Comparison of rhizobia that nodulate *Medicago laciniata* and *Medicago truncatula* present in a single Tunisian arid soil

Y. Badri, K. Zribi, M. Badri, T. Huguet, P. van Berkum, and M.E. Aouani

**Abstract:** The rhizobia present in a single arid region Tunisian soil that nodulate *Medicago laciniata* and *Medicago truncatula* were compared. All isolates, 40 from each host, were *Sinorhizobium meliloti* based on 16S rRNA polymerase chain reaction reaction restriction fragment length polymorphism (PCR–RFLP) patterns and subsequent confirmation by sequence analysis of the 16S rRNA genes in four representatives from each host species. There was no apparent relationship between *Medicago* host species of isolation and the nodulating rhizobial genome as determined by repetitive extragenic palindromic PCR. The isolates of *M. laciniata* were distinguished from those of *M. truncatula* present in the same soil by variation in PCR–RFLP of *nifDK*, indicating that this dissimilarity is originally genetic and not geographic. While forming effective symbioses with their own respective isolates, both *M. laciniata* and *M. truncatula* formed ineffective true nodules, nodule-like structures, or no nodules at all in cross-inoculation tests, as confirmed by the histological observations.

**Key words:** *Medicago laciniata*, *Medicago truncatula*, *Sinorhizobium meliloti*, REP-PCR, efficiency.

**Résumé :** Les rhizobiums nodulant *Medicago laciniata* et *M. truncatula* piégés sur un sol appartenant à l’étage bioclimatique aride de la Tunisie ont été comparés. Tous les isolats analysés, 40 de chaque plante hôte, se sont avérés à partir de l’identification moléculaire par PCR–RFLP du gène de l’ARNr 16S, appartenir à l’espèce *Sinorhizobium meliloti*. Ce résultat est confirmé par le séquençage de ce même gène chez quatre issus de chaque plante. L’analyse des profils REP-PCR n’a pas montré de relations phyléenétiques entre le génome des rhizobiums et leurs espèces végétales d’origine. Les isolats de *M. laciniata* se distinguaient de ceux piégés de *M. truncatula* dans le même sol par une variation de la séquence intergénique *nifDK* indiquant que cette différence était d’origine génétique et non géographique. Les observations histologiques ont montré que même si elles établissent des symbioses efficaces avec leurs propres isolats respectifs, *M. laciniata* et *M. truncatula* ont formé des nodules vrais maisinefficients, des pseudo-nodules ou pas de nodules du tout suite à des inoculations croisées.

**Mots-clés :** *Medicago laciniata*, *Medicago truncatula*, *Sinorhizobium meliloti*, REP-PCR, efficience.

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**Introduction**

Species within the legume genus *Medicago* originating from the Mediterranean basin (Heyn 1963) have the ability of establishing a nitrogen-fixing symbiosis with soil bacteria referred to as rhizobia. Three different species of nitrogen-fixing rhizobia that nodulate *Medicago* spp. have been proposed: *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*; Jordan 1984), *Sinorhizobium medicae* (Rome et al. 1996b), and *Rhizobium mongolense* (van Berkum et al. 1998).

The plant host range for effective nitrogen fixation varies among rhizobia originating from different species of *Medicago* (Brockwell and Hely 1966; Eardly et al. 1990; Brunel et al. 1996; Brockwell 2001). Furthermore, it has been suggested that this plant genus may contain more symbiotic specificity groups than originally anticipated (Béna et al. 2005). *Rhizobium mongolense* forms an effective nitrogen-fixing symbioses with its original host of isolation, *Medicago raphanoides*, but with *Medicago sativa* few to many small white nodule-like structures result from the inoculation (van Berkum et al. 1998). Variability in nitrogen fixation effectiveness among strains of *S. meliloti* with different species of *Medicago* has also been reported (Brockwell and Hely 1966). For instance, the rhizobia of *Medicago laciniata* and *Medicago truncatula* do not effectively cross-nodulate even though all these rhizobia have been...
classified as *S. meliloti* (Villegas Mdel et al. 2006). Barran et al. (2002) presented evidence that this specificity is the result of differences in *nodC* on pSymA of rhizobia that nodulate *M. laciniiata*. A further comparison has indicated that there are differences in *nodA* and *nifDK* as well (Villegas Mdel et al. 2006). However, the inferences made in these two studies showing differences in three loci on pSymA are limited because the reference strains and the isolates of *M. laciniiata* originated from different geographic locations and isolations were made at different times. Another potential problem is that these differences on pSymA were established with no more than seven strains of *S. meliloti* from other *Medicago* spp. (Villegas Mdel et al. 2006). Concern about these limitations are relevant because considerable genetic diversity has been reported to exist among the medic rhizobia originating from different regions within the native habitats of *Medicago* spp. (Paffetti et al. 1996, 1998; del Papa et al. 1999; Carelli et al. 2000; Jebara et al. 2001; Roumiantseva et al. 2002; Badri et al. 2003; Biondi et al. 2003; Braietch et al. 2003; Zribi et al. 2004, 2005; van Berkum et al. 2006). Therefore, the possibility exists that these differences were observed because the reference strains represented a limited pool of the existing diversity and originated from geographic locations that were different from those of the isolates obtained with *M. laciniiata*.

The objective of this study was to isolate an equal number of rhizobia of *M. laciniiata* and *M. truncata* from a single Tunisian soil and to characterize them for genetic diversity by repetitive extragenic palindromic polymerase chain reaction (REP-PCR). PCR – restriction fragment length polymorphism (RFLP) analyses of the 16S rRNA genes were used to establish whether the different isolates possess ribosomal genes that align with *S. meliloti* and (or) *S. medicae*. Eight representative isolates, four from each host species, were examined for their 16S rRNA gene sequence to relate the PCR–RFLP patterns to the gene sequence. Also, the isolates were examined for a relationship between their *nifDK* PCR–RFLP patterns to establish whether this region of pSymA is characteristically dependent upon the host of origin. The symbiotic phenotypes of several selected isolates originating from both *Medicago* spp. were examined on both host plants and histological observations of root tissues were done to examine nodule morphologies.

**Materials and methods**

**Soils sampling and rhizobial isolation**

Soil samples taken from a depth of 0–20 cm were collected in the summer of 2005 from the Amra site located in the central region of Tunisia. The Amra site, which is within the arid climatic zone, does not have a history of agriculture. Both *M. laciniiata* and *M. truncata* are native to this location and served as a source of pods with seeds. Based on microsatellite markers, four polymorphic pure lines of each medic species (Badri et al. 2004) were chosen and cultivated on this soil in 500 mL aseptic plastic pots placed in an environmentally controlled greenhouse (16 h light: 8 h dark photoperiod, continuous 25 °C and 60%–80% relative humidity) for 2 months. Plants grown in these pots were used as a source of nodules for the isolation of the rhizobia. Sixty-day-old plants were recovered from the pots and nodules were selected at random for isolation of the 40 rhizobial cultures from each host species using standard procedures and yeast extract mannitol agar (YEMA) (Vincent 1970). Cultures were grown at 28 °C and maintained on YEMA slants at 4 °C. Subsequently the isolates were stored in 25% glycerol at −80 °C.

**PCR-based analyses**

PCR amplification of the 16S rRNA genes was as previously described (Laguerre et al. 1997) using primers FS6 and PS1509 (Willems and Collins 1993) and restriction digestion of the products was accomplished with *RsaI*. This restriction enzyme was used because digestion products are generated that discriminate between the 16S rRNA genes of *S. meliloti* and *S. medicae* (Laguerre et al. 1997; Andronov et al. 1999; Roumiantseva et al. 2002). The restriction fragments were separated electrophoretically on horizontal 3% (w/v) agarose gels that subsequently were stained in an aqueous solution of ethidium bromide (1 μg/mL) and photographed under 312 nm ultraviolet light with a Biodoc® imager (Biodoc2NT/Biometra; Goettingen, Germany). The 16S rRNA gene sequences of the isolates LAIII29, LA129, LAI12, LAO11, I3.19/3, I3.17/3, 3.20/6, and I3.19/6 were determined according to the methods described by van Berkum et al. (1996) using PCR primers 16Sa and 16Sb (van Berkum and Fuhrmann 2000). The sequences of the 16S rRNA genes were aligned in GeneDoc (Nicholas and Nicholas 1997) and were compared with the three copies of *S. meliloti* strain 1021 (GenBank accession No. AL591688).

Genomic diversity of the 80 isolates was established by REP-PCR with REP1R and REP2 primers (Versalovic et al. 1991). Amplification volumes were 13 μL with PCR conditions and data analysis as described by Zribi et al. (2004).

PCR–RFLP analysis also was used to identify the *nifDK* intergenic space region in each of the isolates using the primers FK597 and FD807 (Jamann et al. 1993). Although these primers were originally designed for the analysis of *Frankia*, they have been successfully used to characterize *nif* patterns by PCR–RFLP in rhizobial isolates obtained from the nodules of *Medicago* spp. (Brunel et al. 1996; Villegas Mdel et al. 2006). The PCR protocol was as described for the 16S rRNA gene with the exception that the annealing temperature used was 63 °C. The PCR products were digested with *HaeIII* and restriction fragments were separated and visualized as described for the 16S PCR–RFLP analysis.

**Computer-assisted analysis of genomic fingerprinting**

The commercially available Fingerprinting II® software program version 3.00 (Bio-Rad, Hercules, California) and the Quantity One® imaging software version 4.5 (Bio-Rad) were used in the computer-assisted analysis of REP-PCR genomic fingerprints. Unweighted pair group method with arithmetic mean (UPGMA) was used for cluster analysis of 80 isolates, and reference strains RCR2011 of *S. meliloti* and the local strain A5 (Zribi et al. 2004). Dendrograms were constructed using the Dice similarity coefficient.
Table 1. Rhizobial isolates and *Medicago laciniata* and *Medicago truncatula* lines used in inoculation and cross-inoculation trials.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Line of origin</th>
<th>nifDK type</th>
<th><em>Medicago laciniata</em> lines</th>
<th><em>Medicago truncatula</em> lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNL3.2</td>
<td>TNL3.3</td>
</tr>
<tr>
<td>LAI29</td>
<td>TNL3.3</td>
<td>n1</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>LAIII29</td>
<td>TNL3.7</td>
<td>n2</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>LAII13</td>
<td>TNL3.6</td>
<td>n1</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>I/LAIII7</td>
<td>TNL3.7</td>
<td>n2</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>LA0111</td>
<td>TNL3.2</td>
<td>n2</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>LA014</td>
<td>TNL3.2</td>
<td>n1</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>LAI32</td>
<td>TNL3.3</td>
<td>n1</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>A5</td>
<td>Zribi et al. 2005</td>
<td>n3</td>
<td>I (0.17)</td>
<td>0</td>
</tr>
<tr>
<td>II3.23/5</td>
<td>TN3.23</td>
<td>n3</td>
<td>I (0.17)</td>
<td>0</td>
</tr>
<tr>
<td>3.20/6</td>
<td>TN3.20</td>
<td>n3</td>
<td>I (0.17)</td>
<td>I (0.17)</td>
</tr>
<tr>
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<td>TN3.19</td>
<td>n3</td>
<td>0</td>
<td>I (0.17)</td>
</tr>
<tr>
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<td>TN3.23</td>
<td>n3</td>
<td>0</td>
<td>I (0.17)</td>
</tr>
<tr>
<td>I3.17/3</td>
<td>TN3.17</td>
<td>n3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I3.19/9</td>
<td>TN3.19</td>
<td>n3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RCR2011</td>
<td>Eardly et al. 1990</td>
<td>n3</td>
<td>I (0.33)</td>
<td>I (0.33)</td>
</tr>
</tbody>
</table>

**Note:** Five lines of each *Medicago* species were inoculated with seven isolates each of *Medicago laciniata* and *Medicago truncatula*. Each combination was replicated six times. E, effective symbiosis; I, ineffective symbiosis but the formation of true nodules; 0, no nodules or nodule-like structures. Values in parentheses are number of nodules per plant. Efficiency is estimated by nitrogen fixation (NFE = shoot dry matter of inoculated plant per shoot dry matter of uninoculated plant). Isolates were considered effective when the ratio was significantly different to controls when estimated by Duncan’s multiple range test at a confidence of $P = 0.05$.
Strains and plant material for symbiotic tests and competitiveness
Seeds of five lines each representing the Amra population of Medicago laciniata and Medicago truncatula were surface-sterilized and scarified with concentrated H2SO4 for 13 min, rinsed 10 times with sterile water, incubated in the dark on water agar (0.9T% m/v) at 4 °C for 48 h, and were subsequently kept at 15 °C. Germinated seeds were placed aseptically in tubes with Fahraeus nutrient solution (Fahraeus 1957). Seven isolates from each Medicago species representing the diversity determined with REP-PCR were used separately for inoculation of the germinated seedlings (Table 1). Cross nodulation trials were done with seven isolates of M. truncatula nodulation trials were done with seven isolates of M. truncatula and vice-versa. All the isolates were examined for nodulation with their original host of isolation as the positive control. Uninoculated plants were included as the negative control. The reference strain S. meliloti RCR2011 was included in the nodulation trial and was inoculated to all lines of M. laciniata and M. truncatula. The rhizobia were inoculated as 300 μL YEM broth suspension at early stationary phase (10^8–10^10 bacteria/mL). The plant tests were done in six replications. At 60 days after inoculation the plants were harvested to determine nodule number and to estimate nitrogen fixation efficiency (NFE) by calculating the shoot dry matter of inoculated plants divided by the shoot dry matter of uninoculated plants. Statistical analyses were done with Statistica® version 5.0 software (StatSoft, Tulsa, Okla.) and Duncan’s test.

Histology
Since both nodules and nodule-like structures were produced on the plant roots depending on which isolates were used as inoculum, histological observations were done to distinguish the formation of functional nodules. Nodules were isolated separately from whole root system 60 days after inoculation and cleared for 3 hours in sodium hypochlorite (12% of active chlorine), then rinsed in water and stained with methylene blue (0.01%) for light microscopy observation.

Results and discussion
Genetic diversity of the isolates
Restriction digestion with Rsal endonuclease and subsequent agarose gel electrophoresis of the 16S rRNA gene PCR products with all 80 isolates resulted in fingerprint patterns that were identical to each other and to that obtained with S. meliloti RCR2011 (the parent culture of 1021). The PCR–RFLP patterns of the 80 isolates were different to those of S. medicae A321 (data not shown). The sequence of the 16S rRNA genes of isolates LAIII29, LA129, LA132, LA011, I13.19/3, I13.17/3, 3.20/6, and I3.19/6 that represented the 80 isolates were identical to each other and to the three copies within the chromosome of strain 1021 (GenBank accession No. AL591688). Based on these results, the 80 isolates align with S. meliloti rather than S. medicae irrespective of the host of isolation. Also, this result is consistent with the observation of Villegas Mdel et al. (2006), who reported that isolates of M. laciniata have characteristics aligning them with S. meliloti and not with S. medicae. Zribi et al. (2004) reported that all 32 isolates obtained from Amra soil using M. truncatula as trap host have 16S rRNA genes that align them with S. meliloti, and that the presence of S. medicae could not be established using Medicago polymorpha as trap host. That all the isolates from M. truncatula at the Amra site align with S. meliloti and not S. medicae is in contrast to data leading to the proposal of S. medicae (A321) as a species (Rome et al. 1996b) because their characterization of two genomic species of Sinorhizobium was among 73 M. truncatula isolates that originated from two French soils. The probable reason for the dissimilar results of these two reports is that the climate and soils of France are significantly different from the arid region of the Amra site in Tunisia. The discovery of a predominance of rhizobia with characteristics of S. meliloti that nodulate M. truncatula in the Amra site is consistent with the conclusions made by Garau et al. (2005) that S. meliloti is present in more alkaline and arid soils while S. medicae is mostly associated with moderately acidic soils.

A high degree of heterogeneity among the 40 isolates of M. laciniata was observed using REP-PCR with 39 unique fingerprint patterns. With analysis of the data, two clusters were obtained that diverged at the 20% similarity coefficient (data not shown); however, no clear relationship was found between the REP-PCR fingerprint pattern and the line of M. laciniata that was used as trap host for isolation. A similar result was obtained with the 40 isolates of M. truncatula: 38 unique fingerprint patterns diverged at the 18%
similarity coefficient (data not shown). When the data of the isolates from both *Medicago* spp. was combined no clear relationship was observed between the REP-PCR fingerprint pattern and the host species used for isolation. Therefore, in the Amra site there is no apparent relationship between *Medicago* host species and the nodulating rhizobial genome as determined by REP-PCR.

PCR–RFLP analysis of the *nifDK* intergenic spacer revealed three fingerprint patterns among the 80 isolates. All the isolates originating from *M. laciniata* could be distinguished from those of *M. truncatula* by the presence of the two *nifDK* types n1 and n2, present in 8 and 32 of the isolates, respectively. These two *nifDK* types were different from the type that was observed with the analysis of all the isolates originating from *M. truncatula* (n3). The *nifDK* pattern n3 also was identified in RCR2011 (Fig. 1). Similarly, Villegas Mdel et al. (2006) observed two distinct *nifDK* PCR–RFLP patterns among 17 *M. laciniata* isolates originating from Tunisian soils. However, information was not provided about *M. laciniata* isolates originating from the Amra site nor did Villegas Mdel et al. (2006) compare the *nifDK* fingerprint profiles of *M. truncatula* isolates originating from the same Tunisian soil samples. The analysis of the 80 isolates in the present study clearly demonstrates a difference in the *nifDK* region between rhizobia that nodulate *M. laciniata* and *M. truncatula* that are present in the same soil of the Amra site. Therefore, it is unlikely that the variation in the *nifDK* region between rhizobia of *M. laciniata* and *M. truncatula* is due to differences in geographic origin, as may have been the case with the results of Villegas Mdel et al. (2006).

**Symbiotic properties**

Visually, major morphological differences in nodule formation were evident on roots of the two medic species scored sixty days after inoculation. *Medicago laciniata* and *M. truncatula* plants exhibited well-developed pink nodules within 8–10 days when they were inoculated with their own isolates. However, in cross inoculation tests, *M. truncatula* lines TN3.18, TN3.19, and TN3.23 formed ineffective true nodules 3 weeks after inoculation with five of the seven isolates from *M. laciniata*, while the hosts TN3.17 and TN3.20 produced no nodules with any of the isolates (Table 1). With the exception of line TNL3.6, all the *M. laciniata* lines pro-

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**Fig. 2.** Histology of nodules and nodule-like structures obtained in the symbiotic tests. Effective nodule structures obtained when isolates from *Medicago laciniata* (A) or *Medicago truncatula* (B) were inoculated on their hosts of isolation; ineffective nodule structures observed when *M. truncatula* was inoculated with isolates from *M. laciniata* (C) and when *M. laciniata* was inoculated with isolates from *M. truncatula* (D). Nodule-like structures (E) and (F) showing the absence of peripheral endodermis and vascular bundles (their presence is illustrated by the arrows in A, B, C, and D).
duced ineffective nodules four weeks after inoculation with three or more isolates originating from *M. truncatula* (Table 1). In some cases, at least 2 weeks after inoculation hypertrichoses were observed that seemed to lack the organized structure that is usually associated with a nodule. Some of these structures were examined by light microscopy to better distinguish them from true nodules. The formation of true nodules was established by the presence of peripheral endodermis and peripheral vascular bundles (Figs. 2A–2D). However, the nodule-like structures obtained in the cross-inoculation tests lacked vascularization and a peripheral endodermis and, therefore, could not be considered true nodules (Figs. 2E and 2F). These nodule-like structures have previously been observed in *M. sativa* and *Trifolium repens* L. (Truchet et al. 1989) and might be elicited by Nod factors (Grosjean and Huguet 1997). It is unclear why there was such high variability among different isolates and *Medicago* lines for the production of these nodule-like structures. Also unknown is what effect there would be on nodulation by the effective homologous isolates if the ineffective heterologous rhizobia were present in the rhizosphere of the two *Medicago* species.

**References**


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