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Diversity, phylogeny and distribution of bean rhizobia in salt-affected soils of North-West Morocco

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Abstract

Different molecular methods: BOX-PCR fingerprinting, RFLP-PCR and sequencing of the 16S rDNA as well as the symbiotic genes *nodC* and *nifH*, were used to study the genetic diversity within a collection of nodulating bean rhizobia isolated from five soils of North-West Morocco. BOX fingerprints analysis of 241 isolates revealed 19 different BOX profiles. According to the RFLP-PCR and sequencing of 16S rDNA carried out on 45 representative isolates, 5 genotypes were obtained corresponding to the species *Rhizobium etli*, *R. tropici*, *R. gallicum*, *R. leguminosarum* and *Sinorhizobium meliloti*. The most abundant species were *R. etli* and *R. tropici* (61% and 24%, respectively). A high intraspecific diversity was observed among the *R. etli* isolates, while the *R. tropici* group was homogeneous. Most of the rhizobia studied belong to species known to nodulate common bean, while 2 species were unconventional microsymbionts: *R. leguminosarum* biovar *viciae* and *S. meliloti*. Our results, especially the nodulation promiscuity of common bean and the relation between the predominance of some species of rhizobia in particular soils and the salt content of these soils, indicate that there is a real need for a better understanding of the distribution of common bean rhizobia species in the soils of Morocco before any inoculation attempt.

Keywords: *Phaseolus vulgaris*, rhizobia, diversity, *R. tropici*, *R. etli*, salinity

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is an important traditional food crop in Latin America and Africa. The origin of this legume is thought to be in Latin America and the first areas of its domestication were the highlands of Mesoamerica and the Andean South America, some 5000 years ago (Kaplan, 1965; Kaplan et al., 1973; Kaplan, 1980). Its ability to adapt to different climates and its nutritional properties made it rapidly spread worldwide, with its cultivation extending from Mesoamerica to the Carribean, and then to Brazil. *Phaseolus vulgaris* was introduced from Brazil into Africa in the 16th century, (Odee et al., 2002).

As with most legumes, common bean has the ability to establish a nitrogen-fixing symbiosis with rhizobia. Because *P. vulgaris* is a relatively non-selective host (Michiels et al., 1998), more and more bean nodulating rhizobia have been reported in different parts of the world and their classification is always under revision. Five *Rhizobium* species able to nodulate and fix nitrogen with *Phaseolus* sp. are currently described: *Rhizobium leguminosarum* bv. *phaseoli* (Jordan, 1984), *R. etli* (Segovia et al., 1993), *R. gallicum*, *R. giardinii* (Amarger et al., 1997) and *R. tropici* IIA and IIB (Martínez-Romero et al., 1991). In addition, some strains of *Sinorhizobium* (Sadowsky et al., 1981; Bromfield and Barran, 1990; Herrera-Cervera et al., 1999; Mhamdi et al., 2002) and *Bradyrhizobium* (Michiels et al., 1998) were reported to form root nodules on bean (for a review see Martinez-Romero, 2003).

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The geographical distribution of the nodulating species varies. On the African continent, in West Africa and Kenya, *R. leguminosarum*, *R. tropici* and *R. etli* were isolated from soils (Anyango et al., 1995; Diouf et al., 2000; Odee et al., 2002), whereas in Tunisia, Mhamdi et al. (2002) reported the occurrence of *R. etli* bv. *phaseoli*, *R. gallicum* bv. *phaseoli*, *R. giardinii* bv. *giardinii*, *R. leguminosarum* bv. *phaseoli* and bv. *viciae*. More recently, *R. etli* strains were isolated from Ethiopian soils (Beyene et al., 2004) and Egypt (Shamseldin et al., 2005).

The distribution of rhizobia is dependent on the soil properties and the agricultural history of field sites. The ability of rhizobia to nodulate the appropriate host can be affected by different environmental stresses (Graham, 1992). Reports about common bean rhizobia in Morocco are, however, still limited (Priefer et al., 2001; Bouhmouch et al., 2005) and almost nothing is known about their diversity and distribution in the soil. In this work, we have choosen to study the indigenous rhizobia of different Moroccan soils where non inoculated common bean is cultivated regularly in rotation with different vegetables. Rhizobial isolates were characterised physiologically and genotypically with different molecular methods in order to be classified phylogenetically. Our overall goal is to find out if ecological factors can influence the distribution of common bean rhizobia species in these soils and whether there is an impact of the species distribution on the efficiency of nitrogen fixation by common bean.

2. Materials and Methods

Soil analysis

Soil samples were collected from 5 uninoculated bean fields in North-West Morocco (surroundings of Rabat; 34.02°N, 6.51°W). Soil pH and electric conductivity (EC in mS/m in duplicates) were measured with a glass electrode membrane pH-meter (HI 8314 HANNA Instruments GmbH, Germany) and a conductivity-meter (WTW, Germany), respectively. The C/N ratios, as well as the carbon and nitrogen contents were determined using a computer-assisted elemental analyser (Vario ELIII, Analysensysteme GmbH, Germany).

Bacterial isolation from soil

Seeds of common bean (*Phaseolus vulgaris* L.) cv. Coco Blanc were surface-sterilized as described by Somasegaran and Hoben (1994) and germinated in pots containing sterilized vermiculite, for 2–3 days at 24°C. The seedlings were transfered to a mixture of sterile vermiculite and a soil suspension according to Zhang et al. (2001). Three plants per pot (and 4 pots per soil) were grown and regularly watered with a nitrogen-free plant nutrient solution according to Broughton and Dilworth (in Somasegaran and Hoben, 1994). After 4 weeks, between 40 and 50 nodules were randomly harvested from plants grown on each soil (from the four pots), surface sterilized, crushed and purified on yeast-mannitol-agar plates (YMA) (Vincent, 1970) supplemented with 25 µg/ml congo red.

Molecular characterisation of the isolates

Total DNA for each isolate was extracted following the CTAB protocol described by Ausubel et al. (1987) and stored at –20°C. Reference strains included in this study were the following: *R. tropici* IIB CIAT899, *R. tropici* IIA CFN299, *R. etli* bv. *phaseoli* CFN42, *R. gallicum* bv. *gallicum* R602sp, *R. giardinii* bv. *giardinii* H152, *R. leguminosarum* bv. *phaseoli* ORS663, *R. leguminosarum* bv. *viciae* 3841, *S. meliloti* GR4 and *Agrobacterium* bv. 1 ORS1351.

BOX-PCR fingerprinting

The PCR was performed using the BoxA1R primer according to the conditions described by Versalovic et al. (1991). The PCR amplification was carried out in a thermocycler Triothermoblock (Biometra, Netherlands), and PCR products were separated on Tris-Borate (TB) 2% agarose gels. The fingerprints were analysed using GelCompar II (Applied Maths, Kortrijk, Belgium) to determine potential groups.

ARDRA and sequencing of 16S rDNA

A 1.4 Kb-sized region of the 16S rRNA gene was amplified using 2 primers, 41f (forward) and 1488r (reverse) as described by Herrera-Cervera et al. (1999). PCR products were individually digested with 4 restriction enzymes (*Msp*I, *Rsa*I, *BsuR*I and *Mbo*I). The restriction fragments were separated on TB 2% agarose gels and the patterns recorded were compared to the reference strains mentioned above. The 16S rRNA genes of representative isolates were fully sequenced using the forward primer 27f (5′AGAGTTTGATCCTGGCTCAG) (Lukow et al., 2000) and two reverse primers, 927r (5′CCGCTTGTGCGGGCCC 3′ (Amann et al., 1995) and 1488r (Herrera-Cervera et al., 1999). The PCR products were purified with the Nucleospin Extract Kit (Macherey-Nagel, Germany) and sequenced by Millegen (Prologue Biotech, France). The sequences generated were compared to the NCBI-nr sequence database and aligned using ClustalX 1.81 (Thompson, 1997). A phylogenetic tree was obtained using Treeview 1.6.1 (Page, 1996). The sequences of the 16S rRNA gene of the Moroccan isolates were submitted to the NCBI Nucleotide Database (accession numbers DQ406695 to DQ406713).

Table 1. General soil parameters.

Soil	Origin	Soil type	pH	EC (mS/m)	C (%)	N (%)	C/N ratio
1	Ain Atiq	Sandy	7.2	266 (±28)	0.691	0.077	9.0
2	Skhirat	Sandy	8.2	418 (±43)	0.925	0.091	10.2
3	Salé	Sandy	8.2	96 (±15)	0.846	0.088	9.7
4	Hay Ryad	Sandy	6.7	107 (±30)	0.572	0.058	9.9
5	Ben Slimane	Clay	7.8	176 (±9)	1.701	0.157	10.9

Table 2. BOX genotypes within the bean-rhizobia collection.

BOX genotypes	Number of isolates	Number of isolates chosen for 16S rDNA RFLP
a	57	10
b	1	1
c	1	1
d	1	1
e	12	3
f	1	1
g	1	1
h	1	1
i	2	1
j	1	1
k	23	4
l	7	2
m	2	1
n	37	3
o	9	2
p	4	1
q	76	6
r	4	4
s	1	1
SUM 19	241	45

RFLP and sequencing of nodC *and* nifH *genes*

The nodCF/nodCI and nifHF/nifHI primer couples (Laguerre et al., 2001) were used to amplify fragments of *nodC* (930 bp) and *nifH* (780 bp). The *nodC* PCR products were subjected to a digestion with 5 restriction enzymes (*Hha*I, *Hae*III, *Msp*I, *Hinf*I and *Rsa*I), while 3 enzymes (*Msp*I, *Hae*III and *Hha*I) were used to digest the *nifH* PCR products.

For sequencing, the PCR products of the representative strains were purified and sequenced as described above. The primers used in this case were nodCF and nodCI for *nodC* and nifHF and nifHI for *nifH* fragments, respectively. All the sequences of *nodC* and *nifH* of the Moroccan isolates are stored in the NCBI Nucleotide Database under the following accession numbers: DQ413002 to DQ413013 and DQ413014 to DQ413021, for *nodC* and *nifH* respectively.

Physiological characterisation of the isolates

The isolates were tested for their tolerance to different stresses: pH, NaCl and temperature. A modified YMA medium adjusted at pH ranging from 4 to 9 (Mannitol 10 g/l; K_2HPO_4, 3 H_2O 0.46 g/l; $MgSO_4$, 7 H_2O 0.2 g/l; yeast extract 1 g/l; 1% congo red 2.5 ml/l and agar 15 g/l) was used to evaluate the tolerance of the isolates to different pH. Buffer HOMOPIPES (Research Organics) was used at a concentration of 25 mM for pH 4 and 5, and buffer TAPS (SIGMA Aldrich) 25 mM was added to the medium to reach pH 8 and 9. To test the tolerance of the isolates to salt, different amounts of NaCl were added to a modified YMA medium (Mannitol 10 g/l, KH_2PO_4, 3 H_2O 0.41 g/l; K_2HPO_4, 3 H_2O 0.52 g/l; $MgSO_4$, 7 H_2O 0.2 g/l; yeast extract 1 g/l; 1% congo red 2.5 ml/l and agar 15 g/l) in order to reach the following final concentrations: 0 M, 0.17 M, 0.34 M, 0.51 M and 0.68 M. The temperature tolerance was checked on YMA medium (Vincent, 1970), at 15°C, 20°C, 30°C, 37°C, 40°C and 45°C. For each test, a preculture of each isolate was incubated in YMB (Vincent et al., 1970) at 28°C until a OD_{600nm} of 0.8 to 1 was reached. Then, 10 µl of each culture were dropped on the appropriated medium and incubated at 28°C for 2 to 4 days, except for the temperature tolerance test in which the isolates were incubated at different temperatures.

The pH, NaCl concentration or temperature tolerance were considered as positive when a growth was seen on a plate.

3. Results

The host trap method led to the isolation of 241 isolates that were labelled as follows: RP201 to RP253 isolates were obtained from soil 1, RP254 to RP301 from soil 2, RP302 to RP351 from soil 3, RP352 to RP398 from soil 4 and RP399 to RP446 from soil 5. The physico-chemical parameters of the soils revealed that all but soil 4 of Hay Ryad have neutral to slightly alcaline pHs. The EC values (Table 1) showed that soils 1, 2 and 5 are affected by salt while soils 3 and 4 are non saline.

Phylogenetical analysis of bean-rhizobia isolated from soils of Morocco

The Box patterns of the 241 isolates were visualized using GelCompare II and compared with reference strains. Among the 241 isolates, 19 different Box fingerprint types were identified, from which type *q* was predominant (31%), followed by type *a* (24%) (Table 3). The isolates belonging to Box type *a* were identical to the reference strain *R. tropici* IIB CIAT899 and isolates belonging to type *r* were very similar to *S. meliloti* GR4. The remaining Box types showed no obvious similarities to the Box patterns of the reference strains used in this study (Fig. 1).

Considering the fact that isolates with similar genomic fingerprints produce, in general, the same 16S rDNA restriction patterns (Laguerre et al., 1996), we used 45 randomly chosen isolates representing all Box types and subjected them to ARDRA analysis (Table 2). Five different 16S rDNA ribotypes were recorded and compared to the restriction patterns of rhizobial reference strains. The 10 isolates representing Box type *a* had the same genotype as *R. tropici* strains CIAT899 and CFN299. Twenty-one isolates showed the same 16S rDNA ribotype as *R. etli* CFN42, although they belonged to 9 different Box types. In addition, the isolates of 6 other Box types exhibited 16S rDNA ribotypes that were identical to those of the two *R. leguminosarum* strains ORS663 and 3841. The isolates of Box type *k* produced the same 16S rDNA restriction patterns as *R. gallicum* R602sp and the 2 remaining Box types (*r* and *s*) revealed a 16S rDNA ribotype identical to that of *S. meliloti* GR4.

Nineteen isolates representing the different Box types were selected for direct sequencing of the 16S rDNA PCR fragments. About 1353 to 1369 bp of each sequence were aligned and a bootstrap tree was generated (Fig. 2). The 9 isolates assigned to *R. etli* were 99% nucleotide identity to *R. etli* strains SEMIA384, IE4804, ISM4 and PRF76. The isolates related to *R. tropici* showed 99% identity with *R. tropici* LMG9518 and *Rhizobium* genosp. Q BDV5102. The isolate close to *R. gallicum* showed 99% similarity with *R. gallicum* R602sp and *R. mongolense* USDA1929. All the isolates affiliated with *R. leguminosarum* were 99% identical to *R. leguminosarum* strain USDA2498. The sequences of the isolates related to *S. meliloti* (i.e. RP254 and RP276) aligned at 100 and 99%, respectively, to that of *S. meliloti* strain 1021.

The species identified by ARDRA and sequence analysis exhibited different levels of intraspecific diversity. A high degree of diversity was observed within the 147 *etli*-like isolates, which produced 9 distinct Box fingerprint types different from that of *R. etli* strain CFN42 (Table 3). Similarly, the 9 isolates related to *R. leguminosarum* exhibited 6 Box types which were not related to the two reference strains of *R. leguminosarum* (ORS663 and 3841). However, no obvious intraspecific diversity was observed in the case of the *tropici*-like and *gallicum*-like isolates (data not shown). When the results of the fingerprint analyses (16S rDNA and BOX) were extended to the complete collection of isolates (241), 61% of the common bean rhizobia isolated from Moroccan soils were considered as *etli*-like, 24% as *tropici*-like, 10% as *gallicum*-like, 4% as *leguminosarum*-like, and 1% as *meliloti*-like.

RFLP analysis of symbiotic genes

The same 45 isolates used for 16S rDNA RFLP were used to amplify and digest *nodC* (930 bp) and *nifH* (780 bp) gene fragments. Altogether, 12 *nodC* profiles and 10 *nifH* profiles were recorded (Table 3). The isolates related to the species *R. tropici* (*nodC* type A) and *R. gallicum* bv. *gallicum* (*nodC* type I), shared all their *nodC* RFLP patterns with their respective reference strains. Among the 21 isolates related to *R. etli*, 6 *nod* types (C, D, E, F, H and K) were identified. Type K was typical of bv. *phaseoli* since it was identical to the reference *R. etli* bv. *phaseoli* CFN42. Type H isolates shared three of their restriction patterns with strain CFN42. Type E was different from the *nod* type of strain CFN42 in 3 restriction patterns, whereas types C and D were different in 4 restriction patterns. Finally, type F shared no restriction pattern with *R. etli* bv. *phaseoli* CFN42. Three *nod* profiles (B, G and J) were recorded among the 6 isolates related to *R. leguminosarum*. Type B isolates harboured the same patterns as *R. leguminosarum* bv. *viciae* strain 3841. However, none of the three types shared restriction patterns with the reference *R. leguminosarum* bv. *phaseoli* ORS663. The *meliloti*-like isolates (*nodC* type L) shared no restriction patterns with any of the typical bean rhizobia references. Results of the *nifH* RFLP analysis supported those of *nodC*. The isolates assigned to the species *R. tropici*, *R. gallicum* bv. *gallicum and S. meliloti* harboured the same *nifH* type as their respective reference strains (A, F and G respectively, Table 3). Four *nifH* types (B, C, D and E) were recorded among the *etli*-like isolates. Type C shared all its restriction patterns with *R. etli* bv. *phaseoli* CFN42. In the case of the 3 remaining types, one restriction pattern was different. The *nifH* restriction patterns of the 6 *leguminosarum*-like isolates were again different from those of the *R. leguminosarum* bv. *phaseoli* ORS663.

Sequencing of nodC *and* nifH *PCR fragments*

Twelve isolates representing the 12 *nodC* types and 10 isolates representing the10 *nifH* types were subjected to the sequencing of *nodC* and *nifH* PCR products in order to clarify the phylogenetic position of their symbiotic genes. The phylogenetic trees based on the aligned sequences of *nodC* and *nifH* are presented in Fig. 2. A portion of 801bp of the *nodC* (Position 335–935 of the *S. meliloti* 1021 *nodC*

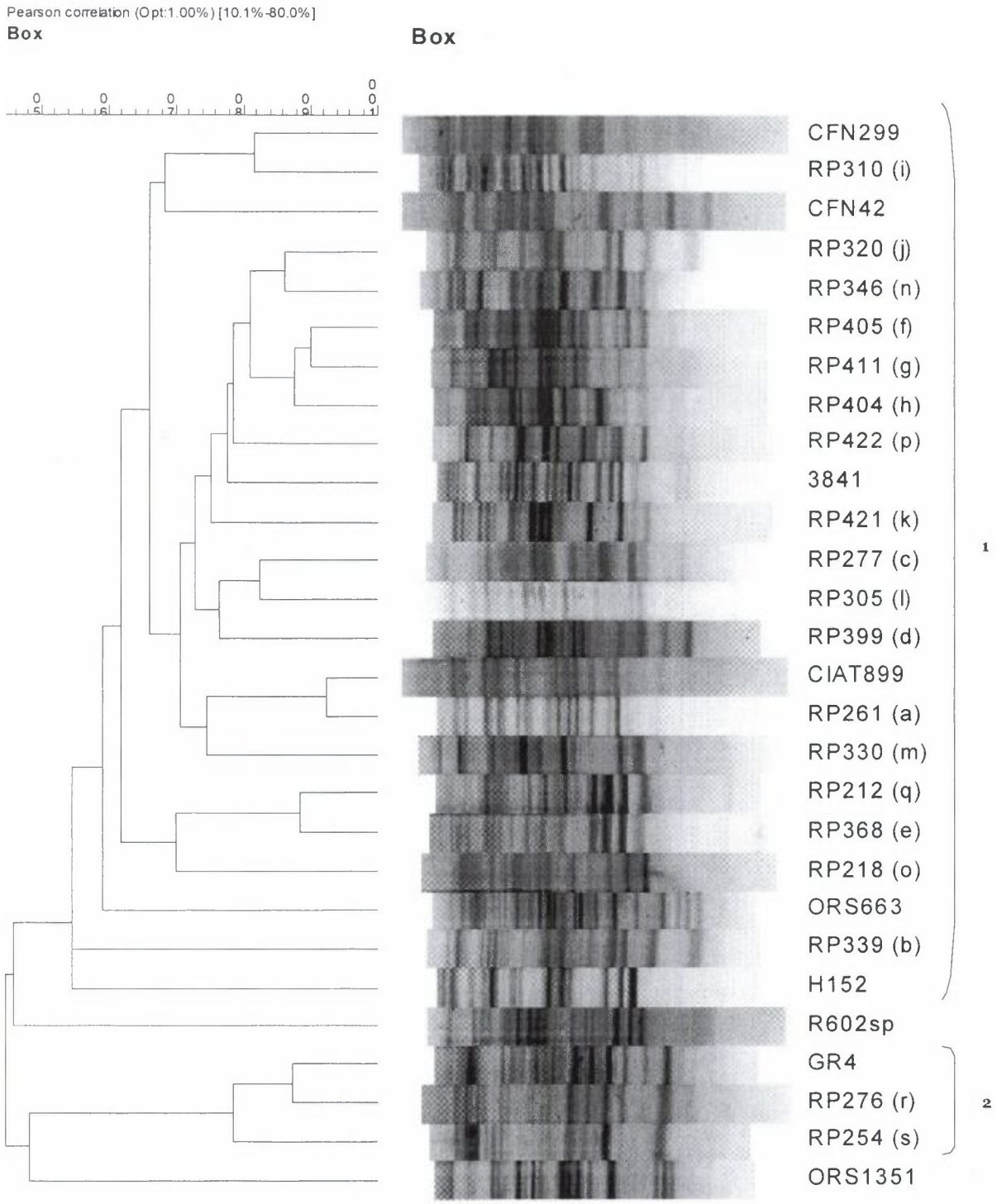

Figure 1. BOX A1R-PCR dendrogram showing 19 representative isolates recovered (by trap host) from 5 Moroccan soils. The following reference strains were included for comparison: CFN299 (*R. tropici* IIA), CFN42 (*R. etli*), 3841 (*R. leguminosarum* bv. *viciae*), CIAT899 (*R. tropici* IIB), ORS663 (*R. leguminosarum* bv. *phaseoli*), H152 (*R. giardinii*), R602sp (*R. gallicum*), GR4 (*Sinorhizobium meliloti*) and ORS1351 (*Agrobacterium* bv. 1). The comparison of the fingerprints was based on the Pearson correlation and the UPGMA grouping method. The letters between brackets correspond to the Box fingerprint types.

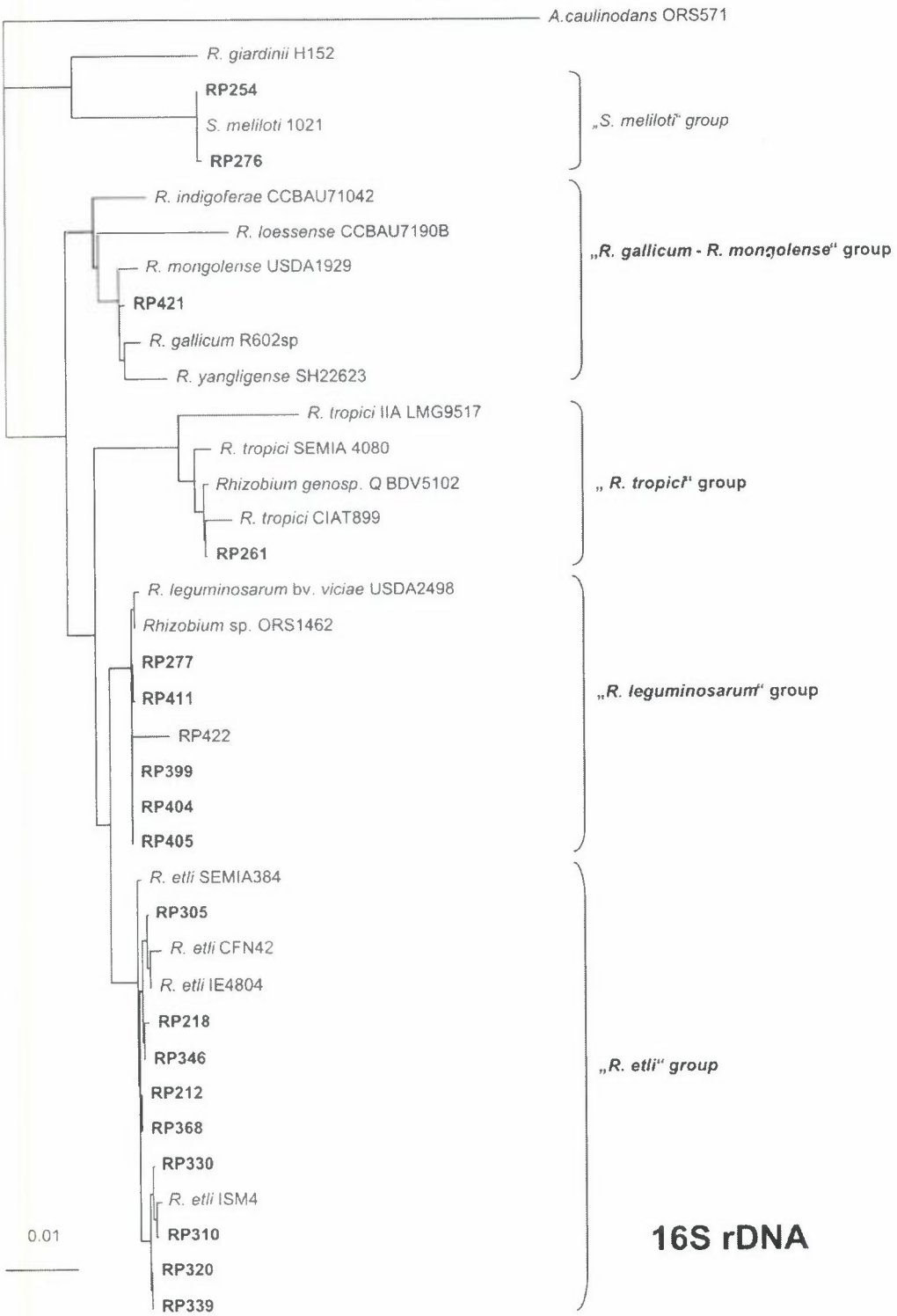

Figure 2A. See legend on p. 91.

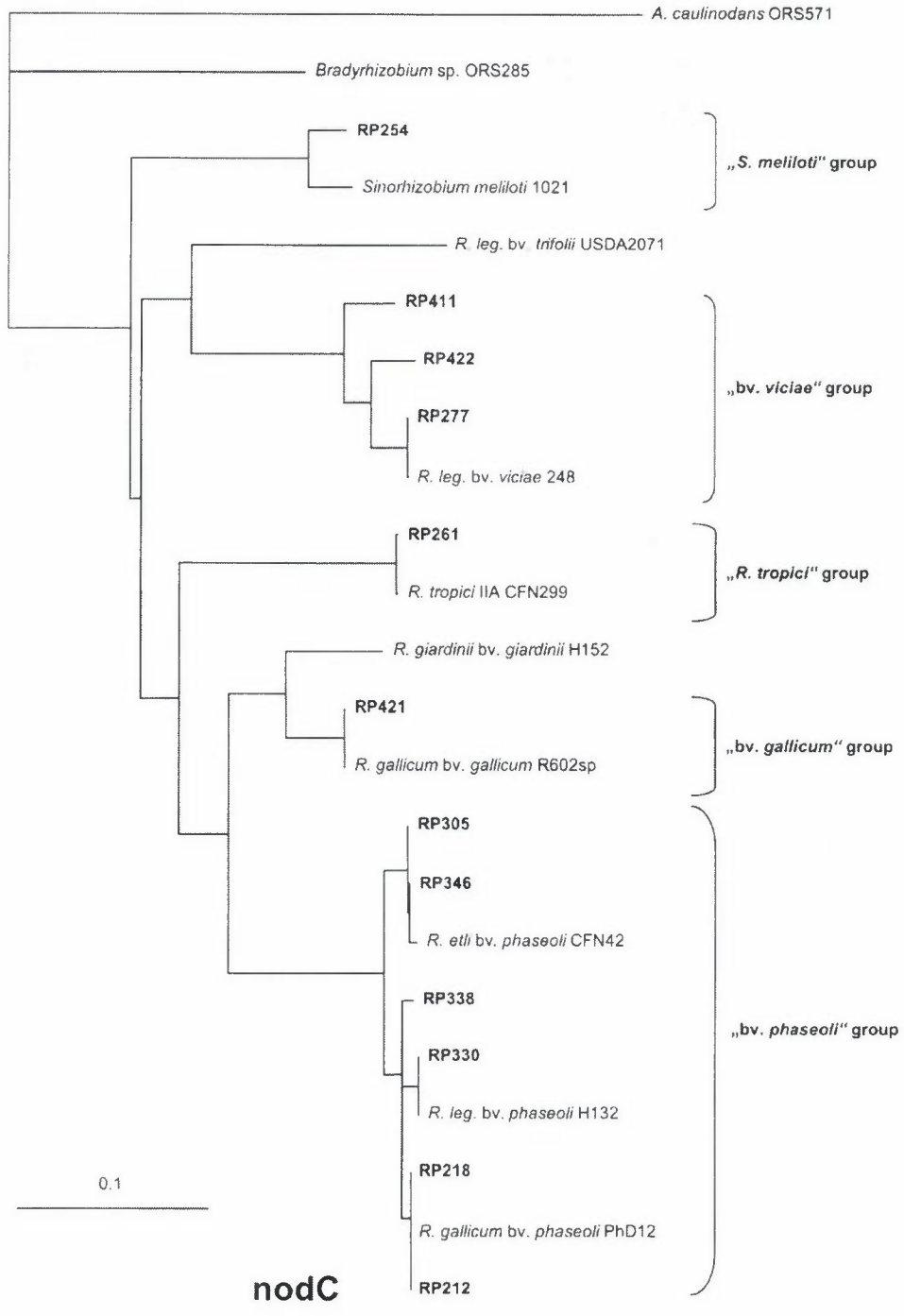
A. caulinodans ORS571
Bradyrhizobium sp. ORS285
RP254
Sinorhizobium meliloti 1021
„S. meliloti" group
R. leg. bv. trifolii USDA2071
RP411
RP422
RP277
R. leg. bv. viciae 248
„bv. viciae" group
RP261
R. tropici IIA CFN299
„R. tropici" group
R. giardinii bv. giardinii H152
RP421
R. gallicum bv. gallicum R602sp
„bv. gallicum" group
RP305
RP346
R. etli bv. phaseoli CFN42
RP338
RP330
R. leg. bv. phaseoli H132
„bv. phaseoli" group
RP218
R. gallicum bv. phaseoli PhD12
RP212
0.1
nodC

Figure 2B.

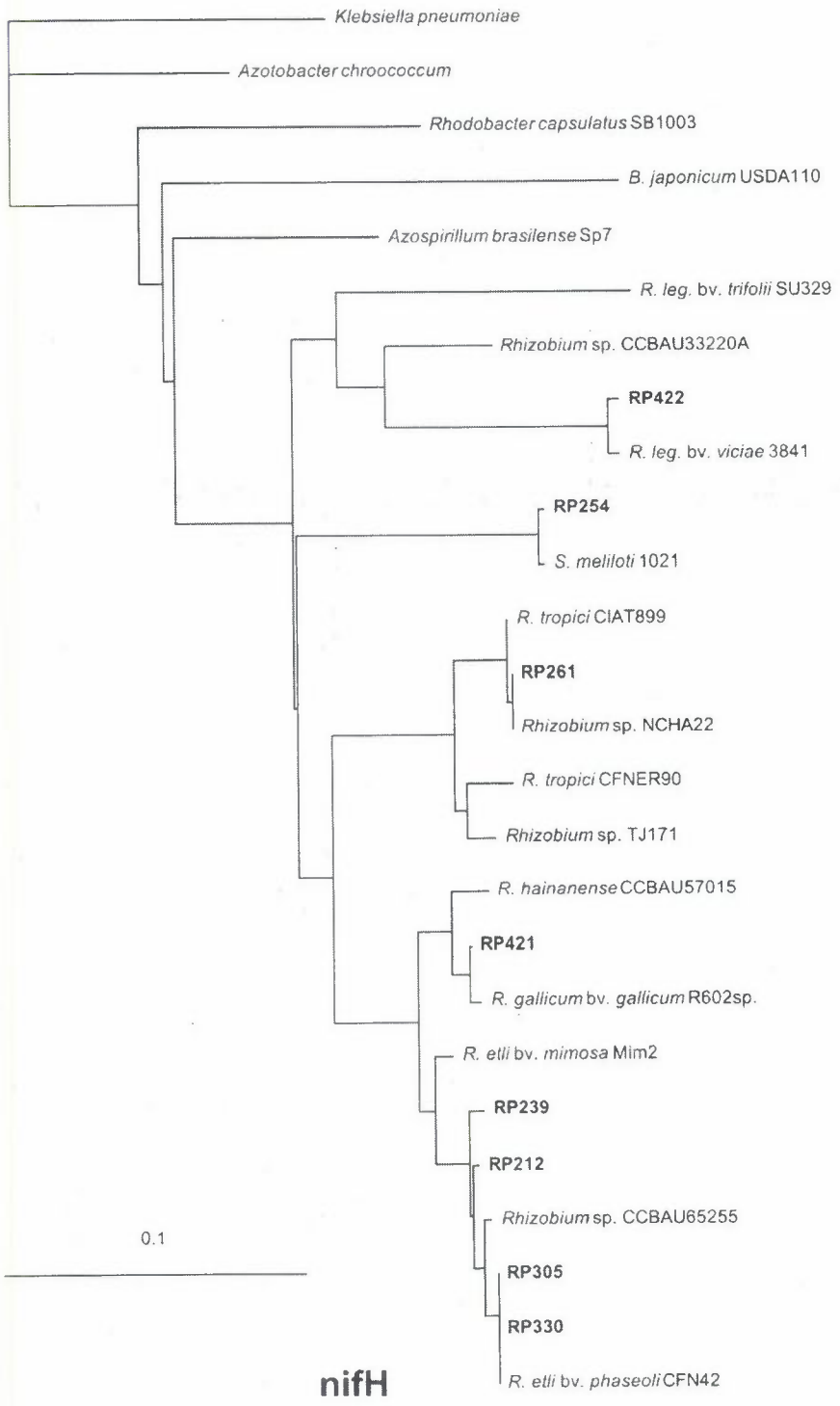
Klebsiella pneumoniae
Azotobacter chroococcum
Rhodobacter capsulatus SB1003
B. japonicum USDA110
Azospirillum brasilense Sp7
R. leg. bv. trifolii SU329
Rhizobium sp. CCBAU33220A
RP422
R. leg. bv. viciae 3841
RP254
S. meliloti 1021
R. tropici CIAT899
RP261
Rhizobium sp. NCHA22
R. tropici CFNER90
Rhizobium sp. TJ171
R. hainanense CCBAU57015
RP421
R. gallicum bv. gallicum R602sp.
R. etli bv. mimosa Mim2
RP239
RP212
Rhizobium sp. CCBAU65255
RP305
RP330
R. etli bv. phaseoli CFN42
0.1
nifH

Figure 2C.

sequence) and 715 bp of the *nifH* genes (Position 310–700 of the *S. meliloti* 1021 nifH sequence) were used to generate the phylogenetic trees.

The *nodC* and *nifH* sequences of the *S. meliloti* representative strain RP254 were found to have a similarity of 95 and 99% with those of *S. meliloti* strain 1021, respectively. Isolate RP422 representing the *leguminosarum*-like group harboured a *nodC* sequence closely related (95%) to *R. leguminosarum* bv. *viciae* strain 248; its *nifH* partial sequence showed the highest similarity (99%) to *R. leguminosarum* bv. *viciae* 3841. Unfortunately, the *nifH* sequencing of RP404, RP405 and RP277 failed, because the gene could not be easily amplified.

The *nodC* sequences of the 6 representatives of the *etli*-like group, namely RP330, RP212, RP218, RP338, RP305 and RP346 were all highly similar to those of reference sequences belonging to the biovar *phaseoli*. For example, the *nodC* partial sequence of strain RP346 was related to *R. etli* bv. *phaseoli* CFN42 with a similarity of 100% and RP330 was by 100% similar to *R. leguminosarum* bv. *phaseoli* H132. Consequently, all the *R. etli* isolates were affiliated to the biovar *phaseoli*. Concerning the *nifH* partial sequences of the 4 *etli*-like representative strains, the sequence of *R. etli* bv. *phaseoli* strain CFN42 was by far the closest, with a similarity value of 98–99%.

The *nodC* partial sequence of strain RP261 representing the *R. tropici* nod type was 99% related to both *R. tropici* CFN299 and CIAT899. The *nifH* sequence was 100% related to that of *R. tropici* IIB CIAT899 sequence and at 97% to that of *Rhizobium* sp. TJ171. The *nodC* and *nifH* partial sequences of strain RP421 representing *R. gallicum* bv. *gallicum* nod and nif types were at 100% identical to those of *R. gallicum* bv. *gallicum* strain R602sp, confirming their affiliation to the biovar *gallicum*.

Reinfection tests were carried out to find out whether the isolates related to *S. meliloti* were indeed able to nodulate bean and alfalfa. All these strains could nodulate *Medicago sativa* efficiently, but induced white nodules on *Phaseolus vulgaris* roots as well. A similar test was carried out with the isolates related to *R. leguminosarum* bv. *viciae*. All the 9 isolates were able to nodulate *Pisum sativum* efficiently and were inefficient on common bean, inducing only small white nodules.

Tolerance to abiotic stresses and distribution of the isolates in the soils

The pH test shows that the optimum range tolerated by the 241 isolates is between 5 and 7 (Table 3). Lower and higher pH are affecting the growth of a great part of the isolates. For example, at pH 4, 22% of the bean-rhizobia isolates are able to grow whether 84% can grow at pH 5. In addition, 33% grow on pH 8 and only 22% tolerated pH 9 (data not shown). All the isolates able to tolerate pHs ranging from 4 to 9 were affiliated to the species *R. tropici*, except RP277 which was identified as *R. leguminosarum* bv. *viciae* (Table 3). The *R. etli* bv. *phaseoli* and the remaining *R. leguminosarum* bv. *viciae* isolates seemed to grow preferentially on pH 5 to 7. *R. gallicum* bv. *gallicum* isolates grow between pH 5 and pH 8, whether neutral to slightly alcaline pHs are tolerated by *S. meliloti* isolates (pH 7 to 8).

The NaCl tolerance test separated the bean-rhizobia isolates into 3 groups: in group 1, the isolates (68%, data not shown) grow only without the presence of NaCl. In group 2, few isolates tolerated 0.17 M and in group 3, the remaining isolates are able to grow until a NaCl concentration of 0.34 M. On this basis, we considered the

See figures on previous three pages.

Figure 2. Neighbor-joining phylogenetic trees based on 1350-bp aligned sequences of 16S rDNA (16S rDNA) (A), 801-bp aligned *nodC* sequences (*nodC*) (B), and on 715-bp aligned *nifH* sequences (*nifH*) (C) of Moroccan rhizobial isolates and reference sequences. The accession number of the 16S rDNA sequences used are: U28916 (*R. etli* CFN42), AY465887 (*R. etli* ISM4), U89822 (*R. mongolense* USDA1929), AL591792 (*S. meliloti* 1021), U89831 (*R. leguminosarum* bv. *viciae* USDA2508), AF364069 (*R. loessense* CCBAU7190B), X67234 (*R. tropici* IIB CIAT899), AF26274 (*R. tropici* SEMIA4080), AF364068 (*R. indigoferae* CCBAU71042), AF003375 (*R. yanglingense* SH22623), U86343 (*R. gallicum* bv. *gallicum* R602sp.), U86344 (*R. giardinii* bv. *giardinii* H152), D11342 (*Azorhizobium caulinodans* ORS571) and D14500 (*Agrobacterium tumefaciens* NCPPB2437). The accession number of the published *nodC* sequences used for comparison are the following: X98514 (*R. tropici* IIA CFN299), AE007235 (*S. meliloti* strain 1021), AF217271 (*R. leguminosarum* bv. *trifolii* USDA2071), Y00548 (*R. leguminosarum* bv. *viciae* strain 248), AF217267 (*R. giardinii* bv. *giardinii* H152), AF217266 (*R. gallicum* bv. *gallicum* R602sp.), AF217268 (*R. etli* bv. *phaseoli* CFN42), AF217265 (*R. gallicum* bv. *phaseoli* PhD12), AF217263 (*R. leguminosarum* bv. *phaseoli* H132), AF284858 (*Bradyrhizobium* sp. ORS285) and L18897 (*Azorhizobium caulinodans* ORS571). The accession numbers of the published sequences used for comparison are as follows: M55225 (*R. tropici* II CIAT899), AE007235 (*S. meliloti* strain 1021), AJ505314 (*Rhizobium* TJ171 (*Mimosa diplotricha*)), K00490 (*R. leguminosarum* bv. *trifolii* SU329), (*R. leguminosarum* bv. *viciae* 3841)*, AF218126 (*R. gallicum* bv. *gallicum* R602sp), AF107621 (*R. etli* bv. *mimosae* Mim2), U80928 (*R. etli* bv. *phaseoli* CFN42), K01620 (*B. japonicum* USDA110), X51500 (*Azospirillum brasilense* Sp7), X03916 (*Azotobacter chroococcum*), J01740 (*Klebsiella pneumoniae*), X07866 *Rhodobacter capsulatus* SB1003). *This sequence was retrieved from the *R. leguminosarum* genome project on the Sanger Website www.sanger.ac.uk/Projects/R_leguminosarum/.

Table 3. Comparison of the relationship of the strains isolated from Moroccan soils to type strains of bean rhizobia, based on all the characteristics analysed.

Strains	Species according to ARDRA and 16S rDNA sequencing	pH range tolerated	Soil pH	$[NaCl]_{max}$ tolerated	Soil EC (mS/m)	T° range tolerated	Growth on LB	BOX geno-type	*nodC* type	*nifH* type
CIAT899	*R. tropici* IIB	4–9	4	0.34 M	–	15–40°C	+	*a*	A	A
RP261^{CH}	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	15–37°C	+	*a*	A	A
RP284	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	20–37°C	+	*a*	A	A
RP290	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	15–37°C	+	*a*	A	A
RP291	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	15–37°C	+	*a*	A	A
RP295	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	15–37°C	+	*a*	A	A
RP296	*R. tropici* IIB	4–9	8.2	0.34 M	418±43	15–37°C	+	*a*	A	A
RP400	*R. tropici* IIB	4–9	7.8	0.34 M	176±9	15–37°C	+	*a*	A	A
RP406	*R. tropici* IIB	4–9	7.8	0.34 M	176±9	15–37°C	+	*a*	A	A
RP418	*R. tropici* IIB	4–9	7.8	0.34 M	176±9	15–40°C	+	*a*	A	A
RP441	*R. tropici* IIB	5–9	7.8	0.34 M	176±9	15–40°C	+	*a*	A	A
CFN42	*R. etli bv. phaseoli*	7–8	–	0 M	–	ND	–	*x*	K	C
RP202	*R. etli bv. phaseoli*	5–7	7.2	0 M	266±28	15–37°C	–	*q*	D	C
RP212^{CH}	*R. etli bv. phaseoli*	5–7	7.2	0 M	266±28	15–37°C	–	*q*	D	C
RP218^{C}	*R. etli bv. phaseoli*	5–7	7.2	0 M	266±28	20–37°C	–	*o*	F	D
RP239^{H}	*R. etli bv. phaseoli*	5–7	7.2	0 M	266±28	15–37°C	–	*o*	F	D
RP247	*R. etli bv. phaseoli*	5–7	7.2	0 M	266±28	20–37°C	–	*q*	D	C
RP302	*R. etli bv. phaseoli*	5–7	8.2	0 M	96±15	15–37°C	–	*n*	K	B
RP305^{CH}	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	30–37°C	–	*l*	H	E
RP310	*R. etli bv. phaseoli*	7–8	8.2	0 M	96±15	15–37°C	–	*i*	C	E
RP320	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	30°C	–	*j*	H	B
RP322	*R. etli bv. phaseoli*	7	7.2	0 M	96±15	30°C	–	*i*	C	B
RP330^{CH}	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	20–30°C	–	*m*	K	B
RP334	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	30°C	–	*l*	K	B
RP338^{C}	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	15–37°C	–	*n*	E	B
RP346^{C}	*R. etli bv. phaseoli*	7	8.2	0 M	96±15	15–37°C	–	*n*	K	B
RP358	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	15–37°C	–	*e*	D	C
RP368	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	15–37°C	–	*e*	D	C
RP370	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	15–37°C	–	*e*	D	C
RP384	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	20–37°C	–	*e*	D	C
RP388	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	15–37°C	–	*e*	D	C
RP396	*R. etli bv. phaseoli*	5–7	6.7	0 M	107±30	15–37°C	–	*e*	D	C
R602sp	*R. gall. bv. gallicum*	7	–	0 M	–	15–37°C	–	*y*	I	F
RP407	*R. gall. bv. gallicum*	7	7.8	0 M	176±9	20–37°C	–	*k*	I	F
RP421^{CH}	*R. gall. bv. gallicum*	7–8	7.8	0 M	176±9	15–37°C	–	*k*	I	F
RP440	*R. gall. bv. gallicum*	7–8	7.8	0 M	176±9	15–37°C	–	*k*	I	F
RP445	*R. gall. bv. gallicum*	7–8	7.8	0 M	176±9	15–37°C	–	*k*	I	F
3841	*R. leg. bv. viciae*	7	–	0 M	–	ND	–	*w*	B	J
RP277^{C}	*R. leg. bv. viciae*	4–9	8.2	0.34 M	418±43	30°C	–	*c*	B	H
RP399	*R. leg. bv. viciae*	7	7.8	0 M	176±9	20–37°C	–	*d*	B	H
RP404	*R. leg. bv. viciae*	7	7.8	0 M	176±9	15–37°C	–	*h*	B	I
RP405	*R. leg. bv. viciae*	7	7.8	0 M	176±9	15–37°C	–	*f*	G	H
RP411^{C}	*R. leg. bv. viciae*	7	7.8	0 M	176±9	20–30°C	–	*g*	G	I
RP422^{CH}	*R. leg. bv. viciae*	5–7	7.8	0 M	176±9	15–37°C	–	*p*	J	J
GR4	*S. meliloti*	7–8	–	0.17 M	–	ND	+	*r*	O	G
RP254^{CH}	*S. meliloti*	7	8.2	0.34 M	418±43	30–40°C	+	*s*	L	G
RP260	*S. meliloti*	7	8.2	0.34 M	418±43	30–40°C	+	*r*	L	G
RP267	*S. meliloti*	7–8	8.2	0.34 M	418±43	30–40°C	+	*s*	L	G
RP276	*S. meliloti*	7–8	8.2	0.34 M	418±43	30–40°C	+	*r*	L	G

ND: not determined; C: *nodC* sequenced; H: *nifH* sequenced; 16Sr DNA representative isolates for sequencing: RP261, RP339, RP277, RP399, RP368, RP405, RP411, RP 404, RP310, RP320, RP421, RP305, RP330, RP384, RP239, RP422, RP212, RP276 and RP254.

isolates of group 2 and 3 as "salt-tolerant" and those of group 1 as "salt-sensitive". The most salt-tolerant isolates were identified by the molecular methods as *S. meliloti*, *R. tropici* and *R. leguminosarum* bv. *viciae* (isolate RP277). The two remaining species identified among the collection were only able to grow without NaCl.

In addition, the majority of the bean-rhizobia isolates (69%) could grow between 15 and 37°C and only 10% could tolerate a temperature of 40°C whether none could grow at 45°C. As for the NaCl tolerance, *S. meliloti* and *R. tropici* isolates were the most tolerant to high temperatures.

On the other hand, Fig. 3 shows the distribution of the isolates with respect to the different soils. All isolates from the soils of Ain Atiq, Salé and Hay Ryad were identified as *etli*-like, while in the other soils a higher diversity was observed. In the saline soil of Skhirat, the isolates were related to 3 species of which *R. tropici* was predominant (90%). It is remarkable that all the isolates coming from this soil are salt tolerant. The moderately saline soil of Benslimane contains also 3 species, but in this case the predominant genotype was related to non salt tolerant *R. gallicum* isolates, representing 51% of the rhizobial population isolated (only strains belonging to *R. tropici* were salt tolerant). In the other salt affected soil (Ain Atiq), we have isolated only NaCl sensitive strains belonging to *R. etli*. All these results indicate that the salinity of the soil is not the only factor that is confering salt tolerance to rhizobia and that most probably salt tolerance is an intrinsic property more related to the species.

4. Discussion

According to Box-PCR fingerprinting, 16S rDNA RFLP and 16S rDNA sequence analysis, five species were able to nodulate common bean in soils of North-West Morocco from which *S. meliloti* and *R. leguminosarum* bv. *viciae* were unconventional bean symbionts (Table 3). The promiscuity of common bean is well established (Bromfield and Barran, 1990; Michiels et al., 1998; Del Papa et al., 1999; Martínez-Romero, 2003) and it was also reported that *Rhizobium* sp. strain OR191 harboured a *nodC* sequence closely related to that of *S. meliloti*. This strain was ineffective on *Medicago* and effective on bean (Eardly et al., 1992; Laguerre et al., 2001). Other studies about bean rhizobia diversity in Mediterranean soils (Herrera-Cervera et al., 1999; Mhamdi et al., 2002) found the same species, except *R. tropici* IIB. Actually, *R tropici* IIB was previously only found in tropical and/or acid soils of Africa (Anyango et al., 1995; Diouf et al., 2000; Odee et al., 2002) and America (Martínez-Romero et al., 1991; Bernal and Graham, 2001; Martínez-Romero and Caballero-Mellado, 1996). In France, the occurrence of members of this species was reported but only those of type IIA (Amarger et al., 1994). We classified the Moroccan isolates as *R. tropici* type IIB, since (i) they showed the same Box profile as *R. tropici* IIB strain CIAT899, (ii) were 100% identical to *R. tropici* reference strains according to 16S rDNA sequencing, (iii) exhibited *nodC* and *nifH* genotypes identical to those of *R. tropici* CIAT899 and (iv) were able to grow on LB medium and at pH 4. Thus, the occurrence of this species in Moroccan soils seems to be an exception in the Mediterranean Basin so far. However, in Morocco, *R. tropici* was found in moderately alkaline soils, as it was also reported by Odee et al. (2002) for Kenyan soils. Although Morocco is not a tropical country, the conditions prevailing in the soils of Skhirat and Benslimane seem to have created microenvironments that were favourable to *R. tropici*. Moreover, this species is known to be very competitive for nodulation in sandy soils but also salt resistant (case of the soil of Skhirat). Indeed, very recently Shamseldin and Werner (2005) showed that *R. tropici* strain CIAT899 was able to grow at concentrations up to 3% NaCl when the incubation was extended to 6 days. In our case, all the *R. tropici* isolates tested were salt tolerant but not depending on the salinity of the soil of origin (Skhirat and Ben Slimane). Thus this property could not correlate directly with the prevailing soil conditions but should be considered as a stable characteristic of this species, the same conclusion could be formulated regarding pH and temperature tolerance (Table 3). Moreover, the stability and absence of intraspecific diversity within the Moroccan *R. tropici* could be either a hint to the recent introduction of this species as seed contaminants (as was suggested for Kenya by Odee et al., 2002) or could be due to the higher genetic stability (Brom et al., 1991; Mostasso et al., 2002) of this species in comparison to other bean rhizobia.

Although the origin of *R. etli* is considered to be in Mesoamerica where commom bean was firstly domesticated, its area of distribution nowadays includes Europe (Herrera-Cervera et al., 1999; Sessitsch et al., 1997; Pérez-Ramírez et al., 1998) and Africa (Mhamdi et al., 2002; Diouf et al., 2000; Anyango et al., 1995; Shamseldin et al., 2005). In Moroccan soils, as in the soils of the Southern Andes in Argentina (Aguilar et al., 1998), Spain (Herrera-Cervera et al., 1999) and Senegal and Gambia (Diouf et al., 2000), *R. etli* was found to be the predominant genotype. In contrast to *R. tropici*-like isolates, the Moroccan *etli*-like strains exhibited a relatively high intraspecific diversity according to their BOX fingerprints and their RFLPs of symbiotic genes, which could be a consequence of the well documented genetic instability of this species (Herrera-Cervera et al., 1999; Brom et al., 1991). In addition, Souza et al. (1992) and Silva et al. (1999) discussed that chromosomal recombination among locally related *R. etli* strains may account for the large genetic variation observed in this species. Moreover, the Moroccan *R. etli* were genomically distant from the reference strain CFN42, as were the Spanish and Tunisian strains (Herrera-Cervera et al., 1999; Mhamdi et al., 2002).

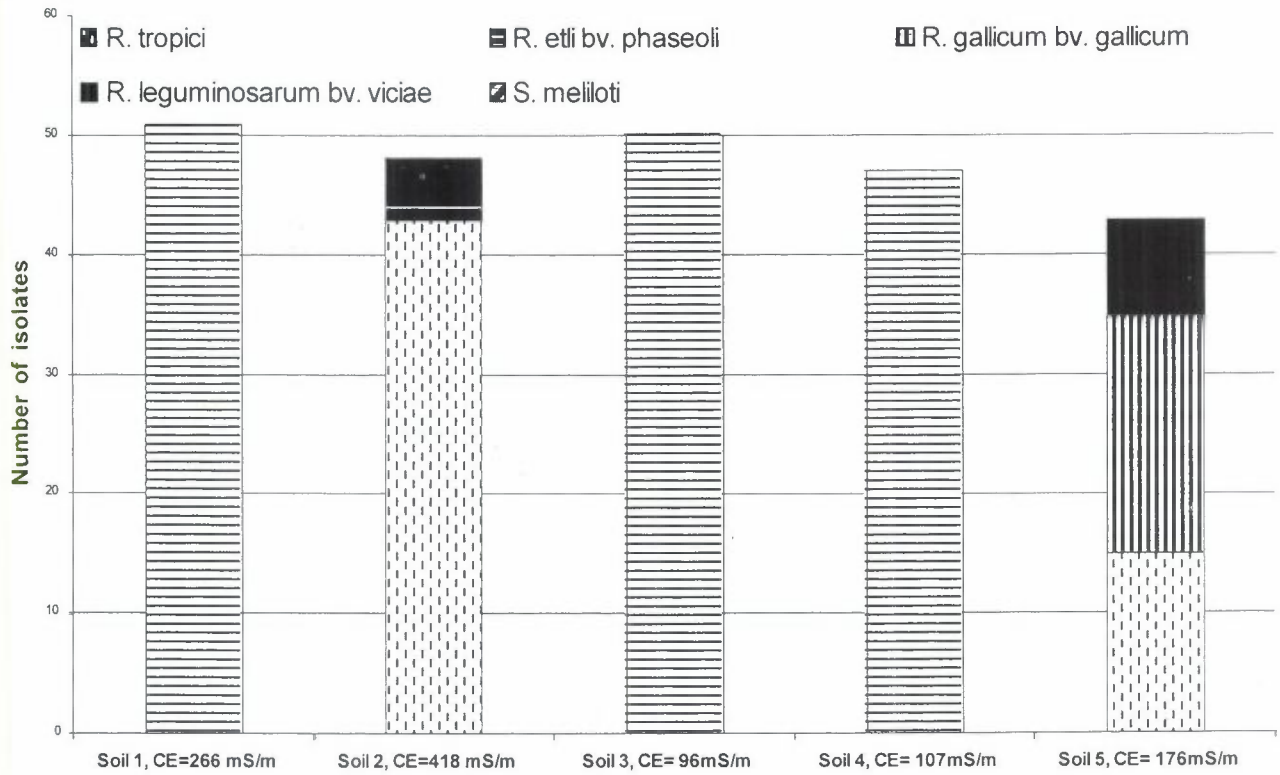

Figure 3. Diagram representing the distribution of the rhizobial species in 5 soils of North-West Morocco. Soil 1: Ain Atiq, Soil 2: Skhirat, Soil 3: Salé, Soil 4: Hay Ryad and Soil 5: Benslimane.

Their exclusive occurence in certain fields of Morocco could be explained by their high competitiveness in comparison to other bean rhizobia species (Martínez-Romero et al., 1991; Pérez-Ramírez et al., 1998) or just by the fact that the conditions in the soils of Ain Atiq, Salé and Hay Ryad were appropriate for their establishment and persistence. In the soil of Ain Atiq, the exclusive occurrence of *R. etli* could also be explained by the fact that in this soil vegetables are usually grown in rotation with common bean. Thus, it is possible to consider that the species *R. etli* was selected by the plant after several years of common bean growth cycles and that it is well established in this soil. Moreover, we noted that in the soils where *R. etli* was found, no *R. tropici* strains were isolated and vice-versa. These two species are known to have very opposite requirements in the soils where they proliferate and each species is able to outcompete the other depending on the conditions prevailing in the respective soils (Peter Graham, personal communication).

Within the *R. gallicum* isolates, no intraspecific diversity was observed. The 23 isolates were identical to the reference strain *R. gallicum* bv. *gallicum* R602sp, according to the molecular methods used in this work. Therefore the isolates were classified as bv. *gallicum*, similar to those found in Tunisian, Austrian and Egyptian soils (Sessitsch et al., 1997; Mhamdi et al., 2002; Shamseldin et al., 2005). This result confirms the recent conclusions of Mhamdi et al. (2002) who reported that *R. gallicum* is not exclusively found in European soils but occurs obviously also in North-African soils. Moreover, Silva et al. (2005) showed that *R. gallicum* encloses two genetically distinct groups that coexist in different geographical regions and though share the same ecological niche and the symbiotic pool of this species. The most recent hypothesis that was emitted about this species was that *R. gallicum* is a cosmopolite species that has a wide geographical distribution and a long history of adaptation to different environments and host-plants (Silva et al., 2005).

The nodulation promiscuity demonstrated in this study and in similar studies in our region could be one of the causes of the known low nitrogen fixation efficiency of common bean. The percentage of plants nodulated by non specific strains or the percentage of nodules induced by non specific (and non efficient) strains on each plant might have a significant effect on the global nitrogen fixation in a given field. Therefore, bean inoculation with bean specific and effective strains in Moroccan soils could be a way to improve bean production at lower costs. In Morocco, the

cultivation of common bean is not extensive and seems to be only occasional. Moreover, wrong irrigation practices and the use of nitrogen fertilisers make difficult to cultivate this legume and take advantage from symbiotic nitrogen fixation. Thus, inoculation of common bean with appropriate inoculum should become a normal practice in Moroccan soils. The 100 years-old experience of soya bean inoculation is a good example of the efficiency of such an approach. However, because that type of inoculation was shown to be generally efficient in rhizobia-free soils (see review Martínez-Romero, 2003) which is not the case of Moroccan soils, we have to take into account the nodulation promiscuity of this legume and especially the competition of non specific rhizobia naturally present in the soil, as well as the prevailing physico-chemical conditions. One of the remaining questions to be verified is to see whether, in soils where specific strains are dominant (Ain Atiq, Salé and Hay Ryad), common bean grows better without an input of combined nitrogen. In this type of soils, we postulate that the predominance of conventional species should be attributed to (i) the host selection after several common bean growth cycles and (ii) favourable physico-chemical conditions prevailing in the soil. In non saline soils, for example, we propose that *R. etli* could be a good candidate for inoculation, as soon as their nitrogen fixation efficiency is confirmed. In salt-affected soils, however, more experiments are needed to select the most competitive and efficient potential inocula, even though *R. tropici* appears to be the best candidate.

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