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Inefficient nodulation of chickpea (*Cicer arietinum* L.) in the arid and Saharan climates in Tunisia by *Sinorhizobium meliloti* biovar *medicaginis*

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Abstract - The aim of this work is to study the genetic diversity and the symbiotic effectiveness of the natural populations of rhizobia nodulating chickpea (*Cicer arietinum* L.) in six locations of South Tunisia, where chickpea had never been cultivated. Nodules were observed only in the two soil samples from Gafsa (0.8 nodules per plant) and Tataouine (2 nodules per plant). PCR-RFLP typing of 16S rRNA genes of 42 isolates indicated that all analysed strains showed the same ribotype as the reference strain *Sinorhizobium meliloti* RCR2011. These isolates induced ineffective nodules on chickpea and *Medicago sativa*; however nodules on *Medicago laciniata* were effective. Analysis of the symbiotic diversity by PCR-RFLP of the *nifDK* spacer suggested that all chickpea isolates from the South belong to the biovar *medicaginis* of *S. meliloti.* The present paper is, to our knowledge, the first report showing that chickpea is selectively nodulated under soil conditions by a specific biovar of *S. meliloti* showing specificity to *M. laciniata*. The specificity of this interaction as well the impact of this inefficient nodulation on chickpea cultivation needs to be investigated further.

Key words: arid climate, genetic diversity, *Medicago laciniata*, *nifDK* spacer, rhizobia.

INTRODUCTION

Rhizobia are useful bacterial resources because of their symbiotic nitrogen-fixing ability. They form nodules on roots or stems of many plant legumes and reduce N_2 to ammonia, which can be used by the host plant. The classification of rhizobia formerly based mainly on symbiotic characteristics is in constant evolution. Cross-inoculation studies revealed that chickpea rhizobia are highly host-specific (Gaur and Sen, 1979). Two species of rhizobia have been described to specifically nodulate chickpea, *Mesorhizobium ciceri* (Nour *et al*., 1994) and *Mesorhizobium mediterraneum* (Nour *et al*., 1995). Recently, high species diversity was found within populations of an agricultural region in the South of Portugal (Laranjo *et al*., 2004). Besides the expected *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*, some isolates were close to *Mesorhizobium loti* or *Mesorhizobium tianshanense* and some formed a clade that may represent a new species (Laranjo *et al*., 2004). In Tunisia, the characterisation of the natural populations of rhizobia nodulating chickpea in soils with humid and subhumid climates in the North of the country showed that only 5% of the nodule isolates were assigned to *Mesorhizobium mediterraneum*. The remaining isolates (95%) were all assigned to *Sinorhizobium medicae*. However, this symbiotic association was ineffective in nitrogen fixation (Aouani *et al*., 2001).

Chickpea is a new crop with highest economic value in the semiarid regions. It is a crop suitable for cereal-pulse rotation systems. With movement of winter-planted chickpea into drier areas of the Mediterranean region, it is expected that locations previously not planted with chickpea will be utilised. In these new areas, inoculation may be necessary if the benefit of winter planting is to be realised. Evaluation of inoculation requirement is an essential component of winter-sown chickpea introduction. Inoculation assays with non-native strains of chickpea rhizobia were used to improve the production of this crop in Northern Tunisia. However, the yield obtained was low (unpublished results). The yield is not only limited by the N_2-fixing potential of the chickpea-rhizobia symbiosis but also by the non-adaptability of both symbionts to the osmotic stress prevailing in these areas. The South Tunisia is a Mediterranean region with arid and Saharan climate and soils are poor in organic matter. In these regions, only few studies addressing the genetic diversity of wild legume rhizobia (Zakhia *et al*., 2004; Ben Romdhane *et al*., 2005, 2006) and grain legume rhizobia (Mnasri *et al*., 2006) were done. Mnasri *et al*. (2006) isolated a highly salt-tolerant and effective strain nodulating common bean from a Saharan soil in the South of Tunisia. This strain constituted a new biovar (bv. *mediterranense*) inside *Sinorhizobium meliloti* (Mnasri *et al*., 2006) and is currently used as inoculant for common bean. This finding underlines the importance of the arid regions as a source of well adapted strains to the prevailing extreme environmental stresses such as drought, high soil temperature and salinity.

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The aim of this research is to characterise rhizobia nodulating chickpea from the arid and Saharan soils in Southern Tunisia and to estimate their symbiotic effectiveness.

MATERIAL AND METHODS

Soil samples and plant material. Soil samples were collected from six different sites in Southern Tunisia (Fig. 1). Some physical and bioclimatic characteristics of the collected soils are shown in Table 1. Soil samples were taken from the 5-20 cm depth and filled in plastic pots of 5 Kg. Four pots were considered for each soil. Precautions were taken during sampling and handling of each soil to avoid cross contamination between soils from different sites. The soil samples were then transferred to the greenhouse. Certified commercial chickpea seeds from the winter cultivar (cv. Chetoui) were used. Five surface-sterilised seeds were sown in each pot. Pots were then covered by a sterile gravel layer to minimise contamination. Irrigation was applied according to plant need through a watering tube installed in each pot. Plants were harvested and scored for nodulation at flowering (seven weeks after emergence).

Bacterial isolates. Rhizobia isolation was performed using the standard procedure described by Vincent (1970). The purity of isolates was checked by repeated striking on YMA medium supplemented with Congo red. One isolate was kept from each single nodule. *Sinorhizobium meliloti* isolates from *Medicago laciniata* and common bean (Mnasri *et al*., 2006) and *S. medicae* isolates from chickpea (Aouani *et al*., 2001) from our laboratory collection were used for comparison. The reference strains *S. meliloti* RCR2011 and *S. medicae* M104 were also included for *nif*DK patterning. All strains were maintained on YMA slants at 4 °C or in 25% (v/v) glycerol at -80 °C for long-term storage.

Plant infection and symbiotic effectiveness tests. All isolates were tested for nodulation ability on their original host. Surface sterilised seeds of *Cicer arietinum* cv. Chetoui were pre-germinated on agar plates (9 g l^{-1}). The experiment was conducted in 0.5 litres plastic pots on sterile perlite. One seedling was deposited in each pot. The seedlings were inoculated with 1 ml (approximately 10^9 cells) of the rhizobial suspension. There were four replicates for each isolate. Non-inoculated controls were also included. The plants were watered with N-free nutrient solution and grown in the growth chamber at 25 °C. The roots were checked for the presence of nodules after seven weeks. Nodulation tests on *Medicago sativa* and *M. laciniata* were carried on agar slopes as described by Vincent (1970). Symbiotic effectiveness of isolates was evaluated according to size and colour of nodules and vigour of plants. Presence of pink, red or brown pigmentation was considered as indicative of nitrogen symbiotic fixation. However, white nodules and yellow leaves were considered as indicative of bad efficiency.

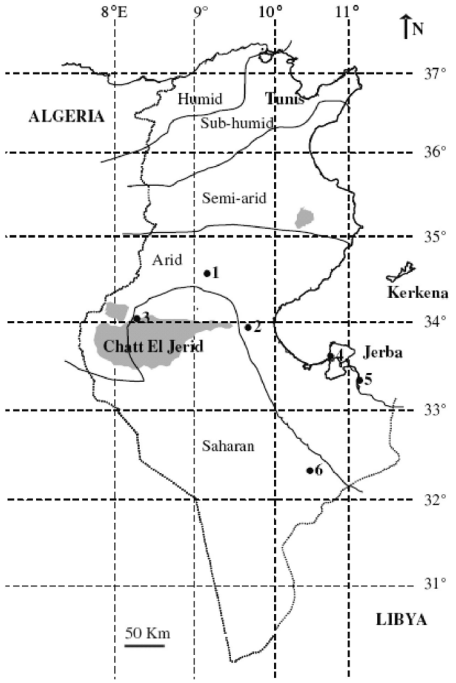

FIG. 1 - Map of Tunisia showing the sites where soil samples were collected. 1, Gafsa; 2, El Hamma; 3, Deguache; 4, Jerba; 5, Zarzis; 6, Tataouine.

PCR-RFLP of 16S rRNA genes and *nifDK* spacers. The prokaryotic specific primers used for 16S rRNA gene amplification were fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGCTTAAGGTGATCCAG-CC-3′) (Weisburg *et al*., 1991). PCR was conducted on cells treated with proteinase K according to the procedure described by Mhamdi *et al*. (2002). The intergenic region between *nifD* and *nifK* genes was amplified by using oligonucleotide primers FGPD 807: 5′-CAC TGC TAC CGG TCG ATG AA-3′ and FGPK 597: 5′-GTG GCT GCC CAC GAA GAA GCT TGG NGT GTG-3′ which were designed from conserved regions of the *nifD* 3′-portion and the *nifK* 5′-portion, respectively (Jamann *et al*., 1993). The PCR was carried out with bacterial cell suspensions as tem-

TABLE 1 - Some physical and bioclimatic characteristics of the soils used in this study

Site	Climate	Annual rainfall (mm)	Altitude (m)	Texture	pH	EC* (mS/cm)	Organic matter (%)
Djerba	Arid	100-200	2	Loamy sand	8.3	0.6	0.7
Gafsa	Arid	100-200	400	Sandy loam	8	0.6	1.4
Zarzis	Arid	100-200	2	Loamy sand	nd	nd	nd
El Hamma	Arid	< 100	20	Loamy sand	8.3	11.4	0.5
Deguache	Saharan	< 100	25	Sand	7	2.5	1.1
Tataouine	Saharan	< 100	600	Sand	7.7	0.5	0.1

* EC, electrical conductivity; nd, not determined.

plate DNAs as previously described by Jebara *et al*. (2001). Aliquots of PCR products were digested with restriction endonucleases (Pharmacia Biotech) as specified by the manufacturer in a total volume of 10 µl. Digestion was performed by five enzymes, *Msp*I, *Nde*II, *Cfo*I, *Hae*III and *Rsa*I, for 16S rDNA and two enzymes, *Hae*III, *Msp*I, for *nifD-nifK* spacer. The enzymes *Hae*III and *Msp*I were the most discriminative and were sufficient to describe all the *nifDK* polymorphism according to Villegas *et al*. (2006), data not shown. The restriction fragments were separated by horizontal electrophoresis in 3% (w/v) ultra-pure agarose (FMC Bioproducts) with TAE buffer. Species assignation was done according to 16S rDNA typing and comparison with the published database of mapped restriction sites in the 16S RNA genes of rhizobia (Laguerre *et al*., 1997).

RESULTS AND DISCUSSION

Six soil samples originating from South Tunisia were tested for chickpea nodulation. All these soils belonged to the Arid and Saharan bioclimatic stages and received limited annual rainfalls (< 200 mm). All these soils were slightly alkaline but with variable salinity as indicated by the electrical conductivity (EC) measures (Table 1). The EC values for non-saline soils in Tunisia usually vary between 0.3 and 0.5 mS/cm. The soils from Deguache and El Hamma are considered saline and highly saline respectively. Chickpea had not been cultivated in all these soils and nodulation was very poor or absent. Nodules were observed only in the two soil samples from Gafsa (0.8 nodules per plant) and Tataouine (2 nodules per plant). These two soils are not saline and are located at higher altitudes, which may contribute to better moistening. The absence of nodulation in the other soils is probably the consequence of the severe bioclimatic conditions (salinity, drought and temperature) that affected survival of rhizobial populations (Zahran, 2001). The absence of the host plant is another important component that may interfere on diversity and density of the natural population of rhizobia (Martinez-Romero and Caballero-Mellado, 1996).

Forty-two isolates were obtained from 42 different nodules, 30 from Tataouine and 12 from Gafsa. All of these isolates showed the same colony morphology and growth rate on YMA medium. They were fast growing rhizobia and formed transparent colonies with 3 mm in diameter after 3 days of incubation at 28 °C. However, *Mesorhizobium ciceri* (Nour *et al*., 1994) and *Mesorhizobium mediterraneum* (Nour *et al*., 1995), which are the specific chickpea symbionts, are moderately slow growing rhizobia.

The molecular characterisation by PCR-RFLP of 16S rRNA genes showed that the 42 isolates analysed had the same ribotype as the reference strain of *Sinorhizobium meliloti* RCR2011 and differed from that of the reference strain *S. medicae* M104 by the *Rsa*I pattern. This enzyme was previously used to differentiate between the species *S. meliloti* and *S. medicae* (Laguerre *et al*., 1997).

During the previous studies conducted in our laboratory, more than 95% of chickpea isolates from the North of the country were assigned to *S. medicae* (Aouani *et al*., 2001). This differential nodulation of chickpea by *S. medicae* and *S. meliloti* between the North and the South of the country is supported by the work of Zribi *et al*. (2004) on *Medicago truncatula*. They found that *S. medicae* was restricted to the superior semi-arid, sub-humid and littoral areas of the country (North) where the soil was generally silty, while *S. meliloti* was widespread all over the country (Zribi *et al*., 2004). The introduction of a new crop may have impact on native bacterial populations, but the extent of these effects has not been evaluated. In some cases, the introduced plants may recruit local "opportunistic bacteria" that have cross specificity to nodulate these plants (Martinez-Romero and Caballero-Mellado, 1996). This seems to be the case with common bean (Mhamdi *et al*., 2002) and chickpea in Tunisia (Aouani *et al*., 2001, this study), in both cases nodules were induced by *Sinorhizobium* strains. In Moroccan soils, Maatallah *et al*. (2002) showed that chickpea rhizobia are both phenotypically and genetically diverse. Most of these rhizobia belong to the *Mesorhizobium* genus as expected. However, some strains originating from a particular soil appeared to have 16S rRNA genes similar to *Sinorhizobium* as well as very distinct auxanographic characteristics compared with *Mesorhizobium* isolates. However, the enzymes used by these authors, *Alu*I, *Hha*I, *Hinf*I, *Msp*I *and Taq*I, could not differentiate between the closely related species, *S. meliloti* and *S. medicae*.

Sinorhizobium meliloti is known to have the ability to form symbiotic relationship with many legumes. It was first isolated from three related genera, *Medicago*, *Melilotus* and *Trigonella* (Jordan, 1984). Later, several studies have shown that *S. meliloti* can spontaneously nodulate many leguminous plants including *Phaseolus vulgaris* (Bromfield and Barran, 1990), *Glycine max* (Gao and Yang, 1995), *Lotus roudairei* (Jeder *et al*., 1996), *Acacia* ssp. (Khbaya *et al*., 1998; Ben Romdhane *et al*., 2006), and some wild legumes in Tunisia: *Argyrolobium uniflorum*, *Hedysarum carnosum*, *Hippocrepis bicontorta* and *Ononis* subsp. *filifolia* (Zakhia *et al*., 2004).

Sinorhizobium meliloti is a very common species of rhizobia in Tunisian soils. It was isolated from *Medicago* species in almost all the territory (Jebara *et al*., 2001; Zribi *et al*., 2004). Recently, Villegas *et al*. (2006) showed that nitrogen-fixing rhizobia on *M. laciniata* differed markedly from the other *S. meliloti* or *S. medicae* isolates and references in their symbiotic traits such as *nifDK* RFLP diversity, *nodA* sequences and nitrogen fixation effectiveness with three other different annual *Medicago* species, *M. truncatula*, *M. polymorpha* and *M. laciniata*. These authors proposed the creation of two new biovars inside *S. meliloti.* The biovar *meliloti* which encompasses the classically known *S. meliloti* strains represented by the strains ATCC9930^T and RCR2011, efficient on *M. sativa* and *M. truncatula*, and forming no nodules or only ineffective ones on *M. laciniata*, and the biovar *medicaginis* which clusters *Sinorhizobium* strains showing nitrogen fixation specificity with *M. laciniata* and *M. sauvagei*, but forms ineffective nodules on *M. sativa* (Villegas *et al.,* 2006). More recently, Mnasri *et al*. (2006) proposed the creation of a third biovar inside *S. meliloti* for isolates recovered from common bean nodules grown on soil samples originating from the Tunisian oases. This biovar was defined on the basis of symbiotic specificity to common bean and phylogenic analyses of *nif*H and *nod*C genes. The isolates of this biovar did not induce nodules on *Medicago*, but induce highly nitrogen-fixing nodules on common bean (Mnasri *et al*., 2006). The question that remains asked is about the specificity of interaction between the *S. meliloti* isolates recovered in this study and *C. arietinum*, to which biovar they belong?

Since the *nifDK* intergenic spacer in symbiotic nitrogen-fixing bacteria is larger and more variable than the other symbiotic genes, it was used for distinguishing closely related strains in PCR-RFLP analyses (Jamann *et al*., 1993). The *nifDK* intergenic region of the strains was successfully amplified, yielding the expected fragment of about 1400 bp (Jamann *et al*., 1993). The independent cleavage by the two enzymes *Msp*I and *Hae*III showed that chickpea isolates from the South belonged to two symbiotic types, N9 (*Hae*III a1 *Msp*I a2) and N10 (*Hae*III b1 and *Msp*I b2) (Fig. 2). These two types were also found with *M. laciniata* isolates from Gafsa and Tataouine, and had been previously found with *M. laciniata* isolates from different locations including soils from Tunisia (Villegas *et al*., 2006). Four isolates of *S. medicae* recovered from chickpea nodules by Aouani *et al*. (2001) were also tested and showed all the same *nif*DK type, N9.

Thirty out of 42 different isolates were tested on their original host, *C. arietinum*, and on two *Medicago* species, *M. sativa* and *M. laciniata* (Table 2). All of our isolates induced nodules on the three hosts; however, nodules induced on chickpea and *M. sativa* were small and white and the plants showed yellow leaves, suggesting that they were ineffective in nitrogen fixation. By contrast, *M. laciniata* plants showed dark-green leaves and pink nodules suggesting that the tested isolates were effective in nitrogen fixation with *M. laciniata.*

In the southern soils where chickpea was inefficiently nodulated, different *Medicago* species (*M. truncatula*, *M. sativa*, *M. ciliaris,* …) were observed in addition to *M. laciniata* and were naturally nodulated. However, in the other regions where chickpea was not nodulated, *M. laciniata* was rare or not found and the soils were degraded and/or with high salinity. The work of Jebara *et al*. (2001) conducted on a set of four *Medicago* species revealed high *nifDK* RFLP diversity in the Tunisian soils. On the other hand, analysis of *nifDK* diversity of *M. truncatula* isolates (K. Zribi, Pers. Com.) indicated the presence of other *nifDK* types in Gafsa than those trapped by Chickpea (N9 and N10). It seems likely that in soil conditions, chickpea is selectively nodulated by *S. meliloti* strains showing specificity to *M. laciniata* and thus belong to the biovar *medicaginis*. Interestingly, the *S. medicae* isolates recovered from chickpea by Aouani *et al*. (2001) in the North of the country showed also the same *nifDK* profile as chickpea isolates from the South suggesting symbiotic plasmid transfer between *S. meliloti* and *S. medicae*. There is evidence that lateral transfer of symbiotic genes did occur during the course of evolution among rhizobia belonging to different species and even different genera (Segovia *et al*. 1993; Amarger *et al*. 1997; Laguerre *et al*. 2001). It seems that the genetic variability of chickpea root nodule bacteria is rather high and more work is needed to precisely locate the taxonomic position and the symbiotic specificity of these bacteria.

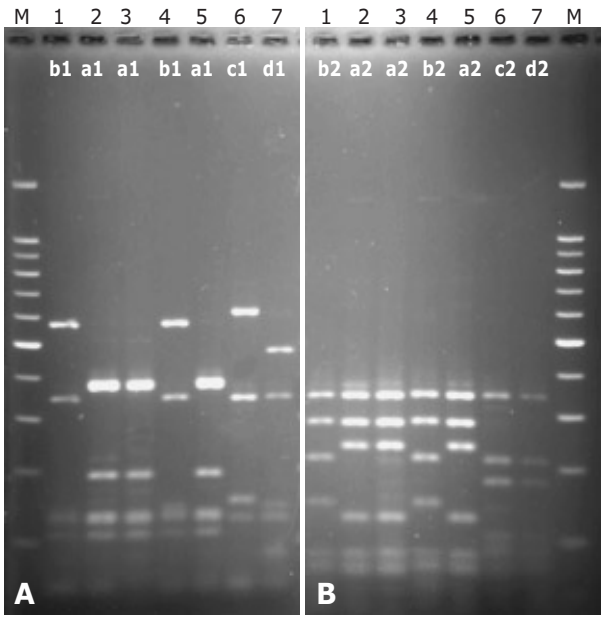

FIG. 2 - Ethidium bromide stained gels showing restriction patterns of PCR-amplified fragment of the *nifDK* region digested by *Hae*III (A) and *Msp*I (B). Lanes 1 and 2, *Sinorhizobium meliloti* isolates from chickpea (this study); lane 3, *Sinorhizobium medicae* isolates from chickpea (Aouani *et al.*, 2001); lanes 4 and 5, *Medicago laciniata* isolates from the South of Tunisia; lane 6, *S. medicae* M104; lane 7, *S. meliloti* RCR2001; M, molecular mass marker 100 bp (Promega).

The present paper is, to our knowledge, the first report indicating that the *S. meliloti* strains that spontaneously nodulate chickpea, albeit ineffectively, show specificity to *M. laciniata* and thus belong to the newly proposed biovar, bv. *medicaginis*. The specificity and the biological implications of this interaction on chickpea need to be explored further through co-inoculation experiments with the specific symbionts, *Mesorhizobium mediterraneum* and *Mesorhizobium ciceri*. The relative competition for chickpea nodulation between *Sinorhizobium* and *Mesorhizobium* could be drastic

TABLE 2 - Symbiotic diversity of chickpea isolates from South Tunisia

Origin	Species[a]	*nifDK*			Isolates	Effectiveness on[c]			Biovar[d]
	(16S rDNA)	*Hae*III	*Msp*I	Type[b]	(no.)	*Cicer arietinum*	*Medicago sativa*	*Medicago laciniata*	
Gafsa	*Sinorhizobium*	a1	a2	N9	2	-	-	+	*medicaginis*
	meliloti	b1	b2	N10	8	-	-	+	*medicaginis*
Tataouine	*Sinorhizobium*	a1	a2	N9	16	-	-	+	*medicaginis*
	meliloti	b1	b2	N10	4	-	-	+	*medicaginis*

[a] The species assignation was done according to 16S rDNA typing by PCR-RFLP using five restriction endonucleases including *Rsa*I.
[b] The *nifDK* types N9 and N10 are those recorded by Villegas *et al*. (2006) among *Medicago laciniata* isolates.
[c] The effectiveness was estimated according to plant vigour and nodule colour: +, effective symbiosis; -, ineffective symbiosis.
[d] The biovar was determined on the basis of *nifDK* type and effectiveness range according to Villegas *et al*. (2006).

for the success of inoculation and deserves a special care. Further taxonomic and phylogenetic studies will be helpful to elucidate co-evolution between *S. meliloti* and its different hosts including *Medicago*, *Phaseolus vulgaris*, *Cicer arietinum*, *Glycine max*, *Acacia*, ...

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