

# Characterization of root-nodule bacteria isolated from *Vicia faba* and selection of plant growth promoting isolates

Sabrina Saïdi · Samir Chebil · Maher Gtari ·  
Ridha Mhamdi

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**Abstract** A collection of 104 isolates from root-nodules of *Vicia faba* was submitted to 16S rRNA PCR–RFLP typing. A representative sample was further submitted to sequence analysis of 16S rRNA. Isolates were assigned to 12 genera. All the nodulating isolates (45 %) were closely related to *Rhizobium leguminosarum* USDA2370<sup>T</sup> (99.34 %). The remaining isolates, including potential human pathogens, failed to nodulate their original host. They were checked for presence of symbiotic genes, P-solubilization, phytohormone and siderophore production, and then tested for their growth promoting abilities. Results indicated that 9 strains could induce significant increase (41–71 %) in shoot dry yield of faba bean. A *Pseudomonas* strain was further assessed in on-farm trial in combination with a selected rhizobial strain. This work indicated that nodule-associated bacteria could be a valuable pool for selection of effective plant growth promoting isolates. Nevertheless, the possible involvement of nodules in increasing risks related to pathogenic bacteria should not be neglected and needs to be investigated further.

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S. Saïdi · R. Mhamdi (✉)  
Laboratory of Legumes, Centre of Biotechnology  
of Borj-Cédria, BP 901, 2050 Hammam-Lif, Tunisia  
e-mail: ridhamhamdi@yahoo.fr; ridha.mhamdi@cbbc.nrnt.tn

S. Chebil  
Laboratory of Molecular Physiology of Plants, Centre  
of Biotechnology of Borj-Cédria, Hammam-Lif, Tunisia

M. Gtari  
Laboratory of Microorganisms and Active Biomolecules,  
Faculty of Sciences of Tunis, University of Tunis El-Manar,  
Tunis, Tunisia

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## Introduction

Although root nodules represent a minor fraction of the rhizosphere, they are a wealthy interface for microbial resources and molecular connections. It was admitted for longtime that root-nodule occupation was restricted to rhizobia. However, an increasing number of  $\alpha$ -  $\beta$ - and  $\gamma$ -Proteobacteria have been isolated from root nodules of a wide range of legumes and reported as nodulating bacteria (Moulin et al. 2001; Sy et al. 2001; Rivas et al. 2002; van Berkum and Eardly 2002; Vandamme et al. 2002; Valverde et al. 2005; Lin et al. 2008) or nodule-associated bacteria (Sturz et al. 1997; Gao et al. 2001; Zakhia et al. 2006; Kan et al. 2007). The non-symbiotic nodule endophytes have been poorly studied compared to symbiotic bacteria. Rhizobia were also frequently isolated from roots of non-legume plants and consequently could also be included as non-symbiotic endophytes (Antoun and Prévost 2005). Generally, endophytes are defined as microorganisms able to successfully colonize tissues of higher plants and cause unapparent and asymptomatic infections. They may establish neutral, detrimental or beneficial interactions with plants. Rhizobacteria that exert beneficial effects on plant development are termed plant growth-promoting rhizobacteria (PGPR). Although the mechanisms by which they promote plant growth are not yet fully understood, multiple modes of action were revealed to be responsible for growth promotion activities. They include direct and indirect mechanisms. Direct effects result from their ability to fix nitrogen, to increase nutrient uptake through solubilization of minerals, or to produce siderophores and phytohormones

(Kennedy et al. 2004; Glick et al. 2007). Indirect effects are expressed when PGPR act like biocontrol agents inducing resistance against plant pathogens, reducing diseases, stimulating other beneficial symbioses, or degrading xenobiotics in contaminated soils (Jacobsen 1997). However, the biological significance and the agronomic implications of nodule endophytism are still not well understood. It was reported that nodule endophytic bacteria might evolve into symbiotic bacteria by acquiring symbiotic genes from rhizobia by lateral gene transfer (Taghavi et al. 2005). It was also reported that an endophytic *Agrobacterium* strain may behave like a PGPR or plant growth deleterious rhizobacteria (PGDR) according to the antagonistic interaction with the rhizobial strain (Chihouai et al. 2012; Salem et al. 2012). Other studies outlined the potential involvement of legume nodules in increasing risks related to human pathogens (Muresu et al. 2010).

This study aimed to characterize the diversity of bacteria associated with root-nodules of *Vicia faba* plants collected from different locations in Tunisia and to assess their ability for promoting plant growth in order to select potential PGPR that perform higher plant growth promotion for use as biofertilizers.

## Materials and methods

### Nodule bacteria isolation

Local *V. faba* plants growing in 20 different sites commonly cultivated with this crop were collected. The collection area covered different bioclimatic regions from the north to the south of Tunisia. The roots were thoroughly washed and five nodules representing size and color variability were sampled in each site from a single plant. Nodules were surface sterilized in ethanol 95° for 15 s and HgCl<sub>2</sub> 0.1 % for 2 min, thoroughly washed with sterile water and then aseptically crushed onto YEM plates according to Vincent (1970). After 4 days of incubation at 28 °C, a single colony representing the major phenotype from each nodule extract was picked and purified by single-colony streaking on YEM plates. To test the efficiency of the surface-sterilization protocol, aliquots of the sterile distilled water used in the final rinse were plated onto YEM plates and incubated at 28 °C for 5 days. Whole sterilized nodules were also checked for surface sterilization by incubation in YEM broth (Vincent 1970).

### PCR–RFLP and sequencing analysis

PCR–RFLP of the 16S rRNA genes was conducted for the 104 isolates using the restriction endonucleases *MspI* and *NdeII* according to Mhamdi et al. (2002). Representative

isolates of the different ribotypes were selected for nearly full-length sequencing of the 16S rRNA gene according to Saïdi et al. (2011). Sequences were checked manually and assembled by the CAP program (<http://pbil.univ-lyon1.fr/cap3.php>). The BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to search for sequence similarities in the DNA databases. Sequences were aligned using the ClustalW2 software (<http://www.ebi.ac.uk/Tools/clustalw2/>). Phylogenetic trees were constructed using the phylogenetic inference package MEGA5 by the Neighbor-Joining method. The trees were bootstrapped with 1,000 replicates to assess the confidence limits of the branching. The evolutionary distance was computed using the Kimura 2-parameter method.

### Symbiotic tests

The nodule formation was checked for all isolates by inoculating faba bean (*V. faba*) and Lentil (*Lens culinaris*) seedlings grown on pots containing sterile gravel with approximately 10<sup>8</sup> cfu as described by Vincent (1970). Nodule formation was checked 40 days after inoculation. The nodulating isolates were typed by PCR–RFLP of the *nodC* gene and the intergenic spacer *nifD*-K using *HaeIII* as previously reported (Saïdi et al. 2009). Effectiveness on faba bean was tested on pots filled with sterile sand using a sample of 10 isolates representing the different *nifD*-K and *nodC* types as previously reported (Mhamdi et al. 2002). After two months, plants were harvested and shoot dry weights were recorded. Data were submitted to mean comparison using the LSD test ( $p < 0.05$ ) by the ANOVA program of the STATISTICA software. The non-nodulating isolates were tested for the presence of *nifH*-like genes using primers *nifH<sub>f</sub>* and *nifH<sub>i</sub>* according to Mhamdi et al. (2002) and by decreasing the hybridization temperature to 55 °C. A subsample of 18 isolates representing the different ribotypes found among the non-nodulating isolates was retained for further analysis.

### Production of indolic compounds

Production of indolic compounds by the selected isolated was analyzed using the PC-colorimetric technique of Pilet and Chollet (1970). TY medium supplemented with 2.5 mM of tryptophan (Trp) was used for preparing bacterial cultures (Glickmann and Dessaux 1995). The stationary phase culture was centrifuged at 6,000 rpm for 30 min and the supernatant was mixed with the Salkowski reagent (1:1 v/v). After incubation in darkness for 30 min at room temperature, color changes were measured at 530 nm. The production of indolic compounds was recognized by the

presence of red coloring. Varied concentrations of pure indole-3-acetic acid were used as standard curve.

#### Phosphate solubilization assay

Selected isolates were screened for their phosphate solubilization ability according to Beneduzi et al. (2008). Three replicates were considered for each strain. The formation of a clear zone around the bacterial colony was considered as positive result.

#### Siderophore production assay

Siderophore production by the selected isolates was tested qualitatively using Chrome Azurol S (CAS) agar as described by Alexander and Zuberer (1991). The bacterial cultures were spread on the CAS agar plates with two replications each. Orange halos around colonies after overnight incubation indicated the production of siderophores.

#### Salt tolerance

In order to select for isolates adapted to saline and arid regions, salt tolerance of isolates was carried out as previously described (Mnasri et al. 2007). Bacterial strains were grown on 1 M NaCl YEM broth medium. The ability to grow was checked visually after 5 days of incubation at 28 °C.

#### Controlled inoculation trial

Representatives of non-nodulating isolates were checked for their PGPR potentialities in a glasshouse trial. The isolates that featured taxa known as human pathogens were excluded from this experiment. Seeds of *V. faba* var. minor were grown in plastic pots (Ø 30 cm) containing soil samples from an experimental field of the Centre of Biotechnology of Borj-Cedria. YEM broth containing flasks were inoculated with a YEM slant pre-culture suspension and incubated on a rotary shaker at 150 rev min<sup>-1</sup> and 28 °C. At mid-exponential growth phase, the equivalent of 20 µl of broth culture for OD<sub>620nm</sub> = 1 was transferred to 250 ml flasks containing 100 ml of YEM broth and grown for 48 h. Emerging seedlings were inoculated with a fresh suspension of the bacterial strain (approximately 10<sup>9</sup> cfu). Nine plants were considered for each treatment (three plants per pot). Uninoculated plants were included as control. After 2 months, plants were harvested and shoot dry weights were recorded. Data were submitted to mean comparison as described above.

#### On-field inoculation trial

A small-scale field inoculation trial was performed in the region of Takilsa (36°47'12.98"N, 10°37'41.83"E) using a selected rhizobial strain (FB206) and a selected nodule endophyte (FB201b). The field was chosen for its proximity and also for the willingness of the farmer to cooperate to the experiment. The experimental design consisted of four treatments: uninoculated, inoculated with FB206, inoculated with FB201b and co-inoculated with both strains. Treatment plots were 10/10 m and were separated by a margin of 3 m. The sowing density was 20 seeds m<sup>-2</sup>. The inoculants were grown separately to late exponential phase in YEM medium and diluted with well water (1/20) and then used to inoculate two-leaf stage seedlings by manually applying about 5 ml on each plant along the seedling line using a watering can. The experiment was conducted under irrigation and without further fertilization, or any other amendment. Weeds were removed manually. Seventy days after inoculation, 30 plants were randomly collected from each treatment and used for the determination of shoot dry weight and grain yield. Data were submitted to mean comparison as described above.

## Results

### Diversity of *V. faba* root-nodule isolates

A collection of 104 strains isolated from surface-sterilized root-nodules of *V. faba* was firstly investigated with PCR-RFLP of 16S rRNA genes. Although the use of two restriction enzymes does not allow full determination of diversity, it allowed as a preliminary screening a quite accurate estimation of 22 ribotypes that were sampled for further analysis. Sequence analysis of nearly full-length 16S rRNA genes of representative isolates revealed a high genetic diversity (Table 1; Fig. S1). The three representative isolates of the dominant ribotype A1 (45 % of total isolates) showed 99.34 % sequence identity with the type strain of *Rhizobium leguminosarum* (USDA 2370<sup>T</sup>). When re-examined for nodulation, all members of ribotype A1 induced nodules on faba bean and lentil. However, all the remaining isolates, failed to induce nodules on both hosts. Non-symbiotic rhizobia lacking symbiotic genes (ribotypes A2, D, E, F, C, B1 and B2) were also frequently isolated (14 strains), their closest relatives were respectively, *Rhizobium grahamii*, *Rhizobium huautlense*, *Rhizobium albertimagni* (formerly *Agrobacterium albertimagni*), *Rhizobium pusense*, *Rhizobium nepotum* (formerly *Agrobacterium tumefaciens* genomovar G14) and *Ensifer meliloti*. These are followed by isolates of ribotype N (9 isolates) and ribotypes L1/2/3 (9 isolates), respectively affiliated to *Pseudomonas*

and *Enterobacter*. Isolates affiliated to *Pantoea* (ribotypes L4 and Q), *Bacillus* (ribotypes O, T and G) and *Rahnella* (ribotype I) were respectively represented by 7, 6 and 5 members. The remaining isolates within the ribotypes H, P, R and S were affiliated to *Staphylococcus*, *Serratia*, *Stenotrophomonas* and *Xanthomonas*.

The non-nodulating bacteria were checked for the presence of symbiotic genes. They failed to amplify *nifH* or *nodC* genes with the primers used even under low stringency conditions except for isolates assigned to *Rahnella* that showed a *nifH*-like amplification product.

#### PGPR abilities

The non-symbiotic isolates were checked for their in vitro abilities to produce indolic compounds, to solubilize phosphates and to produce siderophores. They were then used to

inoculate faba bean plants grown in non-sterile soil under glasshouse conditions. The results are given in Table 2. The shoot dry yield of several inoculated plants increased significantly (41–71 %) comparing to the uninoculated treatment. To confirm these results further, *Pseudomonas* sp. strain FB201b, showing positive results for all screening assays, was retained for a small scale field inoculation trial. Similarly, nodulating representatives of the different *nodC* and *nifD*-K PCR-RFLP groups were evaluated for their symbiotic effectiveness on faba bean under laboratory conditions (data not shown). Strain FB206, inducing the highest shoot dry weight, was also retained for field inoculation. Separate inoculations with strains FB206 or BF201b highly increased shoot dry weight and grain yield, the latter increased by more than thrice (Table 3). Co-inoculation with both strains also significantly increased grain yield comparing to inoculation with the rhizobial strain alone.

**Table 1** Taxonomic affiliation based on nearly full-length 16S rRNA sequence analysis of representative isolates from the different PCR-RFLP ribotypes

Ribotype <sup>a</sup> (RFLP)	Strain	Accession number	Closest relative species	Similarity (%)	Nodulation ability	Amplification of <i>nifH</i>
A1 (47)	FB206	JN558651	<i>R. leguminosarum</i> USDA2370 <sup>T</sup>	99.34	+	+
	FB10061	JN558659	<i>R. leguminosarum</i> USDA2370 <sup>T</sup>	99.34	+	+
	FB14022	JN558660	<i>R. leguminosarum</i> USDA2370 <sup>T</sup>	99.34	+	+
A2 (1)	FB803	JX943843	<i>R. grahamii</i> CCGE 502 <sup>T</sup>	99.52	–	–
D (1)	FB2501	JX943844	<i>R. huaatlense</i> SO2 <sup>T</sup>	100	–	–
E (1)	FB11041	JX943845	<i>R. albertimagni</i> AOL15 <sup>T</sup>	100	–	–
F (1)	FB17031	HQ007645	<i>R. pusense</i> NRCPB10 <sup>T</sup>	99.91	–	–
C (2)	FB1802	HM194623	<i>R. nepotum</i> LMG26435 <sup>T</sup>	100	–	–
	B1 (8)	FB19011	HQ007644	<i>R. nepotum</i> LMG26435 <sup>T</sup>	99.92	–
	FB1406	JX943864	<i>R. nepotum</i> LMG26435 <sup>T</sup>	99.92	–	–
	FB13051	HM194620	<i>S. kummerowiae</i> CCBAU 25048 <sup>T</sup>	98.58	–	–
	B2 (2)	FB2002	JX943861	<i>E. meliloti</i> IAM 12611 <sup>T</sup>	99.62	–
G (1)	FB1502	JX943846	<i>Bacillus megaterium</i> IAM 13418 <sup>T</sup>	99.84	–	–
O (1)	FB705	JX943855	<i>B. muralis</i> LMG 20238 <sup>T</sup>	98.58	–	–
T (4)	1FB2005	JX943862	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> DSM10 <sup>T</sup>	99.92	–	–
H (2)	FB10021	JX943847	<i>S. saprophyticus</i> ATCC 15305 <sup>T</sup>	100	–	–
I (5)	FB303	JX943848	<i>R. aquatilis</i> DSM 4594 <sup>T</sup>	98.77	–	+
L1 (3)	FB1701	JX943849	<i>E. cancerogenus</i> LMG 2693 <sup>T</sup>	99.55	–	–
L2 (5)	FB105	JX943850	<i>E. amnigenus</i> JCM1237 <sup>T</sup>	99.84	–	–
L3 (1)	FB9063	JX943851	<i>E. amnigenus</i> JCM1237 <sup>T</sup>	99.73	–	–
L4 (1)	FB704	JX943852	<i>Pantoea agglomerans</i> DSM 3493 <sup>T</sup>	99.74	–	–
Q (6)	FB2011	JX943863	<i>P. agglomerans</i> DSM 3493 <sup>T</sup>	99.91	–	–
	FB22012	JX943857	<i>Pantoea brenneri</i> LMG 5343 <sup>T</sup>	100	–	–
N (9)	FB201b	JX943854	<i>P. marginalis</i> LMG 2210 <sup>T</sup>	99.77	–	–
P (1)	FB504	JX943856	<i>S. odorifera</i> DSM 4582 <sup>T</sup>	100	–	–
R (1)	FB24052	JX943858	<i>S. maltophilia</i> IAM 12423 <sup>T</sup>	98.99	–	–
S (1)	FB9072	JX943859	<i>Xanthomonas campestris</i> ( <i>X. translucens</i> ) LMG 876 <sup>T</sup>	99.91	–	–

<sup>a</sup> The total number of isolates in each ribotype is indicated between parentheses

## Discussion

Faba bean (*V. faba* var. minor) and fava bean (*V. faba* var. major) are considered major legume crops in Tunisia and had been extensively cultivated as green manure as well as for feed and food. They are extensively used in co-cropping and intercropping systems with different cultures where they are believed to enhance soil fertility through their symbiotic potentialities. These crops are usually well nodulated in Tunisian soils; however, there are no studies about the diversity and the effectiveness of the nodulating rhizobia. In this study a special interest has been focused on microbial diversity of *V. faba* nodule isolates with the aim to select efficient strains for inoculation. A unique isolate representing the major colony-morphology phenotype was retained for each nodule analyzed. Only 45 % of isolates were able to re-nodulate their original host. They were all closely related to *R. leguminosarum*. However, a high proportion of isolates (55 %) could not nodulate *V. faba* and either *L. culinaris*. The most frequent were non-symbiotic rhizobia, mainly taxa previously known as agrobacteria (25 %), and taxa affiliated to *Pseudomonas* (16 %) and *Enterobacter* (16 %), which have been frequently isolated from root-nodules of various legumes (de Lajudie et al. 1999; Ibanez et al. 2009; Ramezanpour 2011). In most cases, isolates were affiliated to type strains with high 16S sequence identity, except for isolates of

ribotypes I, O and R, and two isolates from ribotype B1, whose closest relative species were respectively, *Rahnella aquatilis*, *Bacillus muralis*, *Stenotrophomonas maltophilia* and *Shinella kummerowiae*, with less than 99 % of sequence similarity (Table 1). These strains could probably constitute new species but further analysis such as housekeeping gene sequencing and DNA-DNA hybridization have to be performed.

The prevalence of non-nodulating isolates in nodule extracts may be explained by different alternatives, including loss of symbiotic genes, opportunistic colonization by rhizospheric bacteria or surface contamination of nodules. However, since they survived after chemical surface sterilization of nodules and represented the major phenotype recovered from each nodule they will be considered as putative endophytes. Microscopic observations are required to definitively confirm the endophytic character of these isolates and clarify their localization inside nodule tissues. This had been shown at least for nodule isolates related to the former *Agrobacterium* (Mhamdi et al. 2005). Compared to similar studies on other legumes in Tunisia, such as *Sulla coronaria*, *Cicer arietinum* (unpublished data) *Phaseolus vulgaris* (Mhamdi et al. 2002) that showed that endophytic colonization of nodules does not exceed 25 %; in the present study we found that 55 % of nodule isolates were putative endophytes. This

**Table 2** Plant growth promoting abilities of representatives of the non-symbiotic nodule isolates of *V. faba*

Isolate	Affiliation	Total indoles $\mu\text{g ml}^{-1}$		P-Solubilization	Siderophore production	Growth on 1 M NaCl	Effect on plant growth
		(-trp)	(+trp)				
FB803	<i>Rhizobium</i> sp.	4.6	9.4	–	–	–	NS
FB2501	<i>Rhizobium</i> sp.	3.5	21.7	nt	–	–	S (67 %)
FB19011	<i>Rhizobium</i> sp.	3.3	12.9	–	+	+	S (55 %)
FB1802	<i>Rhizobium</i> sp.	5.2	27.9	–	+	–	S (47 %)
FB1406	<i>Rhizobium</i> sp.	3.9	13.2	–	+	–	S (43 %)
FB17031	<i>Rhizobium</i> sp.	9.4	36.7	–	–	+	NS
FB11041	<i>Rhizobium</i> sp.	11.3	41.5	–	–	+	S (71 %)
FB2002	<i>Ensifer</i> sp.	3.9	11.1	–	–	–	S (50 %)
FB13051	<i>Shinella</i> sp.	6.2	17.9	–	–	–	NS
FB303	<i>Rahnella</i> sp.	6.7	20.9	+	–	+	S (63 %)
FB1502	<i>Bacillus</i> sp.	5.9	3.2	+	–	+	NS
1FB2005	<i>Bacillus</i> sp.	4.8	6.2	–	–	+	NS
FB705	<i>Bacillus</i> sp.	6.3	7.8	nt	nt	–	nd
FB2011	<i>Pantoea</i> sp.	15.4	20.3	+	–	nt	S (57 %)
FB22012	<i>Pantoea</i> sp.	7.8	8.6	+	–	+	NS
FB704	<i>Pantoea</i> sp.	21.7	16.5	+	–	+	NS
FB201b	<i>Pseudomonas</i> sp.	3.9	14.2	+	+	+	S (41 %)
FB9072	<i>Xanthomonas</i> sp.	nt	nt	–	–	+	nt

+ positive result, – test achieved but result is negative, *nd* not tested, *S* significant increase in shoot dry yield comparing to the non-inoculated control as indicated between parentheses, *NS* no significant increase in shoot dry yield comparing to the non-inoculated control

**Table 3** Effect of field inoculation of *V. faba* with selected strains on shoot and seed dry weights

Treatment	Shoot dry weight (g plant <sup>-1</sup> )	Seed dry weight (g plant <sup>-1</sup> )
Uninoculated control	4.67c	1.46c
Inoculated with <i>Rhizobium</i> sp. strain FB206	8.93ab	4.64b
Inoculated with <i>Pseudomonas</i> sp. strain FB201b	7.79b	5.34ab
Co-inoculated with FB206 and FB201b	9.29a	6.81a

Means with different letters are significantly different (LSD test,  $p < 0.05$ ,  $n = 30$ )

high frequency of endophytes in *V. faba* root-nodules prompted the study of their effect on plant growth. However, the strains related to the species *Serratia odorifera*, causing serious disease including pneumonia and bacteremia (Lee et al. 2006), *Staphylococcus saprophyticus*, associated with urinary tract infection (Raz et al. 2005), *S. maltophilia*, an emerging pathogen for nosocomial infections and meningitis (Adamek et al. 2011), *Enterobacter amnigenus*, that can cause opportunistic bacterial infection in human (Capdevila et al. 1998) and *Enterobacter cancerogenus*, that can cause wound infections and septicemia (Abbott and Janda 1997) will not be further considered in this work because of their potential human disease properties. In all cases, it would be necessary to check the pathogenicity of all strains prior to recommend their future use.

Screening for PGPR abilities showed that production of indolic compounds by nodule endophytes varied greatly among different species and strains. The highest producers were strains affiliated to *Pantoea*. However, in presence of Trp, a metabolic precursor of indolic compounds used by most soil bacteria, the largest productions were found among strains affiliated to the *Agrobacterium/Rhizobium* group, then *Rahnella* sp., *Shinella* sp. and *Pantoea* sp. The lowest producers of indolic compounds that seemed unaffected by the addition of Trp were strains affiliated to *Bacillus*. Only strains affiliated to *R. nepotum* and *Pseudomonas marginalis* were positive producers of siderophores. The most efficient phosphate solubilizing strains were affiliated to *Pseudomonas* genus whose strains are well known as efficient phosphate solubilizers (Tilak et al. 2005). Strains affiliated to *Rahnella*, *Bacillus* and *Pantoea* spp. were also phosphate-solubilizing bacteria, but with less efficiency. For the remaining strains, the halo was discreet or absent, their phosphate solubilization ability has to be verified according to the direct measurement of phosphate solubilization in NBRI-BPB broth assay (Mehta and Nautiyal 2001).

The glasshouse trial indicated that 9 strains induced significant increase (41–71 %) in shoot dry yield. These strains were respectively affiliated to *Rhizobium* (5 isolates), *Ensifer*, *Rahnella*, *Pantoea* and *Pseudomonas* species. However, there was no clear correlation between the PGPR abilities observed in vitro and shoot dry yield. Nevertheless, isolates with low production of indolic compounds (<10 µg/ml) usually did not produce significant increase in plant growth. The on-farm trial confirmed the high effectiveness of *Pseudomonas* sp. strain FB201b when inoculated alone or in combination with the selected rhizobial strain FB206. Plants in the control treatment were all highly nodulated, which would indicate the high competitive ability of the selected rhizobial strain against the resident bacterial population. The positive effect observed with the rhizobial strain could be also due to indirect effects on rhizosphere microbial communities. Trabelsi et al. (2012) showed that inoculation of common bean with rhizobia may induce proliferation of bacterial communities with PGPR potentialities. The extent of these changes marked also the second crop season in more ways than just furnishing residual nutrients, but also through stimulating plant growth and enhancing disease resistance. The way that rhizobia enhance proliferation of other microbial communities is not clear and should be investigated further. It seems that nodules may play a crucial role as a niche for proliferation and dissemination seeing their ability to be colonized by different bacterial taxa. This issue raises many questions about the possible involvement of legume nodules in increasing risks related to plant and human pathogens. If this is true, agronomic practices based on co-cropping and inter-cropping with legumes, which had been prompted for a long time, should be reconsidered mainly in soils contaminated with pathogens. Further work should be done to shed more light on this issue.

## Conclusion

Bacteria isolated from root-nodules of *V. faba* grown in different Tunisian soils were highly diverse and could be affiliated to 12 genera, including *R. leguminosarum* and a high proportion of putative nodule endophytes. Some of these endophytes showed PGPR abilities, indicating that legume nodules may be a valuable source for selecting effective microorganisms. However, other endophytes featured taxa known as human pathogens, raising the concern of enhancing the hazards posed by pathogens.

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