# Variability in natural populations of Sinorhizobium meliloti in Morocco

**Thami Alami I., Elboutahiri N., Udupa S.M.**

*in*

**Porqueddu C. (ed.), Ríos S. (ed.).**
**The contributions of grasslands to the conservation of Mediterranean biodiversity**

**Zaragoza : CIHEAM / CIBIO / FAO / SEEP**
**Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 92**

**2010**
pages 265-269

# Variability in natural populations of *Sinorhizobium meliloti* in Morocco

**I. Thami-Alami*, N. Elboutahiri* and S.M. Udupa*'****

*Institut National de la Recherche Agronomique (INRA),
Centre Régional de la Recherche Agronomique de Rabat, BP 415, Rabat (Morocco)
**ICARDA-INRA Cooperative Research Project,
International Center for Agricultural Research in the Dry Areas (ICARDA), BP 6299, Rabat (Morocco)
e-mail: thamialami.ma@gmail.com

**Abstract.** In Morocco, alfalfa (*Medicago sativa* L.) is being grown in harsh environments (such as mountains and oasis) and is frequently subjected to abiotic stresses such as salinity, drought and high temperature. Both alfalfa and its nitrogen fixing symbiotic bacteria *Sinorhizobium meliloti* are affected by these abiotic stresses. Improvements in biological nitrogen fixation could be achieved through selection of tolerant strains of *S. meliloti* to these abiotic stresses and inoculating them to the crop and also growing tolerant cultivars. This study examines phenotypic diversity for tolerance to drought, extremes of temperature and soil pH, soil salinity and heavy metals and genotypic diversity at Repetitive Extragenic Pallindromic DNA regions of 157 *Sinorhizobium* isolates, sampled from marginal soils of arid and semi-arid regions of Morocco. The results revealed high degree of phenotypic and genotypic diversity in *Sinorhizobium* populations. Further more, the isolates which showed tolerance to salinity stress also showed tolerance to water stress, indicating direct relationships between these two physiological pathways. High salt and water stress tolerant strains were isolated and tested for their ability to biological nitrogen fixation. Some of the isolated tolerant strains were also efficient nitrogen fixers, under water and salt stress conditions. The Analysis of Molecular Variance revealed that largest proportion of significant genetic variation was distributed within regions than among regions.

**Keywords.** *Sinorhizobium meliloti* – Phenotypic diversity – Genotypic diversity – Abiotic stresses.

***La variabilité des populations naturelles de* Sinorhizobium meliloti *au Maroc***

***Résumé.*** *Au Maroc, les populations locales de luzerne (*Medicago sativa *L.) sont cultivées dans des montagnes et des oasis présahariennes. Dans ces environnements, la luzerne et son microsymbiont* Sinorhizobium meliloti *se heurtent à des stress abiotiques tels que la salinité, la sécheresse et les températures élevées. L'amélioration de la fixation symbiotique pourrait être atteinte grâce à la sélection des souches de* S. meliloti *tolérantes à ces stress et son utilisées dans des essais d'inoculation sous conditions des stress abiotiques. Cette étude examine, d'une part, la diversité phénotypique de 157 isolats de* S. meliloti *échantillonnés à partir des sols marginaux des zones arides et semi-arides du Maroc vis-à-vis de leur tolérance au stress hydrique, aux températures élevées, au pH du sol, à la salinité et aux métaux lourds ainsi que leur résistance intrinsèque aux antibiotiques. Et d'autre part cette étude examine la diversité génétique de ces isolats en utilisant la Rep-PCR. Les résultats révèlent une grande diversité phénotypique et génotypique entre les isolats étudiés. En plus, les isolats qu'ont montré une tolérance au stress salin, sont également tolérants au stress hydrique. Les souches sélectionnées tolérantes au stress salin et hydrique, ont été testé pour leur efficience de fixation de N.*

***Mots-clés.*** Sinorhizobium meliloti – *Diversité phénotypique – Diversité génotypique – Stress abiotiques.*

## I – Introduction

The impact of climate change on biota has recently gained prominence, given the significant concern towards global warming, or local reduction of rainfall in many parts of the world. The resulting land degradation is a major constraint of crop yield worldwide, with salinization, drought and desertification as important consequences (Rozelle *et al.*, 1997).

Leguminous plants are frequently used for cultivation in degraded soil sites of arid and semi arid regions because they can grow in barren soils that are unsuitable for most crops (Pereira *et al.*, 2008). Many biotic and abiotic factors affect the growth and survival of rhizobia in soil and also its leguminous host. In the absence of a legume host, rhizobia manage to survive and hence must have evolved strategies to adapt to diverse environmental conditions (Rinaudi *et al.*, 2006).

The abiotic factors such as high salt, water stress, pH, and temperature stresses affect dinitrogen fixation in root nodules of legumes and hence their productivity. For the good growth of legumes in arid and semi arid regions where fertilizers are unavailable, it seems deemed necessary to plants, being nodulated by an effective strain of rhizobia that tolerate these adverse environmental conditions (Athar and Johnson, 1997).

Alfalfa (*Medicago sativa* L.) is a deep-rooted, perennial legume capable of producing high yields of high-quality forage. Its excellent nutritional value makes this crop ideal for hay and silage. Alfalfa also has the ability to use atmospheric nitrogen ($N_2$) and deposit significant amounts of N in the soil during growth (Zeng *et al.*, 2007). The gram-negative bacteria *Sinorhizobium meliloti* and *S. medicae* are able to interact with roots of alfalfa to form nitrogen-fixing nodules (Elboutahiri *et al.*, 2010). Based on their genetic relationships, it was suggested that *S. medicae* may originate from an ancestral *S. meliloti* population (Biondi *et al.*, 2003). Phenotypic and genotypic diversity of some species of rhizobia are available (Vinuesa *et al.*, 1998; Delorme *et al.*, 2003; Wei *et al.*, 2006), little is known about such diversity in natural populations of *Sinorhizobium* nodulating alfalfa in the marginal soils of arid and semi-arid regions, which are affected by salinity and frequent droughts. In this study, we have sampled *Sinorhizobium* isolates nodulating alfalfa from marginal soils affected by salt and frequent droughts in arid and semi-arid regions of Morocco where alfalfa is being grown with the aims to characterized phenotypic diversity of the sampled isolates for tolerance to water and salinity stresses, extremes of temperature and pH, heavy metals and antibiotics *in vitro* and to estimate genetic diversity and genetic structure of the rhizobia populations in marginal soils of arid and semi-arid regions of Morocco.

## II – Materials and methods

### 1. Physiological characterization

The 157 rhizobia isolates used in this study were isolated either from nodules sampled in the field or from root nodules of young alfalfa plants grown in soil samples collected from the drought and salt affected areas of southern Morocco (isolated by a trapping method). Rhizobia were isolated using standard procedures (Vincent, 1970) from all the collected nodules. All 157 isolates were Gram-negative, fast-growing rhizobia, formed single colonies with diameters of 2-3 mm within 2-3 days on Yeast Extract Mannitol agar (YEM) plates.

The physiological tests were carried out on YEM plates, except for water stress (Elboutahiri *et al.*, 2010). The following treatments (with three replications) were applied: salt tolerance at 0-10% NaCl (at increments of 1%); temperature tolerance at 28, 32, 36, 40 and 44°C; pH tolerance at pH 3.0, 3.5, 4.5, 5.5, 7.0, 9.0 and 9.5; intrinsic antibiotic and heavy metal tolerance were determined on solid YEM medium containing the following filter-sterilized antibiotics or heavy metals (all µg/ml): chloramphenicol (25 and 100), spectinomycin (15 and 50), streptomycin (10 and 25) and tetracycline (10 and 25), $CdCl_2.2H_2O$ (5 and 20), $MnCl_2$ (300), $HgCl_2$ (20) and $ZnCl_2$ (200). Water stress imposed using PEG 6000 in YEM broth at a level of -0.25, -0.5, -1 and -1.5 MPa. After 7 days of incubation at 28°C, the bacterial growth was compared to controls.

### 2. Genotypic characterization

Bacterial DNA was extracted by a simple boiling method. For the rhizobia species assignment,

the 16S rDNA gene of the isolates was amplified using primers fD1 and rD1 with an annealing temperature of 58°C and restricted with *Rsa*I. PCR targeting repetitive DNA sequences (rep-PCR) were performed according to de Bruijn (1992) with minor modifications (Elboutahiri *et al.*, 2009). PCR amplified fragments were electrophoresed in an agarose gel (1.5%) and visualized using ethidium bromide staining (Elboutahiri *et al.*, 2009).

### 3. Data analysis

Comparison of all physiological traits was performed on the basis of growth (1) or no growth (0) for each of the isolate. Comparison of amplified DNA profiles for each of the primers was performed on the basis of the presence (1) or absence (0) of REP and ERIC fragments. The binary data was used for estimation of shared allele distance and the shared allele distance was further used for cluster analysis based on the unweighted paired-group method using arithmetic averages (UPGMA) using the software program PowerMarker Version 3.25 (http://statgen.ncsu.edu/powermarker/).

## III – Results and discussion

The rhizobial species assignment based on *Rsa*I digestion of PCR amplified 16S rDNA of the 157 sampled isolates, assigned 136 isolates as *S. meliloti* and 21 isolates as *S. medicae*. The phenotypic characterization of the sampled 157 isolates for above characters revealed a large degree of variation. For salinity tolerance, we observed a wide variability for tolerance at 171-1711 mM (1-10%) NaCl; even isolates sampled from the same area/region showed variation for NaCl tolerance. 55.41% of the isolates had good tolerance to NaCl (>513 mM), indicating that the rhizobia nodulating alfalfa are more tolerant compared to other rhizobia species (Struffi *et al.*, 1998; Zahran, 1999). Salinity imposes both ionic and osmotic stresses. Indeed, the imposition of any stress to rhizobia results in adaptive responses, which lead to changes in the regular metabolic processes that are then reflected in protein profiles With regard to water stress, 82.16% of the isolates grew at level of -1.5 MPa. The tolerant rhizobia to osmotic stress accumulate the osmolytes, and changes their morphology and dehydration of cells (Buss and Bottomley, 1989; Smith and Smith, 1989). For the most rhizobia, optimum temperature range for growth of culture is 28-31°C, and many cann ot grow even at 37°C (Graham, 1992). At 28, 32 and 36°C, respectively, 100, 96.81 and 87.26% of the isolates grew well. However, at 40°C, only 57.96% of the isolates grew and these highly tolerant isolates were sampled from hot and dry regions of southern Morocco. There was a varied response of the isolates tested to pH. All the isolates tested grew in alkaline pH (pH 9 and 9.5). At very low pH (pH 3.5), only 3.18% of isolates grew normally. Our study further confirmed that the alfalfa rhizobia are acid-sensitive. The sampled isolates showed good tolerance to heavy metals such as Mn, Zn and Cd. The highest number of isolates grew well in 5 µg/ml Cd (92.99%), followed by 300 µg/ml Mn (90.44%) and 200 µg/ml Zn (85.35%); and the growth of almost all isolates was inhibited by Hg (0.69%). Our study showed that *S. meliloti* and *S. medicae* were more tolerant to the heavy metals than the other rhizobia species (Angel *et al.*, 1993). The evaluation of intrinsic resistance to antibiotics showed that most tested isolates (>85%) had high resistance to streptomycin, tetracycline, chloramphenicol and spectinomycin. However, the degree of resistance to antibiotics was higher than in other species of rhizobia (Wei *et al.*, 2003), indicating that *S. meliloti* and *S. medicae* had higher levels of tolerance to these antibiotics.

Isolates with different phenotypes were observed within a sampling location. The cluster analysis based on phenotypic data further revealed that these isolates represented phenotypically diverse populations. The 157 isolates formed 11 clusters. Each cluster showed tolerance to the multiple environmental stresses which are common in marginal soils of arid and semi-arid regions. This kind of phenotypic diversity observed in the rhizobia populations could offer selective advantages in survival and adaptation to these harsh environments.

Rep-PCR analysis revealed high intraspecific diversity among the isolates and classified the

isolates into 148 genotypes. The dendrogram constructed based on the genotype profiles provided more information on the specific variability of the strains. At 84% level of similarity, there were 13 definitely separated and delimited clusters of strains. Each cluster was formed by strains from different areas of collection and with different phenotypic traits.

## Acknowledgements

This research work was supported by a EU-FP 6 project, PERMED.

## References

**Angel J.S., Mc Grath S.P., Chaudri A.M., Chaney R.L. and Giller K.E., 1993.** Inoculation effect on legumes grown in soil previously contaminated with sewage sludge. In: *Soil Biol. Biochem.*, 25, p. 575-580.

**Athar M. and Johnson D.A., 1997.** Effects of drought on the growth and survival of *Rhizobium meliloti* strains from Pakistan and Nepal. In: *Journal of Arid Environment*, 35, p. 335-340.

**Beringer J.E., 1974.** R factor transfer in *Rhizobium leguminosarum.* In: *J. Gen. Microbiol.*, 84, p. 188-198.

**Biondi E., Pilli E., Giuntini E., Roumiantseva M., Andronov E., Onichtchouk O.P., Kurchak O.N., Simarov B.V., Dzyubenko N.I., Mengoni A. and Bazzicalupo M., 2003.** Genetic relationship of *Sinorhizobium meliloti* and *Sinorhizobium medicae* strains isolated from Caucasian region. In: *FEMS Microbiol. Lett.*, 220, p. 207-213.

**Buss M.D. and Bottomley P.J., 1989.** Growth and nodulation responses of *Rhizobium meliloti* to water stress induced by permeating and nonpermeating solutes. In: *Appl. Environ. Microbiol.*, 55, p. 2431-2436.

**de Bruijn F.J., 1992.** Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergenic Consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. In: *Appl. Environ. Microbiol.*, 58, p. 2180-2187.

**Delorme T.A., Gagliardi J.V., Angle J.S., Van Berkum P. and Chaney R.L., 2003.** Phenotypic and genetic diversity of rhizobia isolated from nodules of clover grown in a Zinc and Cadmium contaminated soil. In: *Soil Soc. Am. J.*, 67, p. 1746-1754.

**Elboutahiri N., Thami-Alami I. and Udupa S.M., 2010.** Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco. In: *BMC Microbiology*, 10, p. 15.

**Elboutahiri N., Thami-Alami I., Zaïd E. and Udupa S.M., 2009.** Genotypic characterization of indigenous *Sinorhizobium meliloti* and *Rhizobium sullae* by rep-PCR, RAPD and ARDRA analyses. In: *African Journal of Biotechnology*, 8, p. 979-985.

**Graham P.H., 1992.** Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. In: *Can. J. Microbiol.*, 38, p. 475-484.

**Pereira S.I.A., Lima A.I.G. and Figueira E.M.A.P., 2008.** *Rhizobium leguminosarum* isolated from agricultural ecosystems subjected to different climatic influences: The relation between genetic diversity, salt tolerance and nodulation efficiency. In: *Soil Ecology Research Developments*. Tian-Xiao Liu (ed.), p. 247-263.

**Rinaudi L., Fujishige N.A., Hirsch A.M., Banchio E., Zorreguieta A. and Giordano W., 2006.** Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. In: *Research in Microbiology*, 157, p. 867-875.

**Rozelle S., Park A., Huang J. and Jin H., 1996.** Dilemmas in reforming state-market relations in China's grain economy. Mimero, April 1996.

**Struffi P., Corich V., Giacomini A., Benguedouar A., Squartini A., Casella S. and Nuti M.P., 1998.** Metabolic proprieties, stress tolerance and macromolecular profiles of rhizobia nodulating *Hedysarum coronarium*. In: *J. Appl. Microbiol.*, 84, p. 81-89.

**Vincent J.M., 1970.** *A Manual for the Practical Study of Root Nodule Bacteria*. IBP Handbook, No. 15. Oxford: Blackwell Scientific Publications Ltd.

**Vinuesa P., Rademaker J.L.W., de Bruijin F.J. and Werner D., 1998.** Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. In: *Appl. Environ. Microbiol.*, 64, p. 2096-2104.

**Wei G.H., Tan Z.Y., Zhu M.E., Wang E.T., Han S.Z. and Chen W.X., 2003.** Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the loess Plateau

of China and description of *Rhizobium loessense* sp. In: *Nov. Int. J. Syst. Evol. Microbiol.*, 53, p. 1575-1583.

**Wei G.H., Zhang Z.X., Chen C., Chen W.M. and Ju W.T., 2006.** Phenotypic and genetic diversity of rhizobia isolated from nodules of the legume genera *Astragalus*, *Lespedeza* and *Hedysarum* in northwestern China. In: *Microbiological Research*, 163, p. 651-662.

**Zahran H.H., 1999.** Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in arid climate. In: *Micobiol. Mol. Biol. Rev.*, 63, p. 968-989.

**Zeng Z.H., Chen W.X., Hu Y.G., Sui X.H. and Chen D.M., 2007.** Screening of highly effective *Sinorhizobium meliloti* strains for 'Vector' alfalfa and testing of its competitive nodulation ability in the field. In: *Pedosphere*, 17, p. 219-228.