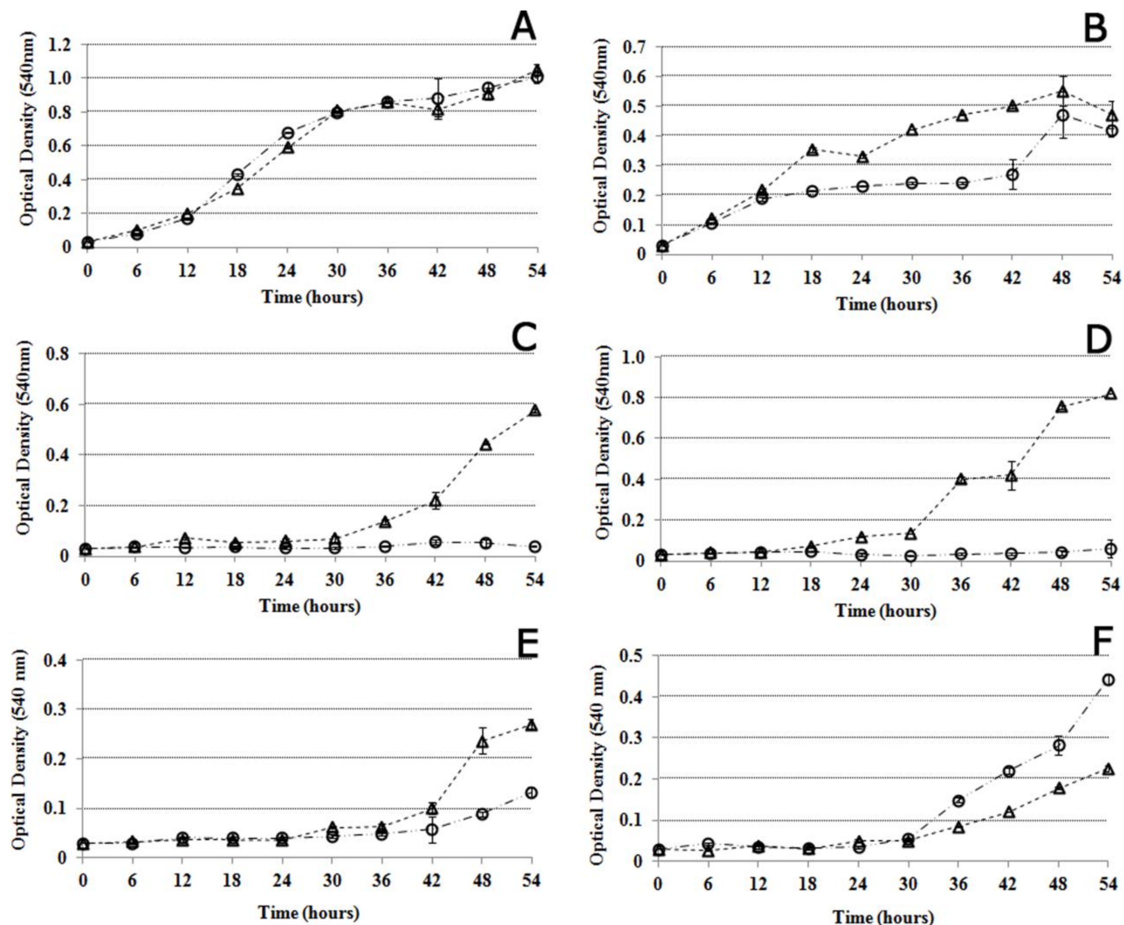
***ΔclpB* mutant phenotype under environmental stresses**

To characterize the phenotype of the ClpB mutant strain, designated as *M. ciceri* LMS-1 Δ*clpB*, its growth was evaluated in liquid medium under several conditions, namely heat, cold, acidity and salinity stresses. To evaluate the phenotype under different stresses, strains were subjected to both shock and stress conditions (Fig 6.3).

No significant differences between the growth rates of the complemented and the wild-type strains, under the different conditions, were found (*data not shown*).

In control conditions (28ºC; pH 7; 0% NaCl), *M. ciceri* LMS-1 Δ*clpB* showed a similar growth curve to the wild-type strain (Fig. 6.3 A).

Under heat stress (37ºC for 54 h), the Δ*clpB* mutant showed a lower growth rate when compared to the wild-type (Fig. 6.3 B), suggesting the involvement of the ClpB in the adaptation to heat stress. To further determine whether ClpB is important for thermotolerance, the strains were subjected to different heat shock conditions, namely 45ºC for 1h and 48ºC for 15 min, followed by growth at control temperature (28ºC, 54 h). In both experiments, the wild-type strain showed ability to grow after heat shocks while the Δ*clpB* mutant was not able to grow (Fig. 6.3 C, D). Evaluation of growth after a heat shock of 50ºC for 5 min showed similar results (data not shown). However, under cold temperatures, no difference was observed between the Δ*clpB* mutant and the wild-type *M. ciceri* LMS-1 strain (*data not shown*).



Stress/shock treatments	*ΔclpB* mutant phenotype
15ºC for 54h	No difference
37ºC for 54h	Higher sensitivity
pH 5 for 54h	No difference
1.5% NaCl for 54h	No difference
Heat shock (45ºC, 1h; 48ºC, 15 min; 50ºC, 5 min)	No growth
Cold shock (4º C, 1h)	No difference
Salt shock (5% NaCl, 1h)	No difference
Salt shock followed by growth with 1.5% NaCl for 54h	Higher tolerance
Acid shock (pH3, 1h)	No difference
Acid shock followed by growth with pH 5 for 54h	Higher sensitivity

Figure 6.3 Growth curves of wild-type (open triangles) and Δ*clpB* mutant (open circles) strains under different conditions during 54 hours. A) Control conditions: 28ºC, 0% NaCl, pH 7; B) Growth curves under heat stress at 37ºC; C) Growth curves after a heat shock of 45ºC for 1 hour D) Growth curves after a heat shock of 48ºC for 15 min; E) Growth curves under pH 5 after a shift of pH 3 for 1 hour; F) Growth curves under salt stress after a salt shock of 5% NaCl for 1 hour. Data represents the mean and standard deviation of three independent biological replicates. Table summarizes the stress/shock experiments tested and indicates the effects in the Δ*clpB* mutant phenotype.

Taking into account these results, the production of ClpB protein in the wild type strain *M. ciceri* LMS-1 after heat shocks (45ºC, 1h; 50ºC, 5min) was evaluated. Total proteins from wild-type strain grown under control conditions and after heat shocks were extracted and ClpB was detected by immunoblotting assay. A protein with the expected molecular weight (±96 kDa) was found in all conditions (Fig. 6.4 lanes 1-3). However, a higher amount of the ClpB protein was detected in *M. ciceri* LMS-1 cells submitted to a heat shock in comparison to cells grown under control conditions, confirming its involvement in heat shock response. As expected, no band was detected in the Δ*clpB* mutant strain, confirming the ClpB absence in the mutant strain (Fig. 6.4 lanes 4 to 6).

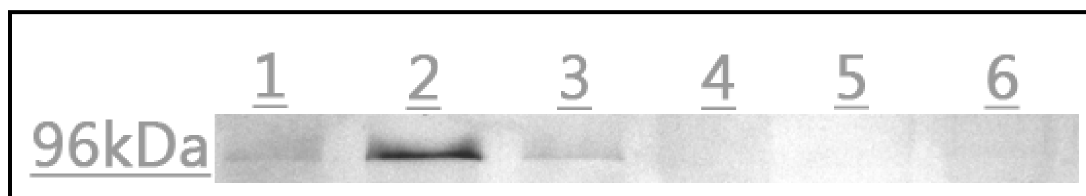


Figure 6.4 Western blot analysis of ClpB production levels with polyclonal antibody against *E. coli* ClpB (1:500). Total protein lysates (15 µg per lane) were obtained from wild-type (1-3) and Δ*clpB* mutant (4-6) strains under control and heat shock conditions. Control conditions (28ºC) (lane 1 and 4), heat shock conditions: 45ºC for 1h (lane 2 and 5) and 50ºC for 5min (lane 3 and 6).

To investigate the involvement of the ClpB chaperone in acid tolerance, the wild-type and mutant strains were submitted to different acid conditions. Both strains were grown under pH 5 for 54 h and were also submitted to a pH 3 shock for one hour and then grown at pH 7 or pH 5, for 54 h. No differences were observed between Δ*clpB* mutant and wild-type strains when grown at pH 5 for 54 h or when submitted to a pH 3 shift and then grown at pH 7 (*data not shown*). In contrast, when the cells were subjected to an acid shock (pH 3) and then were grown at pH 5, a lower growth rate was observed with the Δ*clpB* mutant strain when compared to the wild-type strain (Fig. 6.3 E), suggesting the involvement of the ClpB chaperone in acid-tolerance.

The wild-type *M. ciceri* LMS-1 and Δ*clpB* mutant strains were also tested to salt stress. At continuous growth with 1.5% NaCl, no difference between Δ*clpB* mutant and the wild-type strains growth was observed. Furthermore, no differences between strains growth after a salt shock followed by growth in control conditions (0% NaCl) were observed. Unexpectedly, after a salt shock (5% NaCl for 1h) followed by growth with 1.5% NaCl, the Δ*clpB* mutant growth was two times higher than the wild-type growth after 54 hours (Fig. 6.3 F).

Evaluation of the symbiotic performance of the *ΔclpB* mutant strain

To examine the involvement of the ClpB chaperone in the chickpea-*rhizobium* symbiosis, the symbiotic performance of the Δ*clpB* mutant strain was evaluated. A plant growth assay was performed in a plant growth chamber, using vermiculite as substrate. The chickpea plants were harvested 8 weeks after inoculation and several parameters were measured (Table 6.2). The shoot and root dry weights of chickpea plants inoculated with the Δ*clpB* mutant strain were lower compared to the ones obtained from plants inoculated with the wild-type strain, however, these differences were not statistically significant. Although no differences on the total number of nodules per plant formed by either strain were obtained, a difference in the nodule development was observed. A low free-oxygen level in the infected nodule parenchyma is a prerequisite for the activity of the oxygen-labile nitrogenase in bacteroids, the oxygen-binding plant protein *leghemoglobin* accumulates gradually in the host tissue and the nodule becomes pink to bright red (Fisher, 1994). Concomitantly, the absence of this protein results in small and white, ineffective nodules (Ott *et al.*, 2005). Despite both strains were able to form pink colored nodules, the number of white/pink colored nodules induced by each strain was different (Table 6.2). From the total number of nodules formed by the wild-type, 84,5% were fully pink effective nodules whereas only 61,2% of the total number of nodules induced by Δ*clpB* mutant were effective nodules. The number of small and white, ineffective nodules produced by the Δ*clpB* mutant strain was significantly higher compared to those formed by the wild-type,

suggesting that nodule development was delayed in plants inoculated with the Δ*clpB* mutant strain. Due to the higher proportion of ineffective nodules, a lower nodule dry weight as well as a lower average weight per nodule was obtained in plants inoculated with the Δ*clpB* mutant strain (Table 6.2).

Table 6.2 Symbiotic performance in chickpea plants after inoculation with wild-type LMS-1 or Δ*clpB* mutant strain. Results obtained from a plant growth assay using chickpea plants inoculated with *M. ciceri* LMS-1 and *M. ciceri* LMS-1 Δ*clpB* strains under controlled conditions in a growth chamber. No nodules were obtained in uninoculated plants. Data correspond to the mean ± standard deviation values from four plants for each treatment after eight weeks of inoculation.

Strain	SDW (g/plant)	RDW (g/plant)	Total biomass (g/plant)	NDW (g/plant)
Uninoculated	0.155±0.034	0.327±0.058	0.482±0.082	0
LMS-1	0.768±0.136	0.445±0.104	1.213±0.235	0.224±0.143
LMS-1 Δ*clpB*	0.638±0.146	0.382±0.048	1.019±0.189	0.126±0.023

Strain	Number of nodules	Pink nodules	White nodules	Average weight per nodule (mg/nodule)
Uninoculated	0	0	0	0
LMS-1	84±26	71±19	13±8	2.454±0.792
LMS-1 Δ*clpB*	85±11	52±9	33±2#	1.520±0.387

Statistical significant differences ($P < 0.05$) SDW-shoot dry weight; RDW-root dry weight; NDW- nodule dry weight

The Δ*clpB* mutant shows a delay in nodule development

In order to determine in which step of the symbiosis process the ClpB is involved, further studies were conducted. A hydroponic plant assay was performed to compare the nodulation kinetics of the Δ*clpB* mutant and wild-type strains. After 10 days of inoculation, the chickpea plants inoculated with the *M. ciceri* LMS-1 showed the first nodules (Fig. 6.5). A significant increase of the number of nodules was observed in the first two weeks after the nodules appearance, and the number of nodules stabilized 30 days after inoculation. On the other hand, the first nodules formed by the Δ*clpB* mutant appeared 16-18 days after inoculation, with a 6-8 day delay compared to the wild-type strain. The increase

in the number of nodules was slower than with the wild-type strain. However, 35 days after inoculation, a similar number of nodules was observed in plants inoculated with either strain. In spite of the similar total number of nodules per plant after 35 days of inoculation, the number of effective were significantly less in plants inoculated with the Δ*clpB* mutant strain compared to those formed by the wild-type strain (21 ±4 pink nodules formed by Δ*clpB* mutant strain versus 35 ±5 pink nodules induced by wild-type strain).

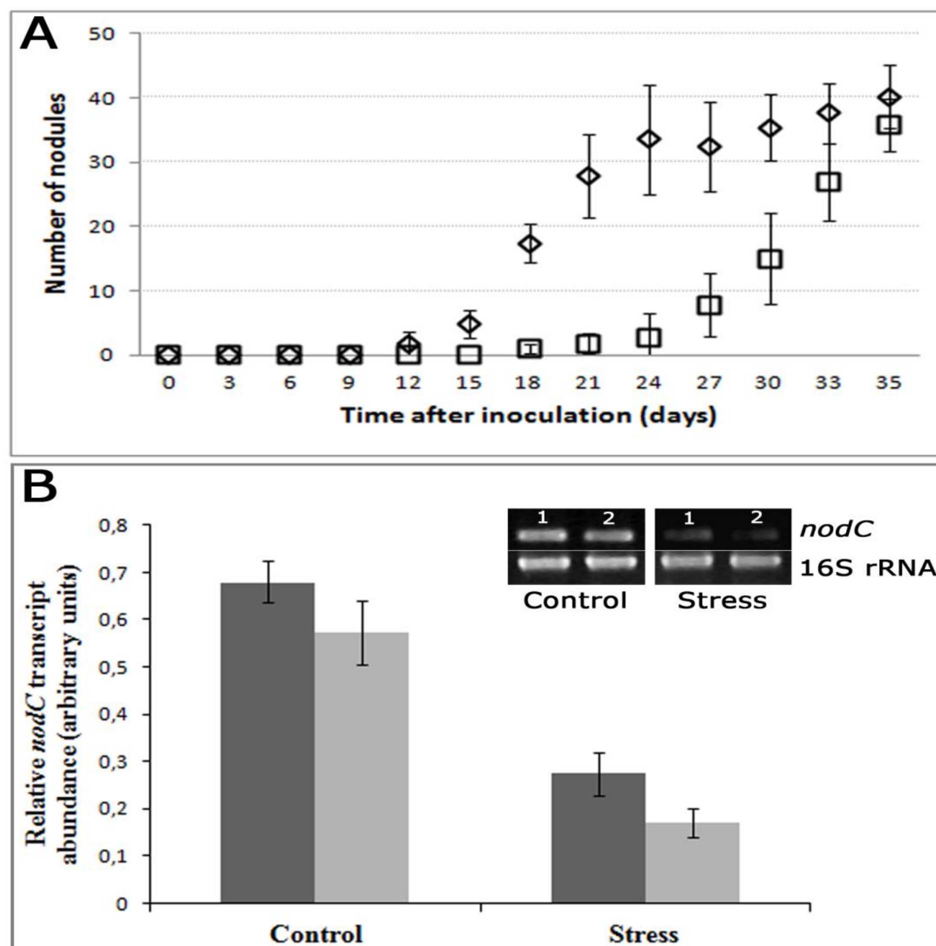


Figure 6.5 A- Nodulation kinetics of chickpea plants inoculated with either wild-type (open diamonds) or Δ*clpB* mutant (open squares) strain during 35 days after inoculation. Each point represents the mean of 5 plants per treatment. Standard deviation is represented by bars on each data point. B- Analysis of *nodC* gene expression by semi-quantitative RT-PCR during control and heat shock conditions in *M. ciceri* LMS-1 (lane 1, dark grey bars) and Δ*clpB* mutant (lane 2, light grey bars) strains, after 6h treatment with chickpea root exudates. Relative *nodC* transcript abundance was normalized against 16S rRNA. Data are the means ± standard deviation of three independent biological replicates.

To compare the level of expression of the nodulation (*nod*) genes in the wild-type and in the Δ*clpB* strains, analysis of the *nodC* gene expression was performed by semi-quantitative RT-PCR. Compared with the wild type strain, the Δ*clpB* mutant displayed a lower amount of *nodC* transcript, particularly under the heat shock condition (Fig. 6.5B). The lower level of *nod* genes expression can account to the delay observed in root nodulation by the mutant strain (Fig. 6.5A).

To investigate potential differences in the nodule formation and development induced by Δ*clpB* mutant and wild-type strains, nodules obtained 25 days after inoculation, in the hydroponic plant assay, were collected. Histological sections of nodules induced by either strain were compared. The main differences observed between the nodules formed by either strain were the size (Fig. 6.6 A-C) and color. The majority of the nodules formed by the Δ*clpB* mutant strain were smaller than the ones formed by the wild-type strain. All nodules induced by the wild-type strain showed the characteristic zones, namely meristematic, invasion, and fixation zones (Fig. 6.6 A). The senescent zone was not visible in any of the nodules analyzed. In contrast, the fixation zone in the majority of the nodules formed by the Δ*clpB* mutant strain showed scarce content in bacteroids. Indeed, the nodules formed by the Δ*clpB* mutant strain contain a higher proportion of undifferentiated bacteria, namely immature bacteroids (Fig 6.6 C). However, no significant differences between the invasion threads and release pockets were observed between the nodules formed by either strain (Fig. 6.6 D, E). Moreover, in the fixation zone found in some nodules induced by the Δ*clpB* mutant strain, the bacteroid organization within the cortical cells was well defined and similar to the wild-type strain (Fig. 6.6 F).

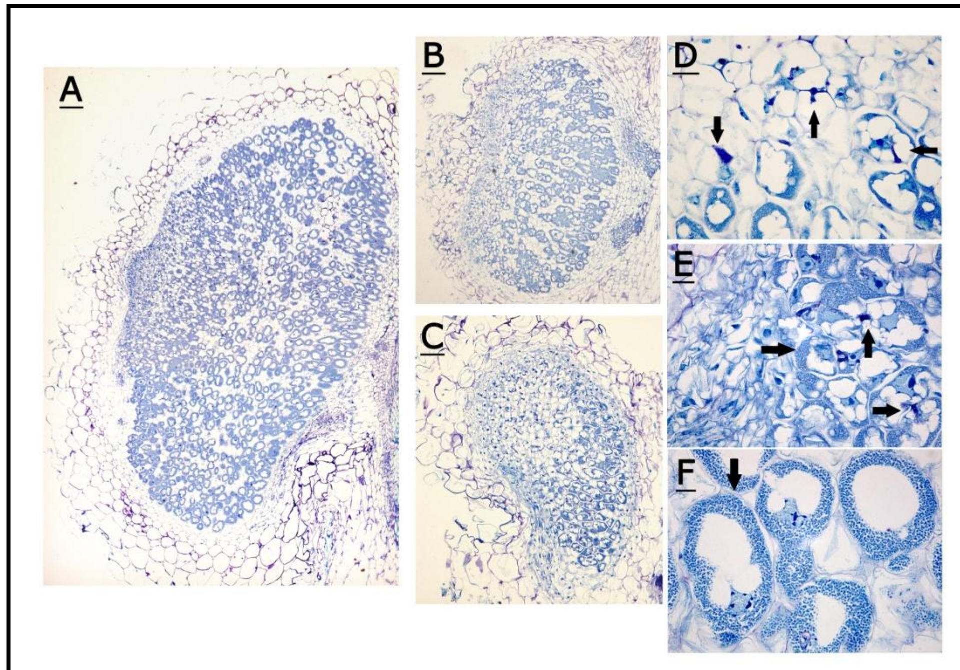


Figure 6.6 Bright field micrographs of 2 μm histological sections of representative embedded pink nodules, stained with Toluidine Blue, formed by the wild-type *M. ciceri* LMS-1 (A), or the Δ*clpB* mutant strains (B-F). A-C, sections of entire nodules; D-F, higher magnification of Δ*clpB* mutant nodules, invasion zone (D), interzone (E), and fixation zone (F). Black arrows in D show pockets of intercellular bacteria and infection threads emerging from them. Black arrows in E show infected cortical cell and bacteria released from the infection threads into the root cortical cells. Black arrow in F shows the bacteroid differentiation and indicates the complete symbiosome organization. Magnification: A-C: 50x; D-F: 630x.

Initial interaction of the Δ*clpB* mutant with chickpea roots

In order to determine if the delay in the nodules appearance observed in plants inoculated with the Δ*clpB* mutant strain, in the hydroponic assay, could be due to a failure in the first stages of the mutant strain's interaction with the plant roots, the infection processes by the Δ*clpB* mutant and wild-type strains were compared. To visualize root hair attachment of rhizobia and their ability to grow on the root surface and form infection threads in root hairs, the *M. ciceri* LMS-1 and *M. ciceri* LMS-1 Δ*clpB* strains, harbouring plasmid pMRGFP, containing the constitutively expressed *gfp*, were used. Four days after chickpea roots

inoculation with either Δ*clpB* mutant or wild-type strain, the roots and root hairs were visualized using confocal spectral microscopy.

Root hair attachment of rhizobia and their ability to grow on the root surface and form infection threads in root hairs were compared. Four days after chickpea roots inoculation, attachment of numerous cells to roots and root hairs was visible with both strains (Fig. 6.7). Bacteria were located mainly on roots, but were also found on root hair tips (Fig. 6.7 A, B) and forming caps (Fig. 6.7 A, B). Curled root hair, infection threads initiation and extension to root epidermal cells inside of some of the root hairs were also observed with both strains (Fig. 6.7 A, B). Overall, no differences in the infection abilities of wild-type and Δ*clpB* mutant cells were detected.

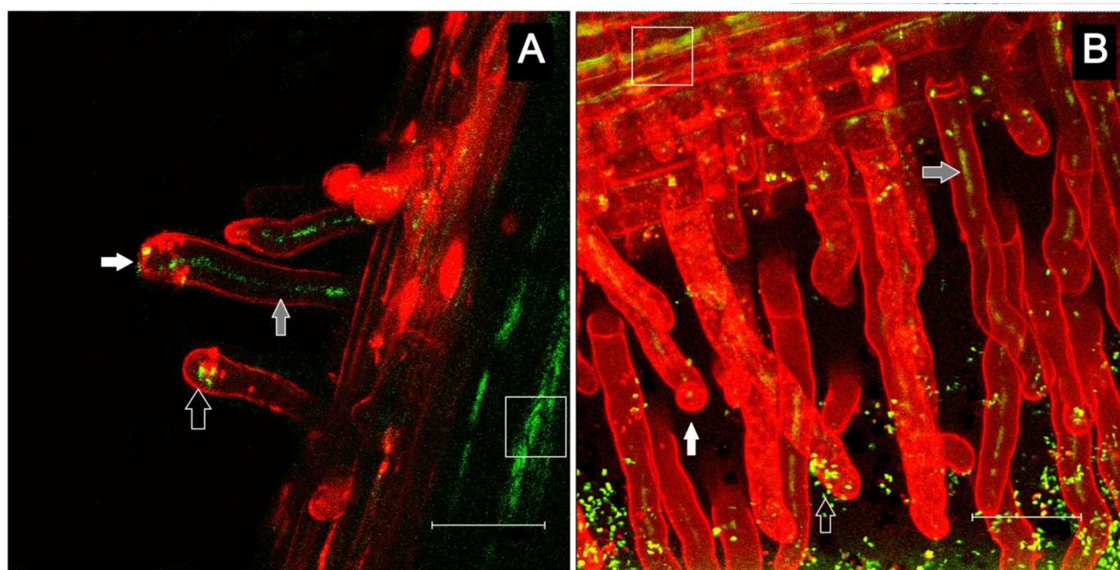


Figure 6.7 Confocal laser scanning micrographs of propidium iodide-stained roots inoculated with GFP-tagged LMS-1 and Δ*clpB* mutant strains showing the initial infection process in chickpea roots. Images from chickpea root and root hairs were obtained 4 days after inoculation with either wild-type (A) or Δ*clpB* mutant (B) strains. Root hair curling formed by either strain (closed white arrows); Infection threads on root hairs (closed grey arrows); caps on root hairs tips (open white arrows), rhizobial attachment on roots (square). A, B scale bars correspond to 30μm and 40μm, respectively.

6.5 Discussion

In the present work, a ClpB chaperone knockout mutant from the strain *M. ciceri* LMS-1 was obtained. Our results suggest that *clpB* is a single gene in the *M. ciceri* LMS-1 genome. Similarly the completely sequenced genomes of *Mesorhizobium* strains, namely *Mesorhizobium* sp. MAFF303099 (Kaneko *et al*., 2000), *M. opportunistum* WSM2075^T (NC_015675), *M. australicum* WSM2073^T (http://www.ncbi.nlm.nih.gov/nuccore/354575234) and *M. ciceri* bv. *biserrulae* WSM1271 (NC_014923) show also a single *clpB* copy gene. Furthermore, the similarity and identity in the *clpB* nucleotide/amino acid sequence between *Mesorhizobium* strains is extremely high (>90%).

The Δ*clpB* mutant strain tolerance to environmental stress conditions as well as its involvement in the symbiotic process with chickpea plants was evaluated. The evaluation of the ClpB mutant phenotype under abiotic stresses showed that it was unable to grow after a heat shock and it was more sensitive to acid shock than the wild-type strain. Our results confirm that ClpB is essential in heat shock tolerance in *M. ciceri* LMS-1. This is in agreement with the transcriptomic study conducted by Sauviac *et al*. (2007), which showed the *clpB* gene up-regulation by heat shock in *Ensifer meliloti* cells. Similar results were observed in diverse organisms, such as *E. coli* (Squires *et al*., 1991; Zolkiewski, 1999), *Vibrio cholera* (Nag *et al*., 2005), *Synechococcus* spp.(Eriksson and Clarke, 2000) and *Brucella suis* (Ekaza *et al*., 2001). In contrast, no difference between wild-type and Δ*clpB* mutant strains growth under cold temperatures was found. Similar results were obtained previously in *Listeria monocytogenes* (Chastanet *et al*., 2004) and *E. coli* (Strocchi *et al*., 2006). Strocchi *et al*. (2006) suggested that low temperatures may lead to inactivation of a few key cold-sensitive chaperone-interacting proteins, such as ClpB, DnaK and GroEL, in *E. coli*. It is possible that the absence of differences under low temperatures between wild-type and Δ*clpB* mutant strains may be due to the cold inactivation of chaperone-interacting proteins, such as ClpB, covering the lack of ClpB in the mutant strain.

Under acid or salt continuous stress conditions, no difference between the growth curves of the wild-type and Δ*clpB* mutant strains was observed. Similar results were obtained when cells were subjected to an acid or salt shock. In contrast, when the cells were subjected to an acid shock followed by growth under continuous mild acid conditions, the Δ*clpB* mutant displayed a higher sensitivity compared to the wild-type strain, indicating the involvement of ClpB in acid response. This result corroborates previous studies (de Lucena *et al.,* 2010) suggesting that ClpB is involved in acid-response in *Ensifer meliloti* cells. Moreover, Reid *et al*. (2008) identified several genes, including *clpB*, which may be involved in acid tolerance of *Campylobacter jejuni*.

Interestingly, the Δ*clpB* mutant showed a higher ability to overcome salt stress followed to a salt shock, compared to the wild-type strain. This may result from the induction of genes upon salt-shock that after were useful to overcome the salt-stress, in a higher extent in the mutant than in the wild-type strain. Nevertheless, further studies are required to clarify this unexpected result.

Our results indicate that ClpB is essential for heat shock response and is involved in the acid-tolerance, suggesting that both stress responses involve the action of this chaperone. Interestingly, Reid *et al*. (2008), using transcriptomic analysis in *Campylobacter jejuni in vivo* and *in vitro* conditions, suggested a common response to acid stress and heat shock involving the up-regulation of most heat shock genes, namely *clpB*, *dnaK*, *groEL* and *groES*.

In the present study, the role of the chaperone ClpB in the chickpea-*mesorhizobium* symbiosis was also examined. Although the shoot and root dry weights of chickpea plants inoculated with the Δ*clpB* mutant strain were lower compared to the ones obtained from plants inoculated with the wild-type strain, these differences weren't statistically significant, suggesting that ClpB is not essential for the successful symbiotic nitrogen-fixation. This is supported by previous studies in *Ensifer meliloti* cells showing that a mutation in *rpo*H1, which regulates the ClpB expression (de Lucena *et al*., 2010), does not affect the expression of the major nitrogen fixation genes (Mitsui *et al*., 2004).

Although the plant development displayed by the Δ*clpB* mutant and wild-type strain was statistically similar, the nodules induced by the mutant strain showed a slower development. The nodulation kinetics showed a 6-8 day delay in nodule appearance in chickpea plants inoculated with the Δ*clpB* mutant strain when compared to the wild-type strain. Furthermore, a higher proportion of the nodules formed by the mutant strain were small and white. This result was corroborated by the analysis of the histological sections of nodules formed by the Δ*clpB* mutant strain, showing that most of the nodules presented low amount of bacteroids. The delay observed in nodule appearance and development probably contributes to the lower number of effective nodules of chickpea plants obtained with the Δ*clpB* mutant strain. Altogether, our results suggest that ClpB is not essential for the establishment of a successful symbiosis with chickpea plants, but is involved in the process of nodule formation and development.

Furthermore, our results indicated that *nodC* gene expression is lower in the Δ*clpB* mutant, suggesting that ClpB is involved in the molecular signalling between both partners. A previous study of Ogawa and Long (1995) showed that the *Ensifer meliloti* GroEL chaperone is required for regulation of early *nod* genes by the transcription activator NodD. This protein is required for the induction of *nodC* expression, as shown in *Ensifer meliloti* (Mulligan and Long, 1985) and in *Rhizobium leguminosarum* (Rossen *et al*., 1985). The lower levels of *nodC* transcript detected in the mutant strain, particularly under the heat shock condition, suggest that the delay in nodules formation by the Δ*clpB* mutant could be related to its inability to activate properly the expression of the nod genes due to an inappropriate folding of the NodD protein.

Oke and Long (1999) suggested that, among the genes needed for bacteroid formation, some of them are specific for symbiosis and others are involved in the physiological adaptation to the environmental conditions within and outside the nodule. More recently, transcriptomic and proteomic analyses of nodules or bacteroids suggested that chaperone genes, including ClpB, are involved in the symbiosis process (Djordjevic *et al*., 2004; Sarma and Emerich, 2005;

Karunakaran *et al*., 2009). Shimoda *et al*. (2008) suggested the interaction between chaperones during symbiosis, namely ClpB with ClpA, as part of a protease complex that functions in protein processing during symbiosis. Nomura *et al*. (2010) verified, by analysis of differential protein profiles of *Bradyrhizobium japonicum* bacteroids, that a number of chaperone proteins were overexpressed at the onset of the nitrogen fixation. Several studies on the symbiotic performance of strains mutated in other chaperone´s genes suggested their involvement in the symbiosis. For example, 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. The DnaK chaperone seems to be required for optimum symbiotic function in *Ensifer meliloti* (Summers *et al*., 1998). A double mutation on *groEL*3 and *groEL*4 genes affects the symbiotic performance in *B. japonicum* (Fischer *et al*., 1999).

It is acceptable that the symbiotic process is affected by the ability of rhizobial cells to respond to environmental stresses encountered when infecting or within the plant host. Marschner (2006) reported that the environmental pH in the rhizosphere of the leguminous host plant is decreased due to the protons and organic acids excreted by the plants. Moreover, rhizobia have to face acid conditions within the plant cell, due to the transport of protons or ionized acids that acidify the symbiosomes. In addition, rhizobia have to deal with the low oxygen concentration in the nodules, leading to the production of organic acids that inhibit the regulation of the cytoplasmic pH (Perez-Galdona and Kahn, 1994).

Furthermore, several studies in rhizobia showed the induction of genes encoding molecular chaperones and proteases, such as ClpB, under acidity or microaerobic conditions in rhizobia, supporting their involvement in the protection of proteins from denaturation and aggregation (Puskás *et al*., 2004; Uchiumi *et al*., 2004; de Lucena *et al*., 2010).

Take into account all these studies, it is probable that the involvement of ClpB in symbiosis is by disaggregating protein aggregates formed in bacteria subjected to atypical conditions, such as pH shifts or microaerobiosis, within the plant cells

and nodules. Considering the lower *nod* genes expression by the Δ*clpB* mutant strain when compared to the wild-type strain plus the stress effects on Δ*clpB* mutant growth, the delay on nodule formation and development may be due to the inability to properly activate the expression of the *nod* genes as well as the lack of ability to counteract the stress conditions within the host plant and nodule. Furthermore, the lack of ClpB, particularly in the atypical conditions within the root cells, such as pH shifts or microaerobiosis, may result in some aggregation of the NodD proteins that fail to induce the normal expression of the *nodABC* genes thus leading to the delay in nodulation observed with the mutant strain.

Assuming that the ClpB function is protein disaggregation, we can speculate that nodule formation and development involve changes in physiological conditions that lead to protein aggregation, thus requiring the ClpB action. However, further studies are required to clarify the role of this major chaperone in rhizobia during symbiosis.

Our results indicate that, in *Mesorhizobium ciceri*, ClpB is involved in the response to several stresses, namely heat and acid shocks. Furthermore, the ClpB absence caused a delay in the nodule formation and development, despite the ability of the Δ*clpB* mutant strain to establish a symbiotic relationship with chickpea plants. Overall, in *Mesorhizobium ciceri* LMS-1, the chaperone ClpB seems to play an important role in overcoming stress conditions as free-living cells as well as in the symbiosis process. To our knowledge, this is the first report supporting the involvement of chaperone ClpB from rhizobia in the symbiotic nodulation process.

6.6 References

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Chapter 7

General discussion

The demand for a more effective utilization of biologically-fixed nitrogen in agricultural systems as an alternative to the chemical nitrogen fertilizers has increased the number of studies on rhizobia diversity and tolerance to environmental stresses. In contrast to other rhizobia such as soybean or bean microsymbionts, only a few studies have addressed rhizobia able to nodulate chickpea, one of the most important legume crops worldwide. Using chickpea rhizobia as a study model, the present thesis focused three main goals: i) the evaluation of biodiversity and characterization of chickpea rhizobia in an attempt to find new species as potential candidates to be used as field inoculants; ii) the evaluation of chickpea rhizobia tolerance to environmental stresses, namely acidity and salinity, in order to find isolates tolerant to either stress conditions; iii) the study of the molecular bases of acid- and salt-tolerance of these chickpea microsymbionts. Globally, the present thesis aimed to contribute to the development of efficient rhizobia.

The present work contributed to the isolation and characterization of chickpea rhizobia that, presently, are part of a collection composed by a total of 121 native Portuguese isolates collected from 26 soil samples, covering almost the entire country. The isolation and characterization of these native chickpea rhizobia allowed the identification of isolates from *Mesorhizobium* species and brought new insights into species geographic distribution. The collection includes several potential chickpea microsymbionts for agricultural applications.

Analysis based on the 16S rRNA gene sequence revealed that all the native chickpea microsymbionts belong to the *Mesorhizobium* genus. However, most chickpea rhizobia isolates were assigned to different species besides the previously described specific chickpea microsymbionts, namely *M. ciceri* and *M. mediterraneum* (Nour *et al*., 1994; Nour *et al*., 1995). Indeed, only 37% of the isolates belong to the *M. ciceri* and *M. mediterraneum* species. Four main species clusters were generated by the 16S rRNA gene analysis. Considering all 121 chickpea rhizobia isolates, cluster A comprises 60 isolates with high identity with the type strains *M. huakuii*, *M. amorphae* and *M. opportunistum*. Although *M. plurifarium* and *M. septentrionale* type strains are included in this cluster, no isolate was found to group closely to these strains. In cluster B, the 33 rhizobia isolates group closely or were more similar to *M. ciceri* and *M. loti*. Chickpea rhizobia (16 isolates) from Cluster C, which formerly included only *M. tianshanense*, are closer to the new species *M. metallidurans*, *M. caraganae*, *M. gobiense* and *M. tarimense* than to *M. tianshanense*. Cluster D includes 12 isolates together with *M. mediterraneum* and *M. temperatum*.

Previous studies on the diversity of chickpea rhizobia from the southern region of Portugal already suggested that natural populations are diverse (Laranjo *et al*., 2001; Laranjo *et al*., 2002). The present study extended the sampling sites to the entire country and confirmed the high diversity found before. Furthermore, the data obtained in the present study also revealed an unexpected high proportion of isolates unrelated to *M. ciceri* and *M. mediterraneum*, especially the high number of isolates clustering with *M. amorphae*, *M. huakuii* and *M. opportunistum*

(cluster A). This was unexpected, since *M. amorphae* and *M. huakuii* type strains are unable to nodulate chickpea and their hosts, *Amorpha fruticosa* and *Astralagus sinicus* respectively, are unrelated to *Cicer arietinum* and uncommon in Portugal. *M. opportunistum* type strain was isolated from *Biserrula pelecinus* root nodules, and similarly to the two other type strains mentioned above, it is unable to nodulate chickpea. In contrast to *Amorpha fruticosa* and *Astralagus sinicus* plants, *Biserrula pelecinus* is a common pasture legume adapted to acid soils in Mediterraneum and common in Iberian Peninsula (Vicente *et al*., 2009). However, *Biserrula pelecinus* microsymbionts are unable to nodulate *Cicer arietinum* (Nandasena *et al*., 2001; 2007a; 2009).

The chickpea rhizobia diversity was also studied in other countries, such as Morocco and Tunisia (Maâtallah *et al*., 2002a; L'Taief *et al*., 2007). However, these studies revealed that most of the indigenous chickpea rhizobia isolates were assigned to *M. ciceri* and *M. mediterraneum,* showing a low diversity of chickpea rhizobia. The high diversity of chickpea rhizobia found in our study may be due to the differences in the Portuguese soil characteristics, such as soil pH, and to the chickpea cultivation history in those countries.

The biogeography found for the Portuguese chickpea rhizobia isolates revealed an association between province of origin of the isolates and species clusters, suggesting that the geographical distribution of species is not random. Despite several parameters may influence the geographic distribution of isolates in terms of species clusters, the biogeography found herein was associated to the soil pH of the sampling site, indicating that pH contributes to determine the species that prevail in the rhizobia population. This type of association was found in several studies addressing soil bacterial communities, indicating soil pH as the variable that better explains the population diversity and the community composition (Fierer and Jackson, 2006).

In order to investigate whether the symbiotic effectiveness of the chickpea rhizobia isolates is related to their species cluster, the analysis of the isolates symbiotic effectiveness (SE) was performed under control conditions. These

results showed that in each species clusters we can find isolates with high and low SE. However, no correlation was found between SE and species clusters. Interestingly, six of the most efficient isolates belong to cluster B (*M. ciceri* / *M. loti*). For example, isolate S-24-Sintra and isolate G-55-Guarda show SE values above 85%, indicating them as potential candidates for field inoculation.

In order to investigate the molecular determinants for chickpea host specificity, several mesorhizobia isolates belonging to the four main clusters were investigated in terms of symbiosis genes *nifH* and *nodC*. The phylogenetic analyses based on these symbiosis genes indicate that all chickpea mesorhizobia show identical *nifH* and *nodC* genes. These results are in agreement with previous studies (Rivas *et al*., 2007; Laranjo *et al*., 2008), which reported that all isolates able to nodulate chickpea possess identical symbiosis genes to the ones carried by the typical chickpea microsymbionts (*M. ciceri* and *M. mediterraneum*). So, despite that several *Mesorhizobium* species are able to nodulate chickpea, they all share the same symbiosis genes, reinforcing chickpea as a restrict host. Both *nifH* and *nodC* genes can be used as markers for the rapid detection of chickpea microsymbionts. Several studies suggest that many *Mesorhizobium* strains have acquired the ability to nodulate a specific host probably by acquiring a symbiosis island carrying specific symbiosis genes (Sullivan *et al*., 1995; Sullivan and Ronson, 1998; Laranjo *et al*., 2008; Nandasena *et al*., 2006; Nandasena *et al*., 2007b).

In the present work, the detection and sequencing of the ACC deaminase gene (*acdS*) was performed in several mesorhizobia type strains as well as in Portuguese chickpea mesorhizobia isolates. The *acdS* gene was detected in 10 of 12 mesorhizobia type strains and in all of the 17 Portuguese chickpea mesorhizobia isolates tested, suggesting that this gene is a common feature among mesorhizobia. Interestingly, the *acdS*-based phylogeny indicates that this gene seems to be similar among rhizobia nodulating the same host, suggesting that the *acdS* gene, similarly to the symbiosis genes, is likely to be horizontally transferred between *Mesorhizobium* species. The analysis of the symbiosis

islands of mesorhizobia strains (*M. loti* R7A, *M. sp.* MAFF303099, *M. ciceri* bv. *biserrulae* WSM1271 and *M. opportunistum* WSM2075^T), shows that they all include an *acdS* gene closely located to the nitrogen fixation genes. Thus, it is probable that symbiosis islands exchange among mesorhizobia will result in the *acdS* gene transfer together with the symbiotic genes.

The development of rhizobial inoculants requires the selection of isolates that are symbiotically efficient as well as adapted to the local environmental conditions. The present study included the evaluation of tolerance of chickpea mesorhizobia to salinity and acidity, in order to identify isolates tolerant to both stresses and to investigate a possible relationship between stress tolerance and the species or the origin site of the isolates. Tolerance to salinity was evaluated in 106 isolates whereas tolerance to acid and alkaline pH was investigated for 98 isolates, representing the entire country. In general, chickpea mesorhizobia isolates showed a low tolerance to salinity. Nevertheless, a few isolates displayed a considerable growth with 1.5% NaCl compared to the majority of the isolates. For example, isolates BR-8-Bragança, CR-32-Caldas da Rainha, PA-6-Praia do Alemão and SL-2-Salir showed a growth above 30% with 1.5%NaCl. Concerning the tolerance of chickpea mesorhizobia isolates to alkaline conditions (pH 9), isolates revealed to be highly sensitive to this condition. In contrast, almost half of the tested isolates are highly tolerant (growth > 70%) to acidic conditions (pH 5), including 11 moderately acidophilic isolates.

Interestingly, associations between province of origin of the isolates and tolerance to either acid or salt conditions were found. These associations may be related to the characteristics of the origin soil. Supporting this hypothesis, correlations between origin soil characteristics, such as soil pH, electrical conductivity or organic matter levels, and acid or salt tolerance of the isolates were found, indicating that soil may act as a selective pressure in terms of acid and salt tolerance. Thus, several characteristics of the origin soil of the isolates may be involved in determining isolates tolerance to acidity and salinity. Correlations between salt tolerance of rhizobia and some characteristics of the

origin soils were found in other studies (Bezdicek, 1972; Zahran *et al*., 1992; Kulkarni *et al*., 2000; Maâtallah *et al*., 2002b). Furthermore, the association between tolerance to acidity and the origin soil pH found herein suggests that isolates collected from acidic or neutral soils may be more resistant to acidic environmental conditions than the ones from alkaline soils. Similar results were already obtained with a smaller set of Portuguese chickpea rhizobia isolates (Rodrigues *et al*., 2006).

The four species groups were found to differ significantly regarding their ability to tolerate acid stress, suggesting that acid tolerance phenotype is related to the species clusters. For instance, the majority of isolates from cluster B are acid-tolerant whereas isolates belonging to cluster C are acid-sensitive. In contrast, no association between species cluster of the isolates and salt tolerance was found. The analysis of the chickpea Portuguese mesorhizobia tolerance suggests that acid tolerance seems to be species-specific while the salt tolerance is more likely to be strains-specific. Several studies in rhizobia have reported that stress tolerance seems to be species related, namely temperature stress tolerance (Alexandre and Oliveira, 2011), tolerance to copper (Laguerre *et al*., 2006), tolerance to acid pH (Reeve *et al*., 2006) as well as antibiotic resistance (Alexandre *et al*., 2006).

Several studies indicate that strains found to be tolerant to a specific stress display a better symbiotic performance than the sensitive ones when subjected to the same stress condition in plant growth assays (Mhadhbi *et al*., 2004). Take into account these reports, the present study allowed the identification of acid- and salt-tolerant isolates that can be used as potential inoculants with economic and agricultural interest, especially in acid or salt-affected soils. Nevertheless, the symbiotic performance of tolerant isolates in plant growth assays, under stress conditions, must be evaluated prior to implementation of field trials.

In order to contribute to the development of efficient rhizobia and since chickpea mesorhizobia showed high sensitivity to salinity, a strategy was followed to improve their symbiotic performance under salt stress conditions. This strategy

involved the expression of an exogenous ACC deaminase gene in rhizobia. To evaluate the impact of the expression of an *acdS* gene in the improvement of the symbiotic performance of two chickpea mesorhizobia with different salt tolerance phenotype, both isolates were transformed with the exogenous *acdS* gene, from *Pseudomonas putida* UW4. The two mesorhizobia isolates were selected based on their ability to endure salt stress, namely EE-7-ENMP as salt-sensitive and G-55-Guarda as salt-tolerant (see chapter 3). The symbiotic performance of the *acdS*-transformed and wild-type isolates was evaluated. The results obtained with both *acdS*-transformed mesorhizobia revealed that through the use of ACC deaminase, the nodulating abilities of both strains were promoted and the negative effects induced by salt stress in the chickpea growth and development were reduced. These results suggest that the exogenous ACC deaminase gene improves the symbiotic performance of mesorhizobia, thus contributing to help chickpea plants endure the negative effects of salt stress. Interestingly, the symbiotic performance of the salt-sensitive strain, transformed with an exogenous *acdS* gene, was increased in a higher extent than the salt-tolerant one. The different increase of the symbiotic performance obtained for each *acdS*-transformed strain under salt stress conditions, may be due to the different ACC deaminase activity detected in the two strains. Similar results were previously obtained with other plants (canola, tomato, cucumber and red pepper) inoculated with rhizobacteria expressing an ACC deaminase gene and subjected to salt stress (Cheng *et al*., 2007; Gamalero *et al*., 2010; Siddikee *et al*., 2010, 2011). However, this is the first study showing that the expression of an exogenous *acdS* gene in mesorhizobia improved the symbiotic performance of the bacteria when they were used as inoculants of chickpea plants grown under saline conditions, thus almost completely alleviating the negative effects caused by salinity. Our results suggest that the expression of an exogenous *acdS* by *Mesorhizobium* strains may be a useful tool in the development of inocula for agricultural purposes, especially to improve crop productivity in salt-damaged land.

The ability of rhizobia to endure stress is essential to achieve high symbiotic performance in suboptimal conditions. It is important to understand the molecular bases of acid and salt tolerance in rhizobia since this knowledge can be used to improve their symbiotic performance under stressful conditions.

Stress response in bacteria is essential for effective adaptation to changes in the environment. Bacteria have evolved several mechanisms that ensure protein folding and promote homeostasis under stress conditions (Frydman, 2001). Among others, DnaK-DnaJ-GrpE and GroEL-GroES and ClpB are fundamental chaperones systems that assist the proteins folding and disaggregation, ensuring homeostasis in *E. coli* cells, under heat stress conditions. Yet, their involvement in tolerance to salt and acid conditions remains to be clarified. In order to find differences that could be related to the different susceptibility to salt and acid stress, the expression levels of *dnaKJ* and *groEL* genes were analyzed, using tolerant and sensitive isolates within the same species cluster. The expression of these chaperones genes in isolates subjected to acid and salt shock was analyzed by northern hybridization.

The transcriptional analysis of the major chaperones genes using 12 chickpea rhizobia isolates, belonging to the four main species clusters (chapter 2), revealed that, in the majority of the isolates (either tolerant or sensitive), the *dnaK* mRNA levels increased after a salt shock while no change or a slight repression was observed with the *groESL* operon. These results suggest that *dnaK* and *groESL* chaperone genes are differently involved in chickpea mesorhizobia salt tolerance. Similarly to our results, Domínguez-Ferreras *et al*. (2006) reported repression of some *groESL* operon copies in *Ensifer meliloti* cells subjected to salt stress. Additionally, *groESL* operon was slightly repressed or unchanged in some mesorhizobia type strains when submitted to salt shock (Laranjo and Oliveira, 2011). Furthermore, Nogales *et al*. (2002) showed that a mutant with a disrupted *dnaJ* gene presented higher sensitivity to salt stress when compared to the wild type. Nevertheless, no relationship between higher expression levels of *dnaK* chaperone gene and higher ability of isolates to tolerate salt stress was

obtained, suggesting that this gene is not involved in determining the salt tolerance phenotype of chickpea mesorhizobia.

On the other hand, both *dnaK* and *groESL* mRNA levels increased after an acidic shock in acid-tolerant isolates and were slightly repressed in acid-sensitive isolates, with the exception of the moderately acidophilic isolate (AL-13-Alenquer). Considering eight isolates, from cluster A and B, a relationship between higher levels of transcriptional induction of both *dnaK* and *groESL* genes upon acidic shock and a higher ability of mesorhizobia to tolerate acid pH was found. These results suggest that increased expression of these chaperone genes may contribute to a higher tolerance to acid stress in mesorhizobia. Recently, transcriptional analysis using *Ensifer meliloti* 1021 cells following an acidic upshift showed increased *groEL5* transcript levels (Hellweg *et al.*, 2009).

Overall, our results suggest that both chaperone genes are involved in determine chickpea mesorhizobia tolerance to acidity but not tolerance to salinity. Interestingly, another study on chickpea mesorhizobia suggest the existence of a relationship between higher levels of transcriptional induction of both *dnaK* and *groESL* chaperones genes and a higher ability of isolates to endure heat stress (Alexandre and Oliveira, 2011). It seems that *dnaK* and *groEL* genes may be involved in determine acid and heat tolerance in chickpea mesorhizobia. A similar up-regulation of most heat shock genes, namely *clpB*, *dnaK*, *groEL* and *groES,* was detected using transcriptomic analysis of *Campylobacter jejuni in vivo* and *in vitro* conditions after both acid stress and heat shock (Reid *et al.*, 2008). These results support a common molecular role of major chaperone genes in both heat and acid tolerances. To our knowledge, this is the first study focusing the transcriptional induction of chaperone genes under salt and acid shocks, using tolerant and sensitive chickpea mesorhizobia.

The transcriptional analysis described in chapter 5 suggests that major chaperones may be involved in acid tolerance in mesorhizobia. In order to investigate the role of the major chaperones in stress tolerance of mesorhizobia, in free-living conditions as well as in symbiosis with chickpea plants, the

chaperone ClpB was chosen for further studies. Few reports address this chaperone gene, compared to GroEL or DnaK in rhizobia. To our knowledge, there are no reports on a ClpB knockout in rhizobia or any study investigating its role in rhizobia tolerance to abiotic stresses.

To investigate a potential role of the chaperone ClpB in stress tolerance and in the symbiosis process of mesorhizobia, the *clpB* gene of the strain *Mesorhizobium ciceri* LMS-1 was deleted (chapter 6). ClpB mutant was tested under several abiotic stresses and compared to the wild-type. These phenotypic tests showed that the Δ*clpB* mutant was unable to grow after a heat shock and was more sensitive to an acid shock than the wild-type strain. These results agree with previous studies that suggest the involvement of this gene in both heat and acid resistance in *Ensifer meliloti* cells (Sauviac *et al*., 2007; de Lucena *et al*., 2010). In contrast, no growth reduction was obtained upon salt stress or shock with the Δ*clpB* mutant, suggesting that this chaperone is not involved in salt resistance. Interestingly, this result agrees with our preliminary data on transcriptional analysis of the *clpB* gene (chapter 3) that showed no induction of this gene upon salt shock. Unexpectedly, a higher growth was obtained with the *ΔclpB* mutant when exposed to a salt shock followed by growth under salt stress, compared to the wild-type. This may suggest that, in the absence of ClpB, other mechanisms are activated under salt shock that provided a higher tolerance to the continuous salt stress. However, further studies must be performed to clarify this result.

In symbiosis with chickpea plants, the differences noticed between the *ΔclpB* mutant and the wild-type strains were a 6-8 day delay in nodule appearance and a higher proportion of ineffective root nodules in plants inoculated with the *ΔclpB* mutant. These results indicate that although ClpB is not essential for the establishment of the symbiotic relationship with chickpea plants, it is involved in the nodulation process. These results agree with transcriptomic and proteomic studies in nodules or bacteroids that revealed the overexpression of the *clpB* gene in symbiosis (Djordjevic, 2004; Sarma and Emerich, 2005; Karunakaran *et*

al., 2009). It is possible that during the symbiosis process, rhizobia have to overcome changes in physiological conditions, such as low pH and microaerobiosis, which may lead to protein aggregation, thus requiring the disaggregation function of ClpB chaperone. However, further studies are required to clarify the role of ClpB in rhizobia during symbiosis, since the specific function of this chaperone in the symbiosis *per se* still remains to be unravelled. To our knowledge, this is the first report supporting the involvement of the chaperone ClpB in the symbiotic nodulation process.

Future perspectives

The present study contributed to find potential candidates to be used as inocula for agricultural interests and to the improvement of the symbiotic performance of mesorhizobia with chickpea plants. Moreover, this work brings new insights on the molecular bases of tolerance of mesorhizobia to acid and salt stresses. Nevertheless, many questions have arisen concerning the molecular mechanisms of stress tolerance in rhizobia.

In the transcriptomic and proteomic era, it would be interesting to compare the transcriptome of tolerant and sensitive strains subjected to environmental stresses in order to investigate the genes that may be responsible for the differences on tolerance phenotype. On the other hand, analysis of the genome of strains from the same species with different tolerance phenotypes may also elucidate potential genes in which relies the mechanism of stress tolerance. Sequencing data and functional studies of stress response genes should clarify their roles in stress tolerance and establishment of the symbiosis. Altogether, furthers studies in genome composition and evolution in combination with functional studies are likely to shed more light on the genetics of symbiotic nitrogen fixation and stress response in rhizobia.

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