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SHORT COMMUNICATION

Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic N_2-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. *mediterranense*) of *Sinorhizobium meliloti*

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Abstract Nodulation of common bean was explored in six oases in the south of Tunisia. Nineteen isolates were characterized by PCR–RFLP of 16S rDNA. Three species of rhizobia were identified, *Rhizobium etli*, *Rhizobium gallicum* and *Sinorhizobium meliloti*. The diversity of the symbiotic genes was then assessed by PCR–RFLP of *nodC* and *nifH* genes. The majority of the symbiotic genotypes were conserved between oases and other soils of the north of the country. Sinorhizobia isolated from bean were then compared with isolates from *Medicago truncatula* plants grown in the oases soils. All the *nodC* types except for *nodC* type *p* that was specific to common bean isolates were shared by both hosts. The four isolates with *nodC* type *p* induced N_2-fixing effective nodules on common bean but did not nodulate *M. truncatula* and *Medicago sativa*. The phylogenetic analysis of *nifH* and *nodC* genes showed that these isolates carry symbiotic genes different from those previously characterized among *Medicago* and bean symbionts, but closely related to those of *S. fredii* Spanish and Tunisian isolates effective in symbiosis with common bean but unable to nodulate soybean. The creation of a novel biovar shared by *S. meliloti* and *S. fredii*, bv. *mediterranense*, was proposed.

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Introduction

Common bean (*Phaseolus vulgaris* L.) is an important food crop in the world. This legume is considered as a poor nitrogen-fixer in comparison to other grain legumes (Buttery et al. 1987; Ramos and Boddey 1987; Hardarson 1993). This problem is generally attributed to the ineffectiveness of the indigenous rhizobia (Graham 1981; Thies et al. 1991) or to adverse abiotic conditions (Sessitsch et al. 2002). At least six species are able to nodulate and fix nitrogen with bean, including *Rhizobium leguminosarum* bv. *phaseoli*, *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium gallicum*, *Rhizobium giardinii* (see review of Martínez-Romero 2003). Additionally, isolates effective in symbiosis with bean were identified as *Sinorhizobium fredii*, a species known to include soybean symbionts (Herrera-Cervera et al. 1999; Mhamdi et al. 2002). Moreover, promiscuous nodulation of bean with non-specific rhizobia with distinct phylogenic positions frequently occurs (Martinez et al. 1985; Bromfield and Barran 1990; Laguerre et al. 1993; Michielis et al. 1998; Mhamdi et al. 2002). Natural populations of rhizobia nodulating common bean in different geographical regions in Tunisia have been characterized (Mhamdi et al. 1999). Nodulation was found to be correlated to the history of bean cropping. Abundant nodulation was recorded in the north of Tunisia where the soils are traditionally cultivated with beans and five species of bean-nodulating rhizobia, *R. etli*, *R. leguminosarum*,

R. gallicum, *R. giardinii*, and *S. fredii*, were identified (Mhamdi et al. 2002). In contrast, no or scarce nodulation was observed on bean plants grown in soils with no history of bean cultivation in the south of Tunisia. The few nodules formed did not fix N_2 and were caused by promiscuous nodulation of rhizobia that were identified as *Sinorhizobium meliloti* and *S. medicae* and accordingly were effective in symbiosis with alfalfa.

In order to explore new potential areas for bean cultivation in the south of Tunisia, the oases, which have not been studied in the previous work of Mhamdi et al. (2002), were accorded special attention. The characterization of indigenous populations of rhizobia from these regions may lead to the selection of inoculant strains for soils affected by salinity. In this study we have assessed diversity, salt tolerance and symbiotic effectiveness of indigenous populations of rhizobia nodulating common bean in different soil samples recovered from six sites representing the main oases in Tunisia.

Material and methods

Isolation and phenotypic characterization of rhizobia

Soil samples were collected from six sites representing the main oases of the south of Tunisia: Douz, Hammet-Gabes, Kebilly, Nafta, Rjim-Maatoug and Tozeur. All the soils were sandy, slightly alkaline ($7.7 \leq pH \leq 8.2$, 1/2.5 dilution) and saline (EC > 2 mmho/cm, 1/5 dilution). Common bean plants (cv. Royalnel) and *Medicago truncatula* (cv. Jemalong) were grown in greenhouse conditions in the soils sampled from the different oases (three replicate pots and five plants per pot for each soil sample). Nodulation was checked after 50 days of growth. Soil sampling and isolation of nodulating rhizobia from root nodules were carried out as previously described (Mhamdi et al. 1999). Salt tolerance of rhizobial isolates was assessed in YEM broth medium using concentrations of NaCl ranging from 0.2 to 5%. The ability to grow was checked visually after 5 days of incubation.

Plant tests

Nodulation and effectiveness tests on *P. vulgaris* (cv. Royalnel), *M. truncatula* (cv. Jemalong) and *Medicago sativa* (cv. Gabes) were conducted as previously described (Mhamdi et al. 1999). Statistical analysis of data was performed by ANOVA followed by comparison of means by Duncan test ($P \leq 0.05$).

PCR–RFLP, Rep–PCR fingerprinting and DNA sequencing

PCR–RFLP typing of 16S rRNA, *nifH* and *nodC* genes were achieved as previously described by Mhamdi et al. (2002). REP–PCR was conducted as previously described (Zribi et al. 2004). PCR products were purified from agarose gels using the Wizard SV Gel and PCR clean-up system from Promega were used for DNA sequencing of strain LILM4H41. The 16S rRNA gene was sequenced using primers fD1, rD1 (Weisburg et al. 1991) and primers FGPS485-292, FGPS1047-295, FGPS505′-313, FGPS910′-270 (Normand et al. 1996). The *nifH* and *nodC* gene regions were sequenced with primers nifHf and nifHi, and nodCf and nodCi, respectively (Laguerre et al. 2001). The nucleotide sequences were deposited in the GenBank database under accession number DQ333890, DQ333891 and DQ394805 for *nifH, nodC* and 16sRNA gene regions, respectively.

Phylogenetic analysis

Molecular sequence analyses were performed by using programs available in the "Centre de Ressources INFOBIOGEN (http://www.infobiogen.fr)". The BlastN program (Altschul et al. 1997) was used for searching DNA databases for sequence similarities. Nucleotide sequences were aligned with CLUSTALW (version 1.8, June 1999; Higgins et al. 1994). Phylogenetic trees were inferred using the phylogenetic inference package (PHYLIP; Felsenstein 1989) with neighbor-joining analyses from Jukes–Cantor nucleotide distances. Confidence in neighbor-joining trees was assessed by bootstrap analysis with the SEQBOOT and CONSENSE programs of PHYLIP.

Results and discussion

Diversity of natural populations of rhizobia nodulating common bean in the oases

The bean plants cultivated in the soil sample originating from Douz were not able to grow, probably because of the very high salt concentration (EC = 3.76 mmho/cm). In the other soil samples, there was very scarce or no nodulation except in the Rjim-Maatoug soil which showed the lowest salt concentration among the six soils analyzed (EC = 2 mmho/cm). In non-saline Tunisian soils, the range of EC is generally 0.3–0.5 mmho/cm. Big and pink nodules, indicating good effectiveness, were observed on plants grown on the Rjim-Maatoug soil. Seventeen nodule isolates

could be recovered from the Rjim-Maatoug soil, but only one from the Hammet-Gabes and the Tozeur soils. Nodulation also correlated with the history of bean cropping since this crop was not traditionally cultivated in these soils except in the Rjim-Maatoug oasis where beans were introduced 10 years ago. These results corroborate the conclusions of our previous study of Tunisian soils from the north and the south of the country (Mhamdi et al. 1999).

The isolates were categorized in three 16S rDNA types by PCR–RFLP analysis of 16S rDNA genes (Table 1), which corresponded to the species *R. gallicum, R. etli* and *S. meliloti* according to the classification proposed by Laguerre et al. (1997). The majority of the isolates (13/17) from Rjim-Maatoug were classified in the *R. etli* species. The four other isolates from Rjim-Maatoug and the isolate from Hammet-Gabes were classified in the *S. meliloti* species, and the isolate from Tozeur belonged to *R. gallicum*. The classification in the *S. meliloti* species was confirmed by nucleotide sequencing of 1487 bp of the 16S rDNA fragment of a representative strain (LILM4H41) from Rjim-Maatoug. The sequence showed 99.7% of similarity with 16S rDNA of *S. meliloti* strain 1021, its closest relative.

Five composite (*nifH/nodC*) symbiotic genotypes were found among the oases isolates by PCR–RFLP analysis of *nifH* and *nodC* genes, which resulted in the categorization of *R. etli* and *S. meliloti* isolates in two groups (Table 1). The symbiotic genotypes associated with *R. gallicum* and *R. etli* isolates were previously identified among bean isolates from the north of Tunisia, (Mhamdi et al. 2002), the genotype *c/d* being the most frequent among the *R. etli* isolates in the oases and in the north. *Rhizobium etli* is supposed to originate from Latin America (Segovia et al. 1993) and to have been introduced in other continents with the seeds (Pérez-Ramírez et al. 1998). If so, our results suggest that this species is able to proliferate and persist in Mediterranean ecosystems, including under salt stress.

The genotype *i/i* identified in the unique isolate of *S. meliloti* from Hammet-Gabes corresponded to that previously found among *S. medicae* bean isolates from the south of Tunisia and also matched to the type of the reference strain of *S. medicae* strain M1 (Mhamdi et al. 2002). In contrast, symbiotic genotype *p/p* associated with four *S. meliloti* isolates from Rjim-Maatoug constituted a novel type. In order to investigate if common bean has trapped specific symbiotic genotypes among sinorhizobia, rhizobia nodulating *M. truncatula* plants were grown in the same oases soils. The plants were well nodulated (more than 15 nodules per plant) except for those grown in the Douz soil. By sampling 17–15 nodules per soil, 83 *M. truncatula* isolates were collected and characterized by 16S rDNA and *nodC* typing. All the isolates were assigned to the *S. meliloti* species, and were classified in three *nodC* types, *h*, *i* and *k*. These types were identified previously among sinorhizobia isolated from nodules of bean grown mainly in soils from the south of Tunisia and were shown to be typical of *Medicago* symbionts (Mhamdi et al. 2002). Type *i* was predominant, grouping more than 90% of the *M. truncatula* isolates. In contrast, no *nodC* type *p* was identified among the *Medicago* isolates. The four bean sinorhizobia with the *p/p* genotype were tested on *M. truncatula* and *M. sativa*. No nodules were observed on these hosts after 40 days of growth. Two genotypes were differentiated by REP–PCR fingerprinting among the new symbiotypes of *S. meliloti* isolates from Rjim-Maatoug (Fig. 1).

Phylogenetic position of isolates with symbiotic genotype p/p

The phylogenetic analysis of the novel symbiotic genotype *p/p* was performed based on nucleotide sequences of 727 bp of *nifH*, and 891 bp of *nodC* of strain LILM4H41. The *nifH* and *nodC* phylogenetic trees are shown in Figs. 2 and 3, respectively. The analysis of *nifH* sequences indicated that the closest relative of strain LILM4H41 were *S. fredii* strain GR-06 and GR-X8 (similarity values of 97.5%) isolated from common bean in Spain (Herrera-Cervera et al. 1999). These

Table 1 Distribution of isolates from Tunisian oases in species and symbiotic genotypes

Species*	Origin	*nifH* type**	*nodC* type***	Number of isolates
R. gallicum	Tozeur	*a*	*a*	1
R. etli	Rjim-Maatoug	*c*	*c*	1
R. etli	Rjim-Maatoug	*c*	*d*	12
S. meliloti	Hammet-Gabes	*i*	*i*	1
S. meliloti	Rjim-Maatoug	*p*	*p*	4

*Species were identified based on PCR–RFLP typing of 16S rDNA using *Rsa*I, *Nde*II, *Msp*I and *Hae*III restriction enzymes

***Cfo*I restriction pattern

****Msp*I restriction pattern

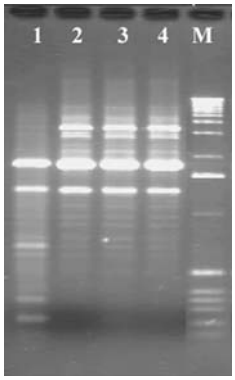

Fig. 1 REP–PCR patterns of *Sinorhizobium meliloti* isolates with *nifH*/*nodC* type *p/p* recovered from common bean nodules in Rjim-Maatoug. *1* isolate 4H7, *2* isolate 4H41, *3* isolate 4H12, *4* isolate 4H5, *M* molecular mass marker 1 kb (Gibco BRL)

S. fredii strains did not nodulate soybean but were effective in N_2-fixation with beans. The *nifH* genes of the *S. fredii* and *S. meliloti* bean rhizobia were also closely related to *nifH* of *S. americanum*, a recently described species that includes rhizobia isolated from native *Acacia* spp. in Mexico (Toledo et al. 2003). The similarity value between *nifH* of LILM4H41 and the *S. americanum* type strain was 96.3%. *S. americanum* strains were reported to form nodules on *Leucaena leucocephala* and *P. vulgaris*, but not on soybean. However, no information is available about their N_2-fixing effectiveness in symbiosis with common beans. The bean sinorhizobia did not cluster with the other recognized common bean symbionts, and the highest similarity value between *nifH* of strain LILM4H41 and of bean rhizobia was only 92% for *R. gallicum* bv. *gallicum* FL27. The *Medicago* rhizobia including *S. meliloti*, *S. medicae* and *Rhizobium* sp. OR191 strains were

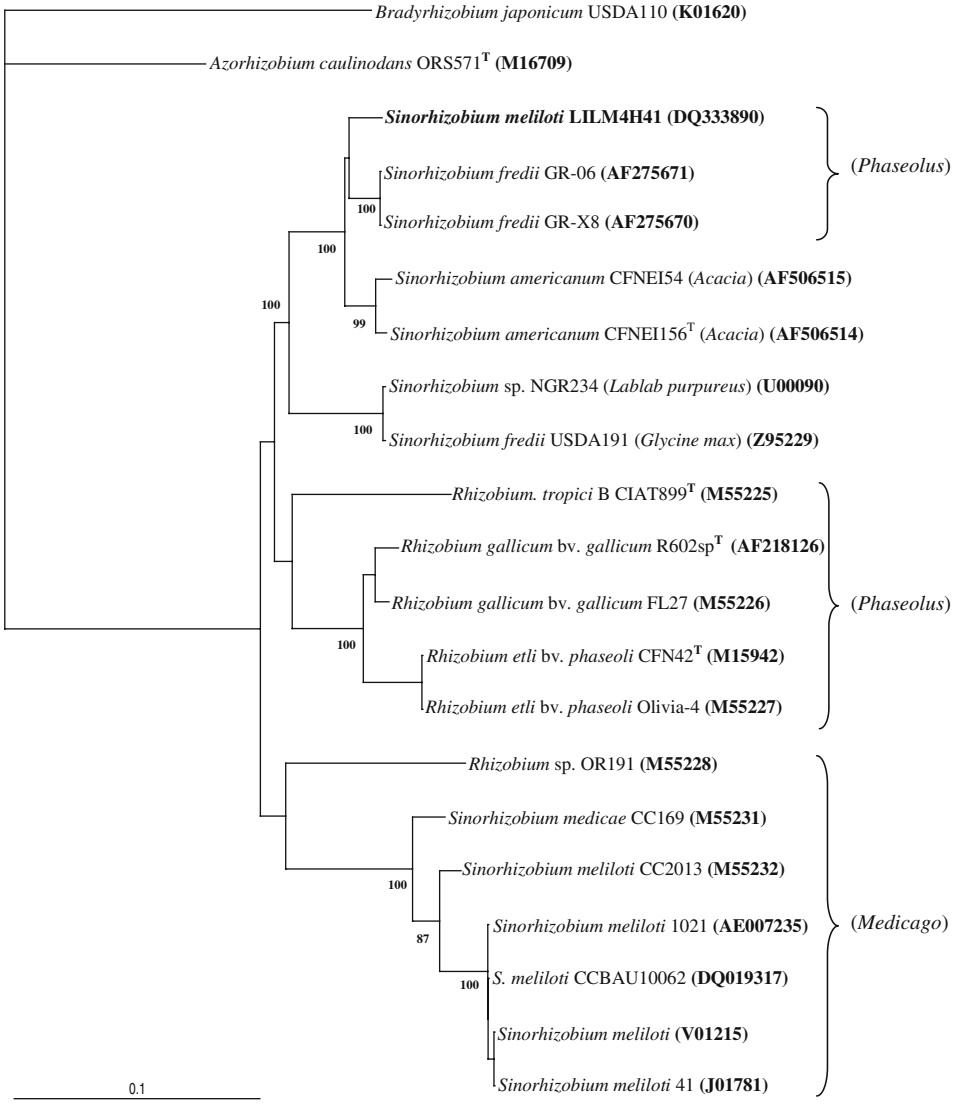

Fig. 2 Phylogenetic (Neighbor-joining) tree based on 469 bp aligned sequences of *nifH* gene. The accession numbers of the strains used are indicated in parenthesis. Only bootstrap probability values greater than 70% (1,000 replicates) are indicated at the branching points. The horizontal branches are drawn proportionally to the number of nucleotide substitutions per site

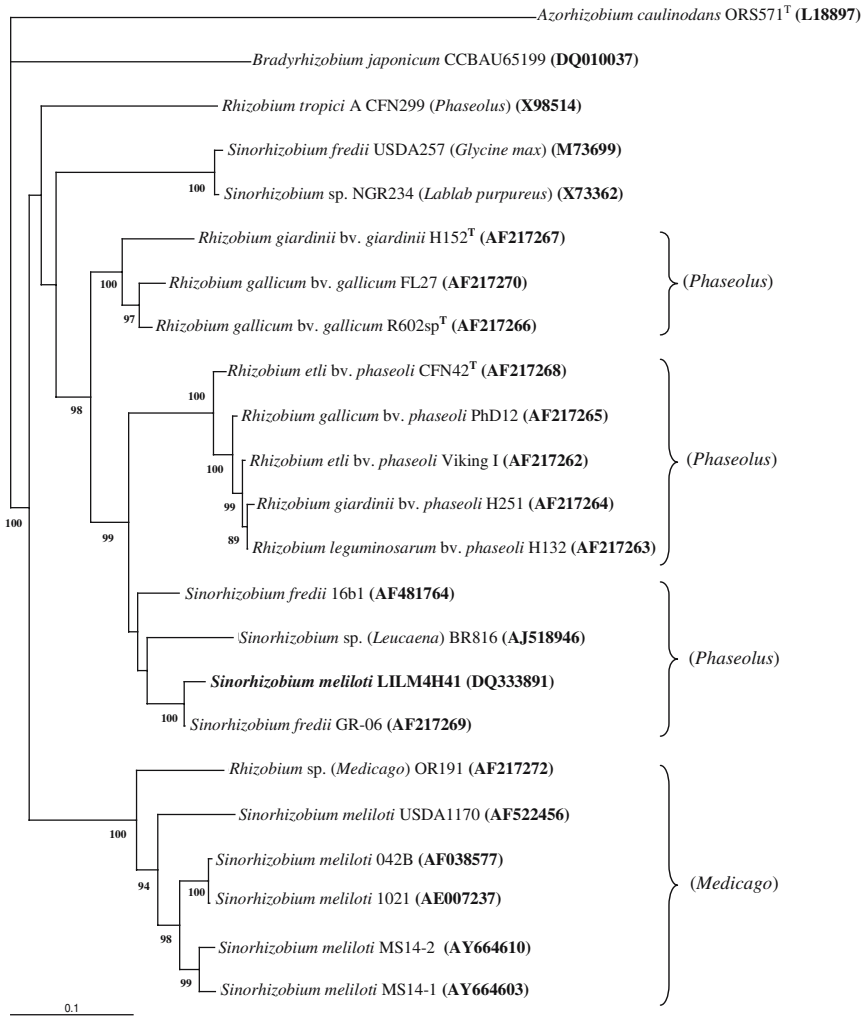

Fig. 3 Phylogenetic (Neighbor-joining) tree based on 523 bp aligned sequences of *nodC* gene. The accession numbers of the strains used are indicated in parenthesis. Only bootstrap probability values greater than 70% (1,000 replicates) are indicated at the branching points. The horizontal branches are drawn proportionally to the number of nucleotide substitutions per site

more distantly related to the bean sinorhizobia (similarity values less than 89%). Sequence comparisons of *nodC* genes confirmed *nifH* gene analysis. The closest relatives of strain LILM4H41 were *S. fredii* strain GR-06 and *S. fredii* strain 16b1 isolated from common bean in Tunisia (Mhamdi et al. 2002) with similarity values of 98.1 and 93.1%, respectively. Like GR-06, *S. fredii* strain 16b1 did not nodulate soybean but was effective in N_2-fixation with beans (Mhamdi et al. 2002). The bean sinorhizobia also clustered with *Sinorhizobium* sp. BR816 based on *nodC* phylogeny. BR816 was isolated from *Leucaena* in Brazil but was selected as a high temperature tolerant strain for bean symbiosis (van Rhijn et al. 1993). Therefore, at least one other *Sinorhizobium* strain that originates from another continent and fixes nitrogen with *P. vulgaris* shares closely related symbiotic genes with the Mediterranean bean sinorhizobia. The bean sinorhizobia clustered with the different rhizobial species harboring the symbiotic genes specific to the biovar *phaseoli*, but the similarity values between strain LILM4H41 and the bv. *phaseoli* *nodC* sequences were not higher than 87.9%. Similarity values between *nodC* sequences of strain LILM4H41 and *Medicago* symbionts were lower than 76%.

Symbiotic effectiveness of oases bean rhizobia

Representatives of the different symbiotic genotypes identified among the oases bean isolates were tested for their effectiveness on common bean. Nodule numbers and shoot dry weights are given in Table 2. The isolates belonging to *R. etli* and *R. gallicum* were as efficient as the reference strain *R. tropici* CIAT899 which is commercially used for inoculant formulation due to its N_2-fixation effectiveness with *P. vulgaris*. The

Table 2 Symbiotic effectiveness of representatives of the different symbiotic types found among oases isolates

Strain	Species	*nifH*/*nodC* type	Nodule number (per plant) (±SD)*	Shoot dry weight (g/plant) (±SD)*
Non-inoculated control	–	–	0[a]	0.33 (±0.02)[a]
LILM6H1	*R. gallicum*	*a/a*	158 (±15)[b]	0.97 (±0.04)[b]
LILM4H14	*R. etli*	*c/c*	229 (±19)[c]	0.99 (±0.04)[b]
LILM4H9	*R. etli*	*c/d*	290 (±21)[d]	1.00 (±0.03)[b]
LILM1H1	*S. meliloti*	*i/i*	293 (±18)[d]	0.30 (±0.03)[a]
LILM4H41	*S. meliloti*	*p/p*	384 (±22)[e]	1.01 (±0.02)[b]
Commercial strain CIAT899	*R. tropici*	*l/l*	289 (±19)[d]	1.07 (±0.06)[b]

SD standard deviation

*Mean values of 12 replicates. The means followed by the same letter are not significantly different ($P < 0.05$)

S. meliloti isolate LILM1H1, with symbiotic genotype *i/i*, induced a high number of nodules on common bean, but the nodules were ineffective based on shoot biomass yield. In contrast, *S. meliloti* LILM4H41 with genotype *p/p* also induced a high number of nodules but this isolate was as effective as strain CIAT899 in N_2-fixation.

The bean sinorhizobia form a novel biovar of the species S. meliloti and S. fredii

Recently, two new biovars, bv. *meliloti* and bv. *medicaginis*, were proposed for *S. meliloti* to discriminate strains showing difference in host specificity toward *Medicago* species (Villegas et al. 2006). However, strains in both biovars can form nodules with *M. sativa* which differentiates them from the Tunisian bean isolates with symbiotic genotype *p/p*. Also, the bean sinorhizobia did not share the symbiotic traits of *M. truncatula* isolates from the oases soils. Accordingly, the symbiotic genes of the bean sinorhizobia did not cluster with those of *Medicago*-nodulating rhizobia. As the term biovar in rhizobia species was coined to refer to taxonomic infrasubspecific divisions with distinct symbiotic properties and symbiotic genes with a similar chromosome background, we propose that the *S. meliloti* rhizobia with symbiotic genotype *p/p* and the previously described *S. fredii* bean rhizobia form a novel biovar, bv. *mediterranense*, shared by *S. meliloti* and *S. fredii* species. This biovar is represented by *S. meliloti* strain LILM4H41 and *S. fredii* strains GR-X8, GR-06 and 16b1 that nodulate and fix nitrogen with *P. vulgaris* but do not nodulate *M. truncatula*, *M. sativa* or *Glycine max*. In the same way, *S. fredii* should be divided into two biovars: (1) the biovar *fredii* which groups the classically known *S. fredii* strains that nodulate soybean and (2) the biovar *mediterranense* which encompasses *S. fredii* strains that do not nodulate soybean but fix nitrogen with common bean and is represented by strains GR-06, GR-X8 and 16b1.

Salt tolerance of isolates from Tunisian oases

To select for superior salt-tolerant strains among natural populations of the oases, NaCl-tolerance was determined for the different isolates that were effective in N_2-fixation with common bean. The maximal concentrations of NaCl permitting growth were 0.4 and 0.6% for the *R. etli* and *R. gallicum* isolates, respectively. However, the four *S. meliloti* isolates with symbiotic type *p/p* were able to grow in 4.4% NaCl, while the commercial strain CIAT899 did not grow in salt concentrations higher than 1.8%.

Because of its effectiveness and high salt tolerance, strain LILM4H41 is a privileged candidate for inoculant formulation in order to promote cultivation of common beans in saline soils. Further field experiments are needed to evaluate improvement of *P. vulgaris* yield by inoculation under salt and drought conditions.

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