RESEARCH ARTICLE

Symbiotic characterization and diversity of rhizobia associated with native and introduced acacias in arid and semi-arid regions in Algeria

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Abstract

The diversity of rhizobia associated with introduced and native *Acacia* species in Algeria was investigated from soil samples collected across seven districts distributed in arid and semi-arid zones. The *in vitro* tolerances of rhizobial strains to NaCl and high temperature in pure culture varied greatly regardless of their geographical and host plant origins but were not correlated with the corresponding edaphoclimatic characteristics of the sampling sites, as clearly demonstrated by principal component analysis. Based on 16S rRNA gene sequence comparisons, the 48 new strains isolated were ranked into 10 phylogenetic groups representing five bacterial genera, namely, *Ensifer*, *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium*, and *Ochrobactrum*. *Acacia saligna*, an introduced species, appeared as the most promiscuous host because it was efficiently nodulated with the widest diversity of rhizobia taxa including both fast-growing ones, *Rhizobium*, *Ensifer*, and *Mesorhizobium*, and slow-growing *Bradyrhizobium*. The five other *Acacia* species studied were associated with fast-growing bacterial taxa exclusively. No difference in efficiency was found between bacterial taxa isolated from a given *Acacia* species. The tolerances of strains to salinity and temperature remains to be tested in symbiosis with their host plants to select the most adapted *Acacia* sp.-LNB taxa associations for further revegetation programs.

Introduction

Owing to their symbiotic association with rhizobia, nitrogen-fixing woody legumes play a key role in the sustainability of many natural ecosystems and agro-ecosystems, particularly in the dry and arid regions of tropical and sub-tropical zones (Brockwell *et al.*, 2005). Many studies have investigated the nitrogen-fixing status of legume trees from natural forests (Moreira *et al.*, 1998; Diabate *et al.*, 2005; de Faria *et al.*, 2010) and agroforestry systems (Bala *et al.*, 2003; Wolde-Meskel *et al.*, 2005; Wang *et al.*, 2006), particularly dry-zone *Acacia* species from Africa (Njiti & Galiana, 1996; de Lajudie *et al.*, 1998; Haukka *et al.*, 1998; McInroy *et al.*, 1999; Ba *et al.*, 2002; Odee *et al.*, 2002) and Australia (Marsudi *et al.*, 1999; Lafay & Burdon, 2001). In the case of Algeria, where the Saharan bioclimatic zone covers 89.5% of the total area in addition to 4.78% and 4.12% of arid and semi-arid regions, respectively (Nedjraoui, 2001), *Acacia* species appear as suitable candidates for thoughtful reforestation programs, with regard to their high resistance to salinity and drought (Brockwell *et al.*, 2005) jointly with their ability to regenerate, stabilize, and fertilize soils through their nitrogen-fixing (Galiana *et al.*, 2002, 2004) and mycorrhizal symbioses (André *et al.*, 2005; Duponnois *et al.*, 2007). In addition, they are considered as excellent sources of fuel, service wood, fodder, tannin, and medicines (Midgley & Bond, 2001). *Acacia* is a member of the

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Mimosoideae subfamily and is represented by about 150 species in Africa and 1100 species in Australasia (Maslin *et al.*, 2003). The native *Acacia* species inventoried in Algeria, mostly distributed in northern and central Sahara, are *A. ehrenbergiana*, *A. laeta*, *A. nilotica*, *A. seyal*, and *A. tortilis* (Ozenda, 2004). *Acacia* species have also been introduced in Algeria, mostly along coastal areas such as *Acacia saligna* for dune stabilization and *A. karroo* that has been essentially planted as living hedges (El-Lakany, 1987), both species being exotics native to Australia and southern Africa, respectively. However, the distribution of *Acacia* species in Algeria remains poorly documented, and a precise cartography is still needed. Similarly, natural rhizobial symbionts of *Acacia* species have never been inventoried in Algeria so far except the recent publication on *A. saligna* by Amrani *et al.* (2010) restricted to bacterial populations from nursery soil. In their natural distribution area, most of the African *Acacia* spp. are associated with *Mesorhizobium* (de Lajudie *et al.*, 1998; Haukka *et al.*, 1998; McInroy *et al.*, 1999; Ba *et al.*, 2002; Odee *et al.*, 2002), *Ensifer* (syn. *Sinorhizobium*) (de Lajudie *et al.*, 1994; Lortet *et al.*, 1996; Haukka *et al.*, 1998; Khbaya *et al.*, 1998; Nick *et al.*, 1999b; Ba *et al.*, 2002) and, to a lesser extent, *Rhizobium* (McInroy *et al.*, 1999; Nick *et al.*, 1999a) and *Bradyrhizobium* (Dupuy *et al.*, 1994; McInroy *et al.*, 1999; Odee *et al.*, 2002). On the other hand, the Australian acacias are mainly associated with *Bradyrhizobium* (Galiana *et al.*, 1990, 1994; Lafay & Burdon, 2001; Rodriguez-Echeverria *et al.*, 2007; Le Roux *et al.*, 2009; Perrineau *et al.*, 2011). There is a major interest to inventory the biodiversity of introduced and native acacias and their associated rhizobia in Algeria to allow for effective selection of both symbiotic partners for use in reforestation programs. For this purpose, and as reported by Zhang *et al.* (1991) and Odee *et al.* (2002) in other arid and semi-arid regions, there is a need for inoculants that are able to survive to the stressful edaphic conditions found in Algeria. Recent field experiments performed on coastal dunes of West Algeria showed that the inoculation at nursery level of *A. saligna* seedlings with native rhizobia improved the survival of trees several months after transplanting (unpublished data). However, and this applies to the other *Acacia* species found in Algeria, the beneficial effect of inoculation on tree establishment and growth in field conditions requires the selection of indigenous rhizobia that are adapted and tolerant to high salinity and temperatures.

The objective of this study was thus to characterize the bacteria associated with *Acacia* spp. in their natural distribution area in Algeria at three different levels: (1) their *in vitro* tolerances to NaCl and high temperatures to find out putative relationships between these features and the corresponding characteristics of the sampling sites, (2) their molecular diversity based on 16S rRNA gene sequence analysis, and (3) testing the efficiency of the bacterial strains toward their host plant of isolation to highlight possible specificities among *Acacia* sp.-LNB (Legume-Nodulating Bacteria, Zakhia *et al.*, 2004) taxa associations.

Materials and methods

Soil sampling and sites characteristics

Soil samples were collected from 13 sites distributed in seven districts (Wilayas) in Algeria, within 50 cm around the *Acacia* trunks and at 20, 40, and 60 cm depth (Table 1, Fig. 1). The sampled sites were distributed from arid to semi-arid zones, with rainfall below 100 mm $year^{-1}$ for the Saharan regions. Electrical conductivity (CW) and pH of soil samples were measured using a conductivimeter, in fivefold diluted soil stirred during 30 min at 25 °C with five repetitions. Nodules and seeds were collected when present.

Isolation of bacterial strains, culture conditions, and symbiotic status

Most of the root nodules used for bacterial isolation were obtained through a preliminary step of trapping from the collected soil samples, using seeds from the different tree species studied so that each soil sample was used with its associated *Acacia* species. Direct bacterial isolation from fresh nodules collected *in situ* was also performed but restricted to all the isolates originating from coastal dunes sites, that is, Bomo-Plage and El Mactaa (Table 2). Seeds were collected at the same time as the soil samples from: (1) five tree species native to Algeria, namely *Acacia ehrenbergiana* Hayne, *Acacia nilotica* (L.) Delile, *Acacia seyal* Delile, *Acacia tortilis* (Forssk.) Hayne, and *Faidherbia albida* (Delile) A. Chev., and (2) two introduced species originating from the Oran region, namely *Acacia karroo* Hayne and *Acacia saligna* (Labill.) Wendl. Scarification and surface sterilization of seeds were achieved by treatment with concentrated (95%) H_2SO_4 for 30, 30, 120, 30, and 120 min for the native species, respectively, and 30 and 90 min for the introduced ones, respectively. Seeds were then rinsed thoroughly with sterile distilled water and germinated on 0.8% (w/v) water agar at 28 °C for 2–5 days. For bacterial trapping, the seeds were then transferred in aseptic conditions into Gibson tubes (Gibson, 1980) containing sterile Jensen nitrogen-free medium (Vincent, 1970). Plants were grown in an incubation chamber as described by Diouf *et al.* (2007). After 1 week of growth, 1 mL of stirred soil suspension was added to each tube. Soil suspension was obtained with 10 g of each soil

Table 1. Geographical origin, ecological characteristics, and properties of sampled soils

Site prospected (Wilaya)	Geographical position (Latitude, longitude, altitude)	Site characteristics	*Acacia* species observed	Soil texture	Annual rainfall (mm)	Air temperature mean range (°C)*	Soil conductivity (mS)†	Salinity status‡	Soil pH
Es-Senia (Oran)	35°63′87″ N 00°61′40″ W 1640 Ft	Vegetation cover	*A. seyal*	Clay	370	0–40	0.600	SS	7.94
Messerghine Farm (Oran)	35°62′66″ N 00°64.90″ W 3280 Ft	Grass farm	*A. karroo*	Rock loam-sand	370	0–40	0.234	NS	8.01
Sebkha Messerghine (Oran)	35°60′81″ N 00°65′75″ W 1640 Ft	500 m from saline depression (sebkha)	*A. seyal*	Loam	372	0–40	1.290	S	7.92
Bomo-plage dunes (Oran)	35°75′12″ N 00°82′92″ W 1640 Ft	Coastal dunes	*A. saligna*	Sand	370	0–40	0.097	NS	8.17
Msila forest (Oran)	35°62′14″ N 00°88.96″ W 6561 Ft	Pine forest	*A. karroo*	Clay	370	0–40	0.140	NS	8.19
El Mactaa dunes (Mostaganem)	35°45′05″ N 00°07′33″ W 2 Ft	Coastal dunes	*A. saligna*	Sand	370	0–42	0.124	NS	8.16
Khemaissa (Relizane)	35°42′98″ N 4°24′53″ E 500 Ft	Vegetation cover	*A. saligna*	Loam	185	−3 to 45	0.280	NS	8.12
Ain Defla	36° 15′ 55″ N 1° 58′ 13″ E –	Vegetation cover	*A. saligna*	Clay	NA	NA	0.38	NS	8.17
Labiod Sidi El Cheikh (El Bayadh)	32°89′46″ N 00°54′55″ E 500 Ft	Orchard	*A. seyal*	Clay-sand	294	−5 to 38	0.290	NS	7.79
Ain Belbel (Adrar)	27°89′97″ N 01°16′72″ E 1000 Ft	*Acacia* forest	*A. ehrenbergiana*	Rock clay-sand	26	−1 to 49	0.075	NS	7.66
Oued In Dalagd (Tamanrasset)	22°56′00″ N 05°52′85″ E 4554 Ft	*Acacia* forest	*A. ehrenbergiana*§ *A. nilotica* *F. albida*	Rock loam-sand	48	−1 to 39	0.105	NS	7.70

Table 1. Continued

Site prospected (Wilaya)	Geographical position (Latitude, longitude, altitude)	Site characteristics	*Acacia* species observed	Soil texture	Annual rainfall (mm)	Air temperature mean range (°C)*	Soil conductivity (mS)†	Salinity status‡	Soil pH
Oued Tassena (Tamanrasset)	35°35′24 N 00°48.53″ W 358 Ft	*Acacia* forest	*A. tortilis*	Rock loam-sand	48	−1 to 39	0.078	NS	8.17
Oued Tin Amezzegin (Tamanrasset)	22°35′22″ N 05°23′81″ E 3740 Ft	*Acacia* forest	*A. ehrenbergiana* *A. tortilis*§	Rock clay-sand	48	−1 to 39	0.149	NS	7.80

NA, not available.
*Temperature range from minimum to maximum annual mean values (source: Organisation Nationale de Météo, 2006).
†Conductivity of water (CW) in millisiemens.
‡based on soil CW scale from Durand (1983): S, saline; SS, slightly saline; NS, nonsaline.
§No strain was isolated from this species in this site.

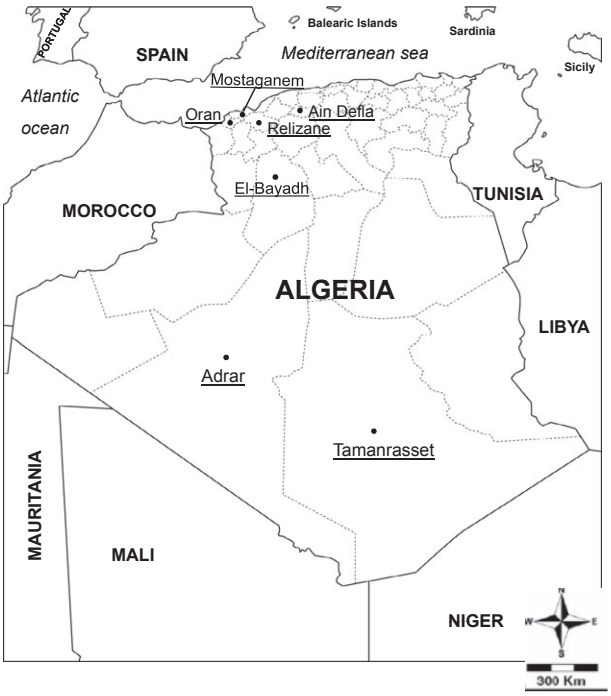

Fig. 1. Geographical location of the sampling sites. The sampling sites were distributed in seven Wilayas (names underlined on the map).

sample added to 90 mL of sterile buffered saline pH 7 (NaCl 0.15 M, KH_2PO_4 0.002 M, Na_2HPO_4 0.004 M) shaken for 1 h. Four replicates were tested for each soil sample. Uninoculated plants served as controls. Plants were observed for nodule formation 6 weeks after germination, and fresh nodules were collected and conserved under desiccated condition in tubes containing $CaCl_2$ crystals. The root nodules were washed by immersion in 70% (v/v) ethanol for 30 s, rinsed with distilled water, then rehydrated for 1 h, individually surface-sterilized by immersion in 35% H_2O_2 for 15 s to 2 min according to their size, and then rinsed in successive baths of sterile distilled water. After crushing, the nodule homogenates were spread onto yeast extract-mannitol agar (YMA) plates (Vincent, 1970) containing Congo red (0.00125%, w/v). One isolated colony per nodule homogenate was obtained from successive streaking on YMA plates, and purity of colonies was checked by phase-contrast microscopy. Colony shape and color were determined using a magnifying glass. Pure cultures were then cryogenically preserved (−80 °C) in yeast extract-mannitol medium (YEM) adjusted to 30% (v/v) glycerol. The nodulation ability of the bacterial isolates obtained was checked by inoculating seedlings of the corresponding *Acacia* species grown on sterilized sandy soil (autoclaving at 120 °C for 20 min, three times at 24-h intervals) in 250-mL plastic pots. One week after germination, three plants per *Acacia*

Table 2. Origin, phenotypic and genotypic characteristics of new strains

Strain	Original host plant	Site of sampling (Wilaya) [Site symbol]*	Maximum temperature† (°C)	Maximum NaCl concentration (mM)†	Bacterial genus affiliation‡	Accession no.
E231a	*A. ehrenbergiana*	Ain belbel (Adrar) [A]	40	680	*Mesorhizobium*	HQ836166
E231c			40	1034	*Ensifer*	HQ836176
E60		Oued Tin Amezzegin (Tamanrasset) [T3]	50	340	*Ensifer*	HQ836175
K31	*A. karroo*	Messerghine Farm (Oran) [O2]	40	680	*Rhizobium*	HQ836160
K32a			35	1034	*Rhizobium*	HQ836161
K32c			45	170	*Rhizobium*	JF810499
K33b			45	102	*Rhizobium*	JF810500
K34a			35	102	*Rhizobium*	JF810501
K1a		Msila forest (Oran) [O5]	35	510	*Ensifer*	HQ836170
K1b			35	1034	*Ensifer*	JF810504
K2b			45	340	*Ensifer*	HQ836171
K2d			35	680	*Ensifer*	HQ836172
K4a			40	1034	*Ensifer*	HQ836173
K4c			35	1034	*Ensifer*	HQ836174
N145	*A. nilotica*	Oued In Dalagd (Tamanrasset) [T1]	40	680	*Rhizobium*	HQ836162
SAB3	*A. saligna*	Bomo-plage dunes (Oran) [O4]	40	860	*Rhizobium*	HQ836156
SAB5			40	860	*Rhizobium*	HQ836157
SAB9			45	510	*Rhizobium*	HQ836155
SAB10			35	340	*Mesorhizobium*	HQ836165
SAB12b			40	860	*Rhizobium*	HQ836158
SAB13			40	860	*Ensifer*	HQ836167
SAB15b			45	510	*Rhizobium*	HQ836154
SAB17a			40	680	*Ensifer*	HQ836169
SABN1a			40	510	*Bradyrhizobium*	HQ836182
SABN1b			45	510	*Bradyrhizobium*	HQ836183
SABN1c			40	340	*Bradyrhizobium*	HQ836185
SABN2a			45	340	*Bradyrhizobium*	HQ836179
SABN2c			40	340	*Bradyrhizobium*	HQ836184
SABN4a			45	510	*Bradyrhizobium*	HQ836178
SABN4b			40	510	*Bradyrhizobium*	HQ836180
SABN5a			40	680	*Bradyrhizobium*	HQ836181
SAB17b			40	680	*Ensifer*	HQ836168
SA13b		El Mactaa dunes (Mostaganem) [M]	35	340	*Bradyrhizobium*	HQ836186
SA15d			35	860	*Bradyrhizobium*	HQ836187
SAKS		Khemaissa (Relizane) [R]	40	170	*Ensifer*	HQ836190
SAAIS		Ain Defla[AD]	40	340	*Rhizobium*	HQ836163
SE21a	*A. seyal*	Messerghine sebkha (Oran) [O3]	45	340	*Rhizobium*	HQ853452
SE21b			45	860	*Rhizobium*	JF810505
SE23bc			35	170	*Rhizobium*	HQ836159
SE24a			50	860	*Rhizobium*	JF810502
SE25a			45	680	*Rhizobium*	JF810503
SE25d			45	680	*Rhizobium*	HQ853453
SE3		Es-Senia (Oran) [O1]	35	1034	*Rhizobium*	HQ836164
SE243a		Labiod Sidi El Cheikh (El Bayadh) [EB]	45	1034	*Ochrobactrum*	HQ836189
SE243c			45	860	*Rhizobium*	JF810506
SE243d			45	1034	*Ochrobactrum*	HQ836188
T82	*A. tortilis*	Oued Tassena (Tamanrasset) [T2]	40	1034	*Ensifer*	HQ836177
A121	*F. albida*	Oued In Dalagd (Tamanrasset) [T1]	40	680	*Mesorhizobium*	HQ836191

*Site symbols were used in Fig. 2.

†Maximum temperature or NaCl concentration for bacterial growth.

‡Blast results from nearly full-length 16S rRNA gene sequences (> 1300 nt).

species were inoculated with approximately 2 mL of bacterial suspension at 10^9 cells mL^{-1} of each isolate. Uninoculated controls were included. Plants were transferred to greenhouse and fed with a sterilized Broughton & Dilworth N-free nutrient solution (1971) once a week. Five weeks after inoculation, plants were harvested to estimate the infectivity and effectivity of bacterial isolates based on the presence of nodules, nodule color, visual observation of plant vigor, and foliage color. The bacterial isolates were recorded as noninfective (0), symbiotically ineffective (I) when nodulated plants were not more developed than uninoculated control plants and nodules had a white color, and effective (E) when plants were greener and significantly more developed than uninoculated plants while having pink nodules.

Tolerances of bacterial isolates to salinity and temperature

All tests were carried out in triplicate. Before inoculation, isolates were grown in YEM liquid medium to log phase (corresponding to approximately 10^9 colony-forming units mL^{-1}). In test plates, inoculation was performed with 10 μL of these cultures. The results were scored after 7-day incubation. Positive results were reported when bacterial colonies were observed under binocular microscope at ×50 magnification. Tolerance to salinity was tested on YEM agar plates containing Nacl concentrations of 170, 340, 510, 680, 860, and 1034 mM incubated at 28 °C. For temperature tolerance, YMA plates were inoculated and incubated at four different temperatures: 35, 40, 45, and 50 °C.

Partial 16S rRNA gene sequencing

Isolated colonies were homogenized into sterile 1.5-mL centrifuge tubes with 50 μL of sterile Milli-Q water; suspensions were centrifuged at 6000 ***g*** for 5 min and washed twice, pellets were re-suspended and then stored at −20 °C until PCR use. The universal eubacterial 16S rRNA gene primers FGPS 6 (5′-GGA GAG TTA GAT CTT GGC TCA G-3′) and FGPS 1509 (5′-AAG GAG GGG ATC CAG CCG CA-3′) (Normand *et al.*, 1992) were used, and PCR was performed as follows: an initial cycle of denaturation at 94 °C for 5 min, 36 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were checked by 0.8% (w/v) agarose gel electrophoresis in Tris/acetate/EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). Bands were excised and DNA purified using a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France) and sequenced using primers FGPS6, FGPS1509, and 16S-1080r (5′-GGG ACT TAA CCC AAC ATC T-3′) (Sy *et al.*, 2001). Sequencing reactions were analysed on an Applied Biosystems model 310 DNA automated sequencer using a BigDye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems). Sequences were corrected using CHROMASPRO 4 (Technelysium Pty Ltd., Tewantin, Australia) before alignment and analysis of the 16S rRNA gene sequences using CLUSTAL X software (Thompson *et al.*, 1997). Phylogenetic analysis was inferred using the neighbor-joining method (Saitou & Nei, 1987) calculated by the Kimura method (Kimura, 1980). The 16S rRNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank/EMBL database and used for cladistic analysis. A bootstrap analysis using 1000 replications was performed. The 16S rRNA gene sequences of 48 new isolated *Acacia* strains, of which 39 were included in the phylogenetic tree, were deposited in the GenBank database under accession numbers HQ836154 to HQ836191, HQ853452, HQ853453, and JF810499 to JF810506 (see Table 2).

Efficiency of bacterial isolates

The most efficient strains identified in the nodulation tests were compared to highlight a possible relationship between strain efficiency and its taxonomic position. As a minimum number of strains was needed for each *Acacia* species to be tested, only three *Acacia* species were tested in this experiment, namely *A. seyal*, *A. karroo*, and *A. saligna*. The test was conducted in the same conditions as those used in the nodulation test described earlier, using triplicates, except that plants were harvested here 7 weeks after inoculation for measurements of shoot height and shoot dry weight.

Statistical analyses

Principal component analysis (PCA) was performed to examine the relationships between soil conductivity and maximal air temperature of the sampled sites (Table 1) and the *in vitro* tolerances of bacterial strains isolated from the corresponding sites to NaCl and temperature. Computations and graphical display were made using the XLSTATTM software package (version 2010.5.04, Addinsoft, Paris, France, http://www.xlstat.com). Factorial correspondence analysis (FCA) was performed to visualize the relationships between rhizobial taxa, as defined from the partial 16S rRNA gene-based phylogeny, and *Acacia* host species, using XLSTATTM software package (see reference above). In the strain efficiency tests, and for each of the three *Acacia* species studied and each parameter analyzed, all the data collected were subjected to a one-way analysis

of variance. When the rhizobium strain factor had a significant effect on a given parameter, the means of the different treatments were ranked into homogeneous groups according to the Duncan's multiple range test (Dagnélie, 1975). The variance analysis was performed using SUPERANOVA software (Abacus concepts Inc., version 1989, CA).

Results

Symbiotic status of isolated strains

Of 288 purified bacterial isolates from surface-sterilized nodules, only 84, that is, 29%, were able to renodulate their host plant of isolation. Among these infective isolates, 48 were further characterized molecularly (Table 2). These 48 isolates were selected based on colony morphology, only one representative per morphological type and per origin (host plant, sampling site, and trapping plant), being retained from the 84 nodulating isolates for molecular analysis and estimation of symbiotic status. Five weeks after inoculation, 25 of these 48 strains were found to be efficient, as estimated by qualitative observation based on nodule color, plant vigor, and foliage color in comparison with uninoculated control plants. In these preliminary nodulation tests, the percentage of effective strains varied according to the *Acacia* species because, among the most represented ones, 100% of the infective strains were effective in *A. seyal* (10 of 10 strains tested) vs. 55% in *A. karroo* (six of 11) and only 33% in *A. saligna* (seven of 21).

Tolerances of bacterial isolates to salinity and temperature

As reported in Table 2, the maximum salinity levels tested varied greatly according to the isolate, that is, from 170 to 1034 mM NaCl. About 20% of the isolates were able to grow at the maximum level tested of 1034 mM NaCl. However, these highly tolerant bacteria were mostly isolated from nonsaline sites except one from Es-Senia (Oran) region that is slightly saline. Isolates from saline sites, especially those from Oran Sebkha, were not particularly tolerant to NaCl under *in vitro* conditions. In the same way, the *in vitro* NaCl tolerance of strains originating from the same site varied greatly. Thus, as clearly confirmed by PCA (Fig. 2), no relationship was found between *in vitro* tolerances of rhizobial isolates to temperature and NaCl, and the edaphoclimatic characteristics of the corresponding sampling sites. Indeed, as shown by direction of variables axes in Fig. 2, no correlation was found between *in vitro* NaCl tolerance of strains and soil conductivity ($r = -0.004$) or

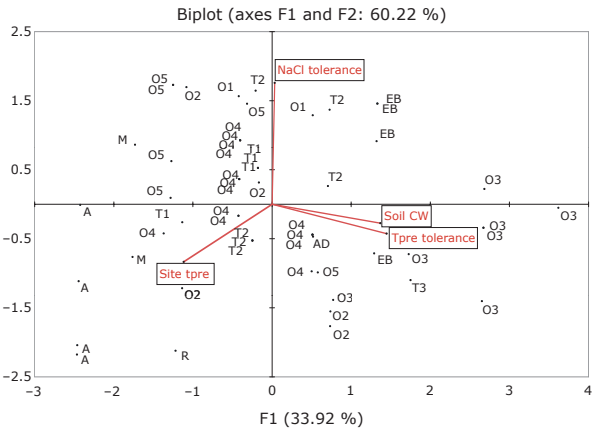

Fig. 2. PCA representing the relationships between *in vitro* tolerance of rhizobial isolates to NaCl (NaCl tolerance) and high temperature (Tpre tolerance) and the edaphoclimatic characteristics of the corresponding sampling sites, *i.e.* soil conductivity (Soil CW) and maximum annual mean temperature (Site Tpre). Each dot corresponds to a single bacterial isolate or several ones superimposed due to similar NaCl and temperature tolerances and their site of sampling is indicated by a symbol as follows: O1: Es-Senia/Oran; O2: Messerghine Farm/Oran; O3: Messerghine Sebkha/Oran; O4: Bomo-plage dunes/Oran; O5: Msila forest/Oran; M: El Mactaa dunes/Mostaganem; R: Khemaissa/Relizane; AD: Ain Defla; EB: Labiod Sidi El Cheikh/El Bayadh; A: Ain Belbel/Adrar; T1: Oued In Dalagd/Tamanrasset; T2: Oued Tassena/Tamanrasset; T3: Oued Tin Amezzegin/Tamanrasset. When they are superimposed on a same dot, the isolates are differentiated and aligned diagonally near the dot.

between *in vitro* tolerance of strains to high temperatures and maximal air temperature of their original site ($r = -0.162$). The main contributors to the structure of this PCA biplot, where both F1 and F2 axes represent 60.22% of the total variability, are bacterial isolates from Sebkha Messerghine/Oran (indicated by 'O3' symbols), which are logically grouped along 'Soil CW' and F1 axes, and those from Aïn Belbel/Adrar (indicated by 'A' symbols), grouped along 'Site Tpre' axis, both sampling sites being characterized by high NaCl soil concentrations and extreme high temperatures, respectively. Despite their proximity in Fig. 2, 'Soil CW' and 'Tpre tolerance' variables were slightly correlated ($r = 0.247$) because F3 axis, representing 21% of the total variability, was not represented. In the same way, the maximum growth temperature varied widely according to the isolate, ranging from 35 to 50 °C with an average of about 40 °C. About 35% of the strains were tolerant to 45 °C while two of them reached a maximal growth temperature of 50 °C. These two latter outstanding strains isolated from *A. ehrenbergiana* and *A. seyal* originated from Tamanrasset and from the Sebkha region (Oran), respectively. However, as clearly shown by the PCA, there was no correlation between the tolerance of bacterial strains to high

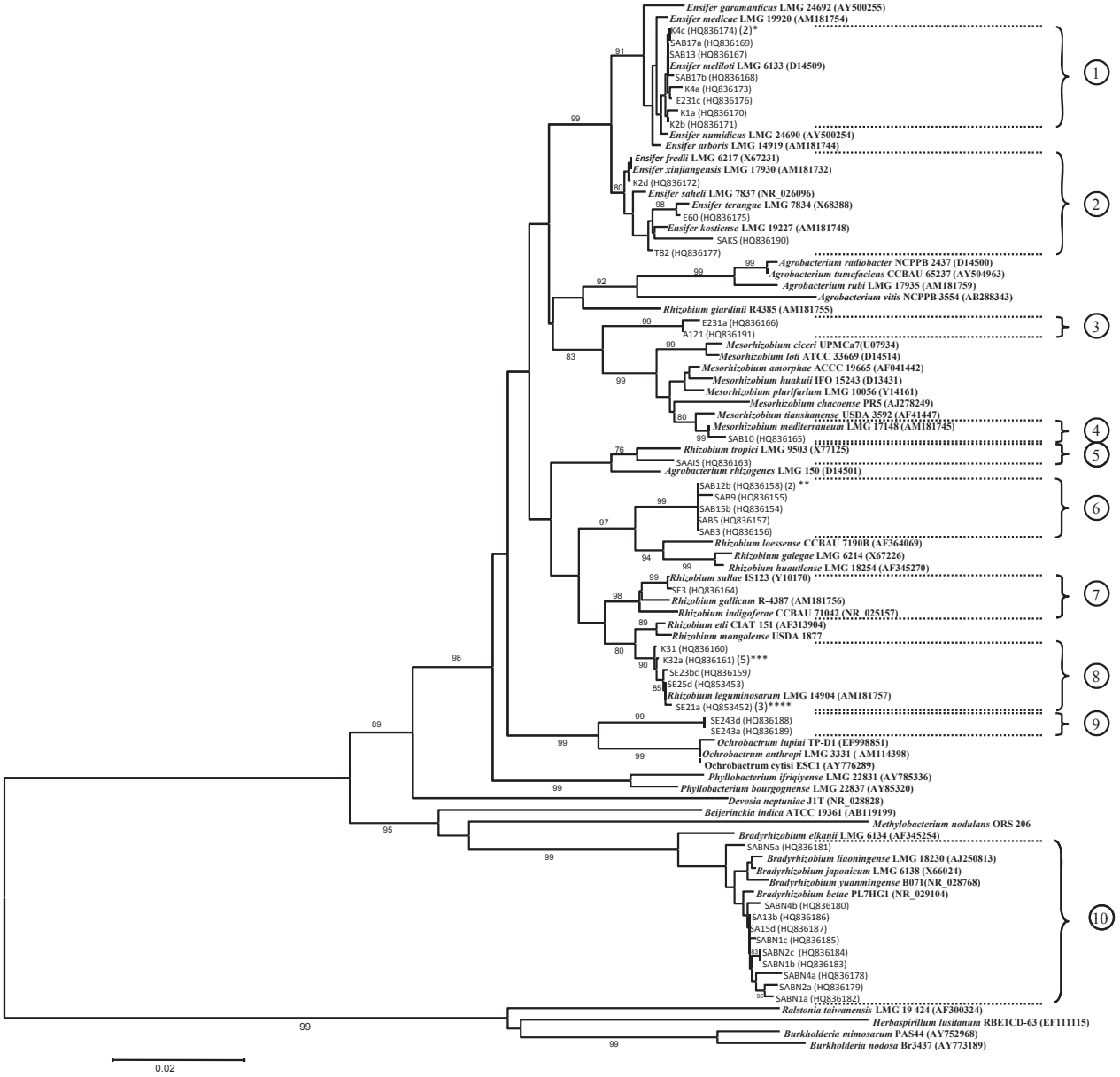

Fig. 3. Phylogenetic tree based on nearly full-length 16S rRNA gene sequence (> 1300 nt) analysis of new and reference (in bold characters) strains. Neighbor-Joining method integrating Kimura 2 distance was used. Data are bootstrap values issued from 1000 repetitions. The ten main groups generated are numbered from 1 to 10. Eight strains listed in Table 2 were not included in the phylogenetic tree since their 16S rRNA gene sequences had 100% similarity with those of representative strains which are indicated with asterisks as follows: *Strain K4c is representative of another strain, *i.e.* K1b, **Strain SAB12b is representative of another strain, *i.e.* N145, ***Strain K32a is representative of four other strains, *i.e.* K32c, K33b, K34a and SE21b, ****Strain SE21a is representative of two other strains, *i.e.* SE24a and SE25a. Between brackets are indicated the numbers of strains having the same sequence.

temperatures in pure culture and the maximal temperatures found in their regions of origin. Thus, none of the strains isolated from the hottest regions of Algeria, Adrar in particular, exhibited any particular tolerance to high temperatures in pure culture in comparison with strains from other cooler regions.

Phylogenetic analysis of bacterial strains

Based on 16S rRNA gene sequence comparisons including reference bacterial sequences, we distinguished 10 phylogenetic groups (Fig. 3) among the 48 new strains studied (Table 2), originating from seven *Acacia* species

and 13 different sites in Algeria. For the sake of clarity, only a limited dendrogram showing the new strains among their closest reference species is presented. Some of the 48 nodulating isolates sequenced were not indicated in the phylogenetic tree because their 16S rRNA gene sequences had 100% similarity with those of representative strains, while only one representative strain per sequence was indicated in the tree (see details in legend of Fig. 3). The 10 groups obtained might be affiliated to five genera: *Ensifer*, *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium*, and *Ochrobactrum*. Group 1, closely related to the *Ensifer meliloti* reference strain, included eight isolates from three *Acacia* species, namely *A. karroo* (K1a, K2b, K4a, and K4c), *A. saligna* (SAB13, SAB17a, and SAB17b), and *A. ehrenbergiana* (E231c). In this group 1, the four *A. karroo* strains were isolated from the same geographical origin, that is, Msila forest (Oran), and the three *A. saligna* strains all originated from Bomo-plage dunes (Oran). The heterogenous group 2 contained four isolates from four different host species, namely *A. karroo*, *A. ehrenbergiana*, *A. saligna*, and *A. tortilis*, all from different geographical origins that were close to *E. fredii* (K2d), *E. terangae* (E60), and *E. kostiense* (SAKS and T82) reference strains. Isolates in *Mesorhizobium* distinguished as group 3, a separate sub-branch consisting of two isolates from *A. ehrenbergiana* (E231a) and *F. albida* (A121) and group 4, represented by a single isolate from *A. saligna* (SAB10), in the vicinity of *M. mediterraneum*. Groups 5, 6, 7, and 8 were affiliated to *Rhizobium*. Groups 5 and 7 represented single isolates, that is, one from *A. saligna* (SAAIS) affiliated to the *R. tropici* clade and the other one from *A. seyal* (SE3) affiliated to the *R. sullae* clade, respectively. On the other hand, group 6, consisting of five bacterial isolates, all from *A. saligna* (SAB3, SAB5, SAB9, SAB12b, and SAB15b) and from the same geographical origin (Bomo-plage Oran), formed a separate clade in the vicinity of the *R. galegae–R. huautlense–R. loessense* branch. Group 8 containing the *R. leguminosarum* reference strain LMG14904 was represented by five *A. karroo* isolates (K31, K32a, K32c, K33b, and K34a) from the same origin (Messerghine Farm-Oran) and five *A. seyal* isolates (SE23bc, SE25d, SE21a, SE24a, and SE25a) from another origin (Messerghine Sebkha-Oran). In group 9, two *A. seyal* strains (SE243a and SE243d) isolated from the same site (Labiod-El Bayadh) were identified as *Ochrobactrum* sp. Lastly, 10 *A. saligna* isolates originating from two different sites, Bomo-plage dune Oran (SABN1a, SABN1b, SABN1c, SABN2a, SABN2c, SABN4a, SABN4b, and SABN5a) and El Mactaa dune Mostaganem (SA13b and SA15d), were affiliated to *Bradyrhizobium* and ranked as group 10.

Host specificity of isolated LNB

The FCA showed that each *Acacia* species had its own specificity in terms of preferential association with taxonomical groups of nodulating bacteria as defined by the phylogenetic analysis mentioned earlier. As shown in Fig. 4, the first factorial plan projection including F1 and F2 axes, which represents about 85% of the total variability, showed that *A. saligna* was nodulated by the highest diversity of taxonomical groups including four single host taxa, namely, *M. mediterraneum*, *R. tropici*, *Rhizobium* sp., and *Bradyrhizobium* sp., as well as a multiple host range taxon, namely, *E. meliloti*, shared with *A. karroo* and *A. ehrenbergiana*. In addition to *E. meliloti*, *A. karroo* and *A. ehrenbergiana* were both nodulated by *E. fredii* while these two host plant species differed in their respective association with *R. leguminosarum* and *Mesorhizobium* sp. Lastly, *A. seyal* was associated with *R. leguminosarum* and two single host taxa *Ochrobactrum* sp. and *R. sullae*. The other *Acacia* host species prospected were not taken into account in this FCA analysis considering the low number of isolates obtained from each of them.

Efficiency of the isolated LNB according to taxonomic affiliation

Strain efficiency tests were performed using a selection of isolates representative of the bacterial taxa found in

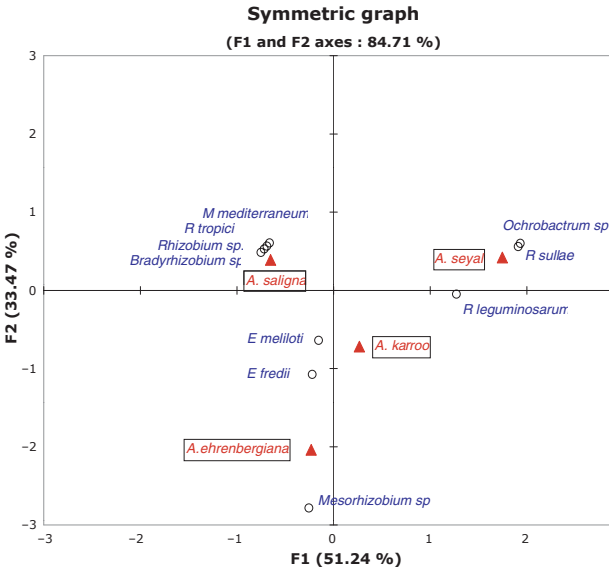

Fig. 4. First factorial plane projection of FCA between bacterial taxa (open circles), as defined from the 16S rRNA gene-based phylogeny, and four *Acacia* species (closed triangles). For clarity, when bacterial taxa were superimposed on a same dot (open circle) due to identical affinities towards *Acacia* species, their dots were slightly shifted and aligned diagonally.

association with each *Acacia* species. As noted earlier, some of the bacterial strains tested, namely, SE243c, SE21b, SE24a, SE25a, K1b, K34a, K33b, and K32c (Tables 3a–c), were excluded from the phylogenetic tree because their 16S rRNA gene sequences were identical to those of other strains already represented in the tree (see details in legend of Fig. 3). Moreover, these experiments were focused on *Acacia* species that had a sufficient number of isolates available. Seven weeks after inoculation and growth, all plants of the three *Acacia* species studied were nodulated for all bacteria isolates tested. All the bacteria tested were efficient in *A. seyal* and *A. saligna* (Tables 3a and c, respectively) while a few nodulating strains were not effective in *A. karroo* (*i.e.* K31, K32c, and K4c) as shown by the absence of significant difference in height and dry weight of shoots between inoculated and uninoculated control plants (Table 3b). In *A. seyal*, the most effective isolate in terms of shoot growth was a *Rhizobium* sp. strain (SE243c), followed by one *Ochrobactrum* sp. strain (SE243a), while another *Ochrobactrum* sp. strain (SE243d) along with four *R. leguminosarum* strains (SE21a, SE21b, SE24a, and SE25a) were ranked in a same group according to the Duncan multiple range test at $P = 0.05$ when shoot dry weight was only considered. The lowest dry weight was observed for a strain of *R. leguminosarum* (SE25d). In *A. saligna*, the seven strains tested had statistically similar growth whatever their taxonomic position and geographical origin. In *A. karroo*, the most effective isolate was an *E. fredii* strain (K2d), for which inoculated plants had a shoot dry weight about three times higher than uninoculated control plants, followed by a very efficient *R. leguminosarum* strain (K34a) and a third group of efficiency – as defined by the Duncan multiple range test applied to shoot dry weight mean at $P = 0.05$ – containing three *E. meliloti* and one *R. leguminosarum* strains (K1b, K1a, K2b, and K33b, respectively). On this same *Acacia* species, an *E. meliloti* isolate was poorly effective (K4a) while two *R. leguminosarum* (K32c and K31) and one *E. meliloti* (K4c) strains were totally inefficient.

Table 3. Efficiency of a selection of new strains on (a) *Acacia seyal*, (b) *Acacia karroo* and (c) *Acacia saligna* grown under greenhouse conditions, 7 weeks after plant inoculation

Strain name	Bacterial species*	Shoot height (cm)	Shoot dry weight† (mg plant^{-1})
(a)			
SE243c	*Rhizobium* sp.	6.5 ab	80 a
SE243a	*Ochrobactrum* sp.	4.8 abc	73 ab
SE243d	*Ochrobactrum* sp.	8.2 a	72 abc
SE21a	*R. leguminosarum*	3.3 c	69 abc
SE21b	*R. leguminosarum*	6.0 abc	58 abc
SE24a	*R. leguminosarum*	5.3 abc	57 abc
SE25a	*R. leguminosarum*	4.0 bc	43 abc
SE25d	*R. leguminosarum*	5.3 abc	32 bc
Uninoculated control	–	3.7 c	24 c
(b)			
K2d	*E. fredii*	7.5 a	107 a
K34a	*R. leguminosarum*	6.2 ab	97 ab
K1b	*E. meliloti*	6.5 ab	84 abc
K1a	*E. meliloti*	4.6 bcd	78 abc
K2b	*E. meliloti*	4.8 bcd	64 abc
K33b	*R. leguminosarum*	5.4 abc	47 abc
K4a	*E. meliloti*	3.0 bcd	42 bc
K32c	*R. leguminosarum*	2.8 cd	33 c
K31	*R. leguminosarum*	2.0 d	29 c
K4c	*E. meliloti*	3.2 bcd	26 c
Uninoculated control	–	3.5 bcd	33 c
(c)			
SAB15b	*Rhizobium* sp.	7.1 a	46 a
SA13b	*Bradyrhizobium* sp.	6.7 a	45 a
SAB3	*Rhizobium* sp.	6.5 a	43 a
SAB5	*Rhizobium* sp.	7.0 a	40 a
SAB10	*M. mediterraneum*	6.5 a	40 a
SAB9	*Rhizobium* sp.	5.8 a	37 a
SA15d	*Bradyrhizobium* sp.	5.5 a	34 a
Uninoculated control	–	3.9 a	13 b

*Bacterial species was determined based on the positioning of a given strain in the phylogenetic tree (Fig. 2).

†Means were obtained from tree replicates per strain tested. Means followed by different letters in a same column are significantly different according to the Duncan multiple range test at $P = 0.05$.

Discussion

The *in vitro* tolerance of rhizobial strains to NaCl in pure culture varied greatly regardless of their geographical and host plant origins but was not correlated with the salinity of the soils from which they were isolated. However, soil salinity was assessed through soil conductivity (CW) that takes into account all types of salts while NaCl might not be dominant in soils of the different sampled sites, even though the latter is the major soluble salt in highly saline soils throughout the world (FAO, 1976). The absence of correlation between laboratory assay and isolation conditions could also be due to the fact that rhizobia are located in microniches at rhizospheric level and inside nodules and are thus protected from soil constraints (Trabelsi *et al.*, 2010). Overall, the *in vitro* tolerance of strains to salinity was quite high because about 20% of them grew at the maximum level tested of 1034 mM NaCl (corresponding to 6% NaCl w/v). Our findings are in agreement with previous studies reporting that rhizobia isolated from woody legumes in general and from dry-zone *Acacia* species in particular tolerated such high salinity levels (Zahran, 1999; Mohamed *et al.*, 2000; Diouf *et al.*, 2007; Essendoubi *et al.*, 2007). In accordance with previous reports (Zahran, 1999), we found *Bradyrhizobium* strains less tolerant, although originating from saline ecosystems in our study (coastal dunes), than fast-growing *Rhizobium*, *Ensifer*, and *Ochrobactrum* strains that tolerated up to 1034 mM NaCl. In the same way, while

the PCA showed that there was no correlation between the tolerance of bacterial strains to high temperatures in pure culture and the maximal temperatures found in their regions of origin, the new isolated strains exhibited a quite high tolerance to high temperatures with about one-third of isolates tolerant to 45 °C and even two strains that grew at 50 °C. There was no link either between the tolerance of rhizobial strains to high temperatures and their taxonomic position because all LNB taxa were indiscriminately represented among these highly tolerant strains. Such high proportion of strains tolerant to high temperatures, as found in our study, is unusual in rhizobial populations isolated from arid-zone tree species (Zhang *et al.*, 1991; Swelim *et al.*, 1997; Zerhari *et al.*, 2000).

The affiliation of strains associated with introduced and native *Acacia* spp. in Africa to *Rhizobium*, *Agrobacterium*, *Ensifer*, *Mesorhizobium*, and *Bradyrhizobium* is well documented (de Lajudie *et al.*, 1994, 1998; Khbaya *et al.*, 1998; McInroy *et al.*, 1999; Odee *et al.*, 2002; Diouf *et al.*, 2007, 2010; Amrani *et al.*, 2010). In this study, *A. seyal* was found to be associated with *R. leguminosarum*, *R. sullae*, and *Ochrobactrum* lineages, in contrast to previous studies that reported that *A. seyal* was preferentially associated with *Mesorhizobium* in Senegal (de Lajudie *et al.*, 1998; Diouf *et al.*, 2007, 2010) and to *Ensifer* spp. in Ethiopia (Wolde-Meskel *et al.*, 2005). To date, only one study performed on *A. mangium* has reported the ability of *Ochrobactrum* (*Brucellaceae* family) to form nitrogen-fixing nodules in legume trees (Ngom *et al.*, 2004). More recent studies also reported the isolation of infective and effective *Ochrobactrum* strains in annual and shrubby legume species (Trujillo *et al.*, 2005; Zurdo-Piñeiro *et al.*, 2007; Imran *et al.*, 2010). In our study, symbionts of *A. karroo* were identified as *E. meliloti*, *E. fredii*, and *R. leguminosarum* while this *Acacia* species was reported to be associated with *R. tropici* in Kenya (McInroy *et al.*, 1999), *S. terangae* biovar *acaciae* in Senegal (de Lajudie *et al.*, 1994), but *E. fredii* in Morocco (Khbaya *et al.*, 1998). Similarly to *A. seyal* and *A. karroo*, *A. ehrenbergiana* was shown to be only associated with fast-growing rhizobia. Although only a few strains were isolated from this latter species, they were quite diverse because they belonged to three well distinct taxa, namely, *E. meliloti*, *E. terangae*, and *Mesorhizobium* sp. Symbiotic status of this species remains to be assessed because rhizobium diversity related to this *Acacia* species has been poorly studied. Among other poorly represented species, *A. tortilis* was found associated with *Ensifer* strains as in other studies, that is, *E. fredii* in Ethiopia (Wolde-Meskel *et al.*, 2005), *E. meliloti* in Morocco (Khbaya *et al.*, 1998), and several *Ensifer* spp. in Tunisia (Romdhane *et al.*, 2006), while *F. albida*, known to be widely nodulated by slow-growing rhizobia, associated with fast-growing *Mesorhizobium*, as already reported (Odee *et al.*, 2002). *Acacia saligna* appeared as the most promiscuous host plant among all *Acacia* species investigated here considering its ability to nodulate with the widest diversity of rhizobial taxa, including both fast-growing ones, namely, *Rhizobium*, *Ensifer*, and *Mesorhizobium*, and the slow-growing *Bradyrhizobium*. Such promiscuity has rarely been reported in other legume species. Other studies also showed that *A. saligna* was nodulated either by fast- and slow-growing rhizobia, either with a predominance of slow-growing ones (Marsudi *et al.*, 1999) or in equal proportions (Amrani *et al.*, 2010) but always with a lower diversity than in our survey. Partial sequencing of the 16S rRNA gene does not allow the identification of *Bradyrhizobium* taxa to the species level, and at least 16S-23S rRNA-ITS gene sequencing is requested (Willems, 2006). We thus considered majority of the new strains as undifferentiated *Bradyrhizobium* sp. Many of them clustered in the vicinity of *B. betae*, similarly to some *A. saligna* strains described by Amrani *et al.* (2010) through 16S rRNA gene sequencing. Concerning the fast-growing strains, our finding that *A. saligna* is associated with *R. tropici* confirmed the report of Marsudi *et al.* (1999), although the latter also found *R. leguminosarum*. Among other *Rhizobium* species, we did not detect any *R. gallicum* strain like Amrani *et al.* (2010), but a cluster of five *A. saligna* strains isolated from Bomo-plage that formed a separate *Rhizobium* sp. subgroup (group 6 in Fig. 3) that was not related to any known referenced type strain, *R. galegae/R. loessense* being the closest ones. *Ensifer meliloti* strains were also isolated from *A.saligna* by Amrani *et al.* (2010), while in addition, we found strains that clustered with *Ensifer kostiense*. Lastly, to our knowledge, no *Mesorhizobium* strain, precisely *M. mediterraneum*, had been isolated from *A. saligna* before this study.

The low number of LNB isolated from the Saharan part of South Algeria, around Tamanrasset in particular, led to a weak representation of the rhizobial diversity in some of the tree species surveyed such as *A. nilotica*, *A. tortilis*, and *F. albida* that were only found in this region. This was mostly because of the inability of many strains isolated from neoformed nodules to renodulate their original host plant after the first step of bacterial trapping from the soil samples collected, these noninfective strains having been further characterized as opportunistic bacteria such as *Agrobacterium*, *Burkholderia* or *Devosia* (results not shown). By contrast, a majority of the strains isolated from the two exotic *Acacia* species found in the Mediterranean coastal zone of North Algeria, namely *A. karroo* and *A. saligna*, did renodulate their original hosts and were identified as rhizobia. The low success of rhizobium isolation from soils of desertic environments could be explained by the very low density of nodulating bacteria that is usually found in such ecosystems, especially at the soil surface around superficial roots of legume trees (Jenkins *et al.*, 1987). Thus, the elevated temperature in the Saharan sampling sites might play a role in rhizobial

nonsurvival. On the other hand, the noninfectiveness of nodule isolates has been observed in many studies (Khbaya *et al.*, 1998; de Lajudie *et al.*, 1999; Odee *et al.*, 2002). Different assumptions have been proposed: the infective bacteria might have lost their symbiotic information or at least their *nod* genes during symbiosis or once purified onto artificial media (Brom *et al.*, 1992), or the true symbiont is unculturable or very slow-growing, and only nonnodulating bacterial endophytes coexisting with rhizobia inside nodules may be isolated (Rosenblueth & Martínez-Romero, 2006; Muresu *et al.*, 2008), which has been clearly established for *Agrobacterium* (de Lajudie *et al.*, 1999) and *Burkholderia* (Diouf *et al.*, 2007) and several other genera (Muresu *et al.*, 2008).

Opposite to other *Acacia* species, such as *A. mangium* and *A. mangium* × *A. auriculiformis* interspecific hybrids (Galiana *et al.*, 1990; Le Roux *et al.*, 2009), the efficiency of rhizobial strains did not vary according to the bacterial taxon in each of the three *Acacia* species tested, *namely*, *A. karroo*, *A. saligna*, and *A. seyal*. Our results also highlight the particularly high promiscuity of *A. saligna* that nodulated and fixed nitrogen efficiently with a wide range of rhizobial taxa. Such promiscuity could also explain the invasive status of *A. saligna* and to a lesser extent *A. karroo* in their introduction zones. However, some authors did not find any clear relationship between rhizobial diversity and the invasiveness status of acacias, in particular the Australian acacias introduced as exotic species in different Mediterranean countries such as *A. longifolia* (Rodriguez-Echeverria *et al.*, 2007). Considering its outstanding promiscuity, *A. saligna* remains an exception because all Australian *Acacia* species investigated so far are known to be preponderantly nodulated by *Bradyrhizobium* spp. (*i.e.* over 90% of nodule isolates).

The collection of new strains showed high phenotypic and genotypic diversity reflecting no particular relationship between the phylogenetic position of bacterial strains and (1) their tolerances to salinity and temperature, and (2) their efficiency toward the different *Acacia* species tested. Moreover, despite the variety of host species and wide range of geographical zones investigated across Algeria, we clearly showed that there was no correlation between the *in vitro* tolerances of strains to salinity and high temperature and the edaphoclimatic characteristics of their regions of origin. This questions the strategies of rhizobium selection based on *in vitro* phenotypic criteria because, in case of incongruence, we should always tend to favor the choice of indigenous strains isolated from the future planting site to be used as inoculants for a given tree species. The tolerance of strains to these factors remains to be further tested in symbiosis with the host plant. In such perspective, parallel extensive research of tree species provenances tolerant to particular edaphoclimatic conditions is also requested. This involves the *in situ* exploration of genetic material for each species of interest to establish a broader genetic basis, keeping in mind that the geographical distribution of *Acacia* spp. in Algeria remains poorly known.

Acknowledgements

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