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Characterization of rhizobia nodulating chickpea in Tunisia

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Abstract – One hundred and fifty rhizobia nodulating chickpea (*Cicer arietinum* L.) were isolated from soils sampled in different regions of Tunisia. Variability in the time at which nodules appeared after plant inoculation was observed among these isolates. Five isolates induced nodules two weeks after inoculation, whereas, with the remaining 145 isolates, nodules were observed after more than four weeks. On the basis of PCR/RFLP analysis of 16S rDNA, isolates from the first group were classified as *Mesorhizobium mediterraneum*, and isolates from the second group as *Sinorhizobium medicae*. The respective taxonomic position of both types of isolates was confirmed by their symbiotic properties. *M. mediterraneum* isolates did not nodulate *Medicago sativa* and formed effective nodules on *C. arietinum* while *S. medicae* isolates were able to form effective nodules on *M. sativa* but formed ineffective nodules on *C. arietinum*, as did reference strains of the species.

Cicer arietinum* / nodulation / 16SrDNA-PCR/RFLP / *Mesorhizobium* / *Sinorhizobium

Résumé – Caractérisation des rhizobia nodulant le pois chiche en Tunisie. Cent cinquante rhizobia nodulant le pois chiche (*Cicer arietinum* L.) ont été isolés à partir de sols échantillonnés dans différentes régions de Tunisie. L'inoculation de la plante hôte avec ces isolats montre une variabilité dans le temps d'apparition des premières nodosités. Cinq isolats induisent des nodosités deux semaines après inoculation alors que pour les 145 isolats restants les nodosités ne sont observées qu'après au moins quatre semaines. L'étude par PCR/RFLP de l'ADNr 16S a permis de rattacher les isolats du premier groupe à l'espèce *Mesorhizobium mediterraneum* et ceux du second groupe à l'espèce *Sinorhizobium medicae*. La position taxonomique des isolats a été confirmée par leurs propriétés symbiotiques. Les isolats de *M. mediterraneum* ne nodulent pas *Medicago sativa* mais forment une symbiose fixatrice avec *C. arietinum*, ceux de *S. medicae*, comme les souches références de l'espèce, forment des nodosités efficientes avec *M. sativa* et inefficientes avec *C. arietinum*.

Cicer arietinum* / nodulation / 16SrDNA-PCR/RFLP / *Mesorhizobium* / *Sinorhizobium

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most cultivated grain legume in the world. In Tunisia, it shares the first rank with faba bean, each crop being grown on 35 000 ha, but it gives low yields that never reach 0.7 $t \cdot ha^{-1}$. The national production does not cover the consumption, thus importation is unavoidable. To increase production, extension of surfaces cultivated with chickpea, presently located in the north of the

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country, is contemplated. This could be achieved by introduction of winter cultivars in areas with more than 400 mm of rainfall in the centre and south of the country [4].

In a previous study [1], we showed that the nodulation of chickpeas in Tunisia varied with the sites. Nodules were not always observed. When they were present, their number never exceeded 10 per plant and most of them were of small size and apparently ineffective. This suggested that compatible effective rhizobia were not present in the soils, which limited symbiotic nitrogen fixation and, as a consequence, the productivity of this legume crop. In order to verify this hypothesis, the presence of rhizobia nodulating chickpea was checked in soils sampled in regions of Tunisia where chickpea is presently or is likely to be grown. When present, rhizobia were isolated, identified, and characterized by their symbiotic properties.

2. MATERIALS AND METHODS

2. 1. Reference strains and growth conditions

Mesorhizobium mediterraneum 918, *M. ciceri* UPMCa7^T were obtained from Ruiz-Argüeso; *Sinorhizobium medicae* WSM533 and M104 and *S. meliloti* 2011 from Cleyet Marel**.** These strains and the nodule isolates were grown on YMA [12]**.**

2.2. Isolation of rhizobia

Soil samples were collected in 76 sites located in the north and the centre of Tunisia (Fig. 1). Surface sterilized seeds of *C. arietinum* cv. Amdoun I were planted at the rate of one per pot in 500 ml pots filled with soil. We prepared 4 pots for each sample of soil. The plants were grown in a greenhouse with a minimum-maximum temperature of 15–28 °C. Water was added when necessary. After 6 weeks of growth, the roots were checked for the presence of nodules. Isolation of rhizobia from nodules was done as described by Vincent [12].

2.3. Nodulation and effectiveness tests

The ability of each isolate to nodulate its host of origin was verified in two series of tests. Surface sterilized seeds of *C. arietinum* cv. Amdoun I were pregerminated on water-agar plates (9 g·l^{-1}). In the first test, cotton-plugged test-tubes containing a mixture of 4 parts of coarse sand, one part of vermiculite and 50 ml of Beck's nutrient solution [2] were sterilized by autoclaving, and

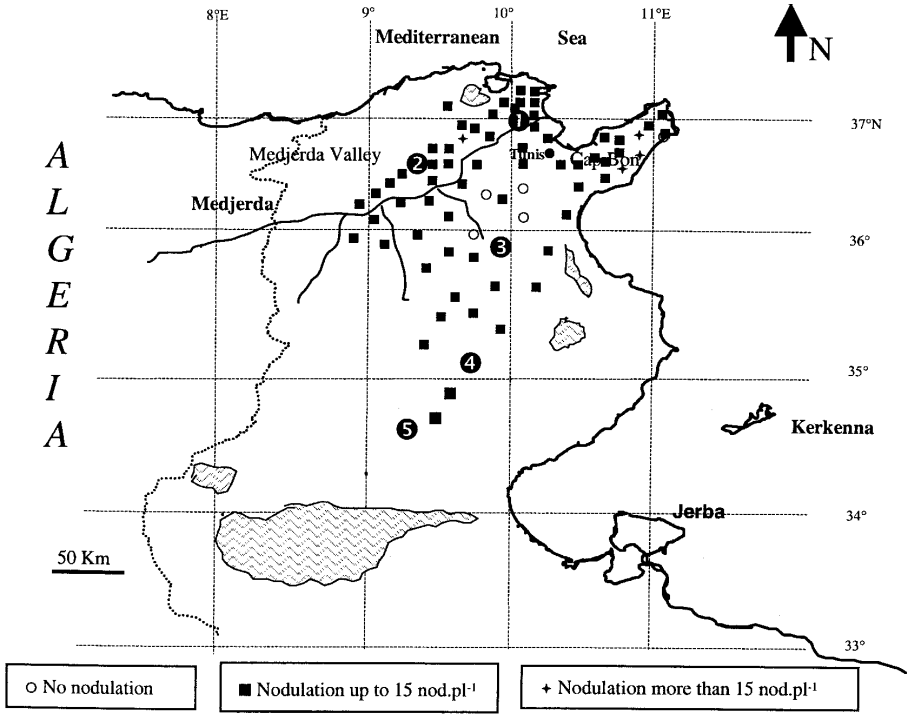

Figure 1. Areas where chickpeas are cultivated and sites prospected for nodulation and used for isolation of rhizobia nodulating chickpea.
❶ ❷ ❸ ❹ ❺: sites where strains 22a12, 48.2, 72c11, 74c10 and 75c21 were isolated from.

one seedling was deposited in each tube. The roots were examined weekly for the formation of nodules. In the second test, each seedling was planted in a 500 ml plastic pot containing heat-sterilized gravel, and the roots were checked for the presence of nodules after 7 weeks. In both tests, each isolate was inoculated into two plants and four plants were left uninoculated for control. Inoculation was performed 2 or 3 days after planting with 10^9–10^{10} rhizobial cells per plant. The plants were watered with Beck's nutrient solution [2] and grown in greenhouse conditions. Nodulation tests on *Medicago sativa* were carried on agar slopes as described by Vincent [12]. For effectiveness tests, the chickpea plants were grown as in the second nodulation test but we used 12 replicates per treatment. Aerial parts were harvested after 7 weeks, dried and weighed.

2.4. Genotypic characterization

PCR/RFLP of 16S rDNA was carried out according to Nour et al. [9] using five restriction enzymes: *Rsa*I, *Cfo*I, *Nde*II, *Msp*I and *Alu*I. Plasmid profiling was done according to the method of Eckhardt [3] as modified by Hynes et al. [5].

3. RESULTS

Soils were sampled from 76 different sites located in the north and centre of the country and were examined for nodulation of chickpea (Fig. 1). The sampled regions included areas where chickpea is traditionally cultivated as well as areas where it has never been cultivated. No nodule could be detected on the roots of the chickpeas grown in 6 soils (Fig. 1). These soils were hydromorphic or very sandy soils. The chickpeas grown on the 70 remaining soils were all nodulated. The nodules were heterogeneous in size and color and their number did not exceed 15 per plant, except for plants grown on 5 soils that presented up to 20 nodules. For isolation of rhizobia, 32 soils were chosen as representing the different climatic areas, from humid to semi-arid. One to 8 nodule isolates were recovered from the plants grown on each of these 32 soils, which gave a total of 150 isolates.

The results of the different tests that we performed for the characterization of these isolates are shown in Table I. When the 150 isolates were tested for their ability to nodulate their host of origin, we could distinguish two groups of isolates according to the time at which nodules appeared and to their size and color. For 5 isolates (group I), recovered from 5 soils, sampled in the north of the Medjerda valley and in CapBon where chickpea is traditionally cultivated, we observed nodules two weeks after inoculation. Two weeks later these nodules were 3 to 4 mm long and had turned red which indicated that haemoglobin had been synthesized and that nitrogen fixation had taken place. For the 145 remaining isolates (group II), recovered from every soil, nodules did not appear before four weeks and remained small and white. No nodules could be found on the roots of the non-inoculated plants which showed that the conditions had remained sterile for the duration of the experiment. These isolates were more mucous and grew faster than the isolates from group I. In the second test carried put in pots, that allowed better plant growth, the aerial parts of plants inoculated with group I isolates could be differentiated from those inoculated with group II isolates and from the uninoculated control plants by their color, which appeared much greener and suggested that these isolates were effective. Differences in nodules were also seen, the nodules from plants inoculated with group I isolates were bigger than those of the other group and they were red in color while those from group II were white. The roots of the control plants did not bear any nodules.

Table I. Characterization of rhizobia nodulating chickpea in Tunisian soils. The number of isolates tested is given in parenthesis.

	Group I	Group II
Time at which nodule appeared after inoculation	2 weeks	4 weeks
	(5)	(145)
Ribotype by PCR/RFLP of 16S rDNA	*M. mediterraneum*	*S. medicae*
	(5)	(61)
Presence of a plasmid band of size > 1000 kb	–	+
	(5)	(27)
Nodulation of *M. sativa*	–	+
	(5)	(10)
Geographic distribution	Only in regions where chickpea is traditionally cultivated	In all prospected sites

PCR/RFLP analysis of 16S rDNA showed that the profiles obtained with the five enzymes tested for the 5 isolates of group I were identical to those of the type strain of *Mesorhizobium mediterraneum.* Since this type of analysis has been shown to allow differentiation between *M. mediterraneum* and other species of *Mesorhizobium* including *M. ciceri* [8], we assigned the 5 isolates of group I to *Mesorhizobium mediterraneum*. The 61 isolates of group II that we tested, and which represented one or two isolates per soil, all showed restriction profiles identical to those of *Sinorhizobium medicae* and were assigned on this basis to *S. medicae*.

To confirm the taxonomic position of the two types of isolates, we then compared a sample of these isolates and the two reference strains, *M. mediterraneum* 918 and *S. medicae* WSM533, on two additional characteristics allowing differentiation between the species: the presence of a megaplasmid in *S. medicae* strains, and the capacity to nodulate *M. sativa*. Plasmid profiles analysis showed that a plasmid band of more than 1000 kb was present in the 27 isolates of group II tested, but absent in the isolates of group I. When checked for their ability to nodulate *M. sativa*, the 10 isolates sampled from group II were shown to be capable of inducing effective nodules on this host whereas group I isolates did not form any nodule. These results agree with the taxonomic position assigned to the isolates by the 16S rDNA-PCR/RFLP analysis. In order to see whether the ability to nodulate chickpea was a common feature among *S. medicae* strains and was also found in strains of the closely related species, *S. meliloti*, nodulation tests were performed with two reference strains of *S. medicae*, WSM 533 and M104, and one of *S. meliloti*, 2011. The three strains were capable of forming nodules on chickpea, which shows that this capacity was not linked to the geographical origin of the strains but was a species characteristic.

The effectiveness of two and three isolates assigned to *M. mediterraneum* and *S. medicae*, respectively, was compared to that of reference strains *M. mediterraneum* 918 and *M. ciceri* UPMCa7^T. The results are shown in Table II. The number of nodules formed per plant by the five Tunisian isolates tested was significantly less than that formed by the reference strains. Nevertheless, the two isolates assigned to *M. mediterraneum* were as effective as the reference strains since the shoot dry matter of the plants inoculated with these isolates and the reference strains did not differ significantly. On the contrary, inoculation with isolates assigned to *S. medicae* induced a plant growth similar to the non-inoculated plants.

4. DISCUSSION

Two species of the genus *Mesorhizobium* [6], *M. mediterraneum* [10] and *M. ciceri* [9], are presently recognized as specific symbionts of *C. arietinum*. These two species do not encompass all the genetic diversity described so far for rhizobia nodulating chickpea, and there are a number of strains which still remain unclassified [10]. One objective of this study was to determine whether rhizobia compatible and effective with chickpea were present or not in Tunisian soils. We found that most soils, in the regions of Tunisia that we prospected, and that includes regions where chickpea is traditionally cultivated, and others where it has never been grown, contain populations of rhizobia that nodulate chickpea. However, among the rhizobia isolated from the nodules of chickpea grown on these soils, chickpea-specific mesorhizobia were identified only from five soils

Table II. Nodulation and effectiveness on chickpea cv. Amdoun I of some local isolates (75c21, 74c10, 72c11, 48.2 and 22a12) and reference strains *M. ciceri* UPMCa7^T and *M. mediterraneum* 918.

Strain	Ribotype as determined by PCR/RFLP of 16S rDNA	Number of* nodules/plant	Shoot dry weight* (g/plant)
48.2	*M. mediterraneum*	26.12 ^{b}	1.04 ^{a}
22a12	*M. mediterraneum*	23.50 ^{b}	1.01 ^{ab}
75c21	*S. medicae*	26.50 ^{b}	0.72 ^{c}
72c11	*S. medicae*	20.30 ^{b}	0.72 ^{c}
74c10	*S. medicae*	25.67 ^{b}	0.81 ^{bc}
918	*M. mediterraneum*	35.17 ^{a}	1.04 ^{a}
UPM Ca7^T	*M. ciceri*	36.83 ^{a}	1.05 ^{a}
Non inoculated			0.75 ^{c}

* Averages with different letters are statistically different with LSD (least significant difference test) at the level 0.05.

sampled in a region where chickpea was traditionally grown. The five isolates belonged to *M. mediterraneum* and represented only a fraction of the isolates identified from each soil, which indicates that they were either not dominant and/or not competitive. These results strongly suggest that the two *Mesorhizobium* species described as being specific to chickpea are not indigenous to Tunisian soils and that the few *M. mediterraneum* detected have been introduced, likely with seeds, since there is no known history of chickpea inoculation in Tunisia.

We also demonstrated that, in Tunisian soils, rhizobia that nodulated chickpeas belonged almost exclusively to *S. medicae*, a *Sinorhizobium* species not considered, up to now, as a natural symbiont of chickpea. *S. medicae* has been described as a specific symbiont of the *Medicago* species [11]. The genus *Medicago* diversified in the Mediterranean basin and several of the more than 60 different species that the genus comprises are indigenous to Tunisia, which could explain the wild distribution of *S. medicae* in the Tunisian soils. However, although *S. medicae* strains were able to nodulate chickpea, the symbiosis they formed took a longer time to establish than when formed with specific mesorhizobia, and was ineffective, which indicated that the two symbionts were not fully compatible. The degree of specificity between leguminous plants and rhizobia is highly variable. Some rhizobial species can have a very wide host range and others a very narrow one. Some plants can be very specific, and others promiscuous. Our results show that the host spectrum of *S. medicae* and *S. meliloti* can be enlarged to *C. arietinum* and that *C. arietinum* might be a rather promiscuous host plant.

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