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DIVERSITY OF BACTERIA ASSOCIATED WITH ROOT NODULES OF *HEDYSARUM PALLIDUM* DESF. GROWING IN DIFFERENT REGIONS OF ALGERIA

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Abstract – This work deals with the study of the bacterial diversity associated with the wild legume *Hedysarum pallidum* nodules collected from six different sites of Algeria along a geobioclimatic level transect. A total of eighty-one bacterial isolates were retrieved from harvested plants nodules. Isolates were subjected to preliminary sorting through internal transcribed spacer 16S-23S rRNA amplification, which allowed the distinction of 20 different polymorphic haplotypes. Thirty-two representative isolates were subjected to species identification using partial 16S rRNA gene sequencing. The identified strains were affiliated to *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* with an uneven distribution between the different sites indicating an important bacterial diversity. The phenotypic characterization carried out illustrated the diversity of the isolates. Principal Component Analysis (PCA) of soil parameters showed a differentiation between soil samples. Denaturing gradient gel electrophoresis analysis (DGGE), highlighted the presence of a band across all investigated samples that showed 100% nucleotide identity to *Mesorhizobium camelthorni*. Such uncultured taxon is envisaged as the responsible of nodule induction in *Hedysarum pallidum* but the role and possible contribution to the symbiosis exerted by culturable bacteria are discussed.

INTRODUCTION

Leguminous plants were considered as an effective pioneer of agriculture in many developing countries. The importance of legumes is evidenced by their capacity to form nitrogen-finxing symbiotic relationship with soil bacteria known as rhizobia, as well as, legumes present an essential source of plant proteins for human and animal nutrition (Graham and Vance, 2003).

Rhizobia are Gram negative bacteria known to occupy root nodule tissues and perform biological nitrogen fixation (BNF), but they are not the unique microbial nodular inhabitant. Several reports indicate that nodules may harbor a wide diversity of symbiotic bacteria and non-symbiotic endophytes known as Non-Rhizobial Endophytes (NRE) (Peix *et al.,* 2012; De Meyer *et al.,* 2015). These endophytic bacteria are able to benefit their host through different mechanisms includes phytohormones synthesis, enhancing nutrient availability, phosphate solubilization, controlling phytopathogens and conferring biotic and abiotic stresses resistance and tolerance, they were described as Plant Growth Promoting Bacteria (PGPB) (Stagkovic *et al.,* 2009; Ibañez *et al.,* 2017; Singh, 2018).

Symbiotic nitrogen fixation relationship contributes to enhance crop and pasture growth and limit the use of chemical fertilizers. In contrast, few studies have been made in rhizobia and non-crop

forage legumes of agricultural significance, despite their agronomic and ecological importance (Bevan, 2006).

Hedysarum pallidum Desf., (Syn: *Sulla pallida*) is an endemic fodder of *Fabaceae* growing in North Africa, which presents an important agro-economic interest due to its ability to improve soil fertility through the nitrogen fixation process (Hannachi-Sahli *et al*., 2004). However, this species was little exploited, particularly in Algeria (Benhizia, 2006).

Previous studies reported the unculturability of rhizobial symbionts of Mediterranean wild species of *Hedysarum* including *Hedysarum pallium* and the occurrence of a vast array of opportunistic endophytes (Benhizia *et al.,* 2004), but their symbiotic properties have not yet been demonstrated.

The 16S rRNA gene and internal transcribed spacer (ITS) were developed for the identification and the classification of microorganisms (Huybens *et al.,* 2009). In fact, several studies have reported the usefulness of ITS analysis in the discrimination of bacterial isolates at the genus/species level (Gürtler and Stanisich, 1996). Moreover, fingerprint profiling and analyses, as cultivation-independent techniques, were commonly used to study the microbiome of environmental samples and to unravel the 16S rRNA gene diversity among different microbial communities (Muyzer *et al.,*1993; Fisher and Triplett, 1999) . Among these methods denaturing gradient gel electrophoresis (DGGE) was efficiently used to detect environmental microbiomes fluctuations (Muyzer *et al.,*1993 ; Li *et al.,* 2005).

This study aimed to identify and compare the bacterial community structures of *Hedysarum pallidum* nodules from different areas of Algeria along a geobioclimatic level transect, using culture-independent and-dependent methods.

MATERIALS AND METHODS

Sites description and sampling

The sampling was carried from sub-humid and arid climatic zones in Algeria: two semi-arid sites located in Oum El Bouaghi: Aïn Babouche (35°56′31′′N, 7°11′35′′E) and Sebkha EL Zemoul of Aïn M'lila (35°54′60′′N, 6°31′43′′E), two sites in Setif : the sub humid site of Amoucha (36°21′1′′ N, 5°24′32′′E) and the arid site of Djbel Boutaleb (35°40′13′′N,5°16′53′′E), a littoral site in Oran (35°51′3725"N,0°18′3146"E) and a semi-arid site of Aïn El Bey in Constantine (36°14'3"N, 6°36'28"E) (Figure 1). Nodules were detached from roots for bacterial isolation and dry stored in tubes containing $CaCl_2$ at 4 °C (Date, 1982).

Physico-chemical soil characteristics

Nine sampled soil were tested from the six sites: Aïn Babouche, Aïn M'lila, Arzew, Djbel boutaleb, Amoucha and Aïn el Bey along latitude ranging from 35°56′31′′ to 36°14'3"N and longitude from 7°11′35′′ to 6°36'28"E (Table 1). Soil samples were sieved through a 2mm sieve, then, they were

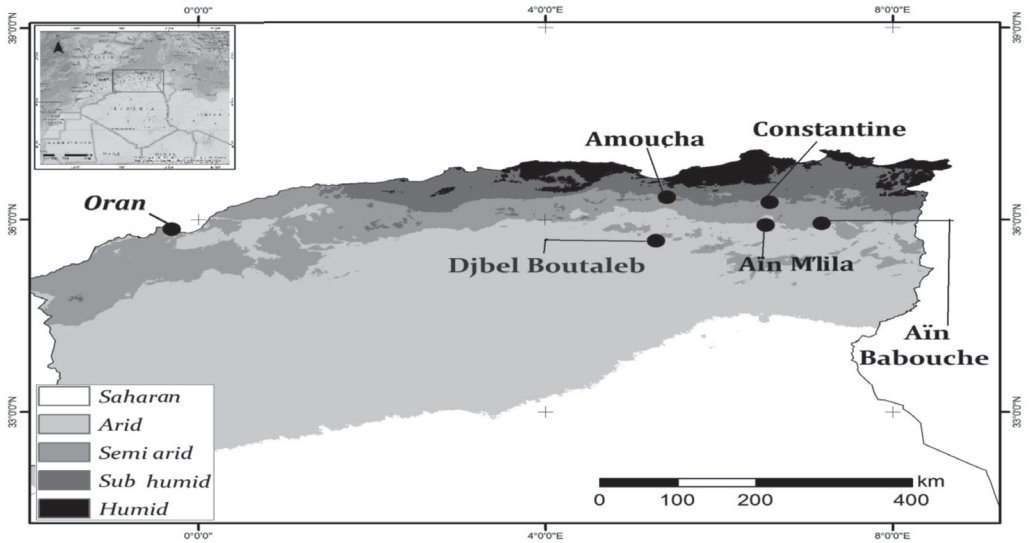

Fig. 1. Bioclimatic zones of Algeria with the collection sites: semi-arid sites (Aïn Babouche, Constantine, Aïn M'lila, Oran) sub humid site (Amoucha), arid site (Djbel Boutaleb)

analyzed for texture, pH, calcium carbonate ($CaCO_3$) and electrical conductivity (EC) as described by Mathieu *et al.* (2003).

Total DNA extraction from nodules

DNA was extracted directly from root nodules using the method modified of Bousquet *et al*. (1990).The nodules of *Hedysarum pallidum* were surface sterilized in 30% H_2O_2 for 10 min and washed with distilled water. Each lobe was crushed with sterile plastic pestle in 600 µL of Cetyltrimenthylammonium bromide buffer extraction (2% Cetyltrimethylammonium bromide, 100mM Tris (pH8), 20mM EDTA, 1.4M NaCl) and incubated overnight at 65°C, then centrifuged at 8.000×g for 10 min to remove cell debris. The supernatant was then extracted twice with an equal volume of chloroform-isoamyl alcool (24:1vol/vol) and centrifuged at 10.000×g for 15 min. DNA from the aqueous phase was precipitated with 1 volume of isopropanol, incubated overnight at -20 °C and centrifuged at 10.000×g for 45min in 4 °C. The DNA pellet was washed with 70% of ethanol, centrifuged at 10.000×g for 10 min, vacuum dried, suspended in 30 µL of TE [10mM (pH8) Tris, 0.1Mm EDTA] and stored at -20 °C.

PCR-Denaturing Gradient Gel Electrophoresis Analysis (PCR-DGGE)

The hypervariable V3 region of 16S rRNA gene (~550 bp) was amplified using primers 907R and 357F containing GC-clamp extension (Muyzer *et al.*, 1993). The reaction mixture was prepared with 1X PCR buffer, 2.5 mM $MgCl_2$, 0.12 mM deoxynucleoside triphosphate, 0.3 µM of each primer and 1U Taq DNA polymerase. The PCR cycles used were as follows : 94°C for 4min, followed by 10 cycles of 94°C for 30s, 61°C for 1min, and 72 °C for 1 min; followed by further 20 cycles of 94 °C for 30s, 56 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 7min. The PCR products were verified by electrophoresis in 1%w/v agarose gel. PCR products were applied on 7% (w/v) polyacrylamide gel in 1X TAE pH 7.4 with denaturing gradient (40-60%) according to Muyzer *et al*. (1993). Gels were run at 90V for 17h at 60 °C. Gels were incubated for 30 min in ethidium bromide solution, rinsed with sterile distilled water and photographed on a UV transillumination table (Mapelli *et al.*, 2013).

DGGE bands were cut from the gel using a sterile scalpel and eluted in 50 µL milliQ water at 37 °C for 6 hours. The eluted DNA was used for amplification using 907R and 357F primers (without the GC-clamp). Positive amplifications were partially sequenced using the primer 357F and they were subjected to a NCBI BLASTN (http://www.ncbi.nlm.gov/ blast/) algorithm and Ez Biocloud (Yoon *et al.*, 2017).

Bacterial isolation

Bacterial strains were isolated from plant root nodules following the standard method described by Vincent (1970). The nodules were surface-sterilized by immersion in 95% ethanol for 10 sec, followed by 0.1% $HgCl_2$ for 3 min and were rinsed ten times, successively, with sterile distilled water to eliminate the toxic traces of $HgCl_2$. The sterilized nodules were transferred on Yeast Extract Mannitol Agar (YMA) plate (Fred and Waskman, 1928) to check the sterilization efficiency. Each nodule was crushed in a drop of sterile distilled water and the suspension was streaked on YMA medium in Petri dishes. The isolates were grown at 28±2 °C for 2-8 days and conserved at 4 °C for short-term storage or at –20°C for long-term storage.

DNA extraction, Ribotyping and identification of isolates

DNA was extracted from bacterial cultures using SDS/CTAB lysis and a phenol/chloroform extraction method (Wilson, 1987) modified by using of 30mg/ml of lysozyme. ITS-PCR amplification was carried using S-D-Bact-1494-a-S-20 (5′- GTCGTAACAAG GTAGCCGTA-3′) and L-D-Bact-0035-a-A-15 (5′-CAAGGCATCCACCGT-3′) universal primers (Daffonchio *et al.*, 1998). PCR amplification consisted of 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs mixture, 0.3 5M of eachprimer, 1U Taq polymerase (Fermentas) and 250 ng of total DNA, using the following program: 94 °C for 3min, followed by 35 cycles of 94 °C for 45s, 55 °C for 1min and 72 °C for 2min, and a final extension step at 72 °C for 7min. PCR products were checked by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. Bacteria redundancy was reduced by checking the ITS-PCR pattern. One to three isolates per each different ITS haplotype was subjected to phylogenetic analysis. Representative's strains were characterized by partial 16S rRNA gene sequencing using the primers S-D-Bact-0008-a-S-20 (5′-AGAGTTTGATCCTGGCTCAG-3′) and S-D-Bact-1495-a-A-20 (5′- CTACGGCTACCTTGTTA CGA-3′) (Daffonchio *et al.*, 1998). PCR amplification

was carried as previously procedure using a thermal cycler (Biometra T3000 Thermocycler.48.Germany). The 16S rRNA PCR amplicons were purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Exo- Sap, Fermentas, Life Sciences). The obtained sequences were compared with those available at the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLASTN) algorithm and Ez Biocloud (Yoon *et al.*, 2017). Phylogenetic analysis was performed with Molecular Evolutionary Genetics Analysis (MEGA) software, version 7 (Kumar *et al.*, 2016) A phylogenetic tree was constructed using Maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The16S rDNA sequences were submitted to the NCBI nucleotide database under the accession number MN945240 to MN945272.

Biochemical and physiological characteristics of culturable bacteria

Bacterial isolates were evaluated for their abilities to produce nitrate reductase (Delarras, 2007), urease (Lindstrom and Lehtomaki, 1988) and endoglucanase (Struffi *et al.*, 1998). Moreover, the tolerance of strains to different abiotic factors was assessed on Yeast-Mannitol Broth at different pH values ranging from 4 to 11(Struffi *et al.*, 1998; Benhizia, 2006), on sodium lactate aspartate medium (Gloux and Le Rudulier, 1989) in the presence of different concentrations of NaCl ranging from 0.01 to 2M (Benhizia, 2006) and on YMA incubated at different temperatures (4 °C to 50 °C) (Struffi *et al.*, 1998; Benhizia, 2006).

Nodulation Test

The isolates were tested for their ability to re-infect their host plant in axenic conditions. Seeds of *Hedysarum pallidum* were surface sterilized in 95% ethanol for 30s, then transferred in concentrated sulfuric acid for 3 min and rinsed thoroughly with sterile distilled water, maintained in sterile water 3 hours and germinated on Tryptone yeast agar (TYA) (Beringer, 1974) in Petri dishes for 2-7 days. Seedlings were aseptically transferred to 250ml flasks containing the N-free Fahraeus nutriment solution (Vincent, 1970; Beck *et al.*, 1993) supplemented with 1% (w/v) $CaCO_3$ and closed with two hole perforated plastic caps, one for inserting the sprout rootlet and the other for inoculation of the seedlings, as previously described by Riah *et al.* (2013). Each seedling was inoculated with 1 mL of a bacterial suspension (10^8–10^9 cells ml^{-1}) and an un-inoculated flask was included as a control. Plants were placed in a growth chamber under bacteriological controlled conditions (16h daylight photoperiod at 23 °C, 8h at 18°C night temperature and 60% constant relative humidity). Plants were harvested eight weeks after planting.

Statistical analysis

Soil parameters were subjected to PCA (Principal Component Analysis) using XLSTAT (Addinsoft, version 2016.05.34949).

RESULTS

Physico-chemical soil characteristics

Nine sampled soil were obtained from the six sites and were physico-chemically characterized by analyzing (EC), pH and Calcium carbonate ($CaCO_3$). The soil texture of six sites studied was variable (Table 1). The soil pH showed values comprised between 7.35 and 7.95, signifying a weakly alkaline soil in the six regions according to Cherni *et al.* (2019). The electrical conductivity varied between 0.15dS/m and 1.95dS/m. Calcium carbonate was variable between the stations, ranging between 9.1% and 39.84%. In fact, Amoucha soil was distinguished

Table 1. Physico-chemical soil characteristics. $CaCO_3$: Calcium carbonate; EC: Electrical conductivity, pH

Samples	$CaCO_3$(%)	EC (dS/m)	pH	Texture
Aïn Babouche 1	15.07	0.16	7.85	Sandy
Aïn Babouche 2	11.84	0.15	7.65	Sandy
Aïn M'lila 1	39.84	0.19	7.54	Silty-clay
Aïn M'lila 2	33.07	0.17	7.91	Silty- clay
Arzew 1(Oran)	18.3	0.45	7.83	Silty, silky, talc
Arzew 2(Oran)	15.22	0.30	7.57	Silty- clay
Djbel boutaleb	18.07	1.95	7.35	silky, talc
Amoucha	9.1	0.18	7.95	Silty
Aïn el Bey (Constantine)	21.5	0.20	7.83	Silty-clay

with the highest value of pH, the soil of Djbel Boutaleb with the most important value of EC and Aïn M'lila1 soil with the highest percentage of Calcium carbonate (Table 1).

A principal component analysis (PCA) showed that soils in the nine locations were different. The first two components were the most important by a cumulative variability of 90.72%. The first axis F1 contributes with 56.15% in the variability, it possible to distinguish the soil of Amoucha with the highest pH value and the soil of Djbel Boutaleb with the highest EC value. The second axis F2 contributes with 34.57% in the variability, it possible to distinguish the soil of Aïn M'lila1 with the highest percentage of Calcium carbonate (Figure 2).

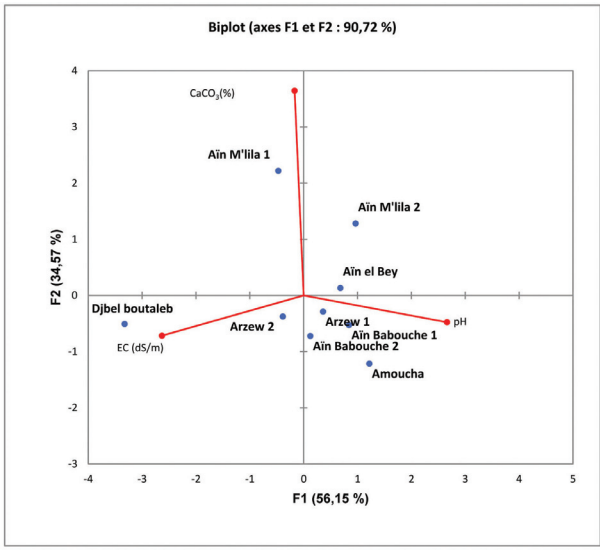

Fig. 2. Principal Component Analysis (PCA) of soils according to their physico-chemical characteristics

Culture-independent analysis of the bacterial Community based on PCR-DGGE

The bacterial diversity of all samples was investigated by DGGE analysis of amplified partial 16S rRNA gene. The obtained DGGE patterns highlighted the presence of a dominant band in all sites (a, b, c, d, e, f) with some faint bands (Figure 3). Partial 16S rRNA gene sequences of the dominant bands (b and d) showed 100% of homology with *Mesorhizobium camelthorni* CCNWXJ 40-4^T (EU169581). However, we were not able to analyze the remaining faint bands because they were very weak.

Analysis and characterization of culturable bacteria

A total of eighty-one bacterial strains were isolated for 2-8 days from the root nodules of *Hedysarum*

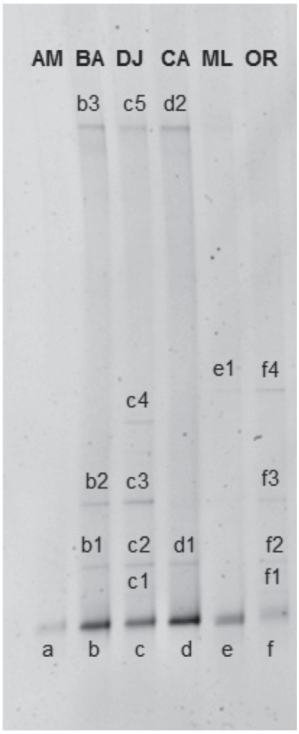

Fig. 3. DGGE patterns of 16S rDNA fragments amplified from nodules roots of *Hedysarum pallidum* in six regions of Algeria: AM (Amoucha), BA (Aïn Babouche), DJ (Djbel Boutaleb), CA (Constantine), ML (Aïn M'lila), OR (Oran)

pallidum harvested from six regions of different geo-climatic areas of Algeria.

The sterility test was positive suggesting that the isolated strains were indigenous symbionts of *Hedysarum pallidum* nodules.

The isolates were classified into 20 groups based on ITS- PCR fingerprinting analysis using FPQuest software. Strains presenting the same profile were grouped in the same ITS haplotype. The suitability of ITS ribotyping for the discrimination of bacterial isolates according to the species and subspecies level was thoroughly reported (Gürtler and Stanisich, 1996; Daffonchio *et al.*, 2000). From each haplotype (n=20) 1 to 3 isolates were selected for partial 16S rRNA gene sequencing. A total of 32 representative isolates were identified, taxonomic identification as detailed in the phylogenetic tree (Figure 4), grouped the bacterial community associated to the nodules of *Hedysarum pallidum* into two phyla *Proteobacteria* [*Gammaproteobacteria* (59%), *Alphaproteobacteria* (18%), *Betaproteobacteria* (3%)] and *Firmicutes* (18%)]. Culturable bacteria diversity was observed between the different sites suggesting an influence of the geo-

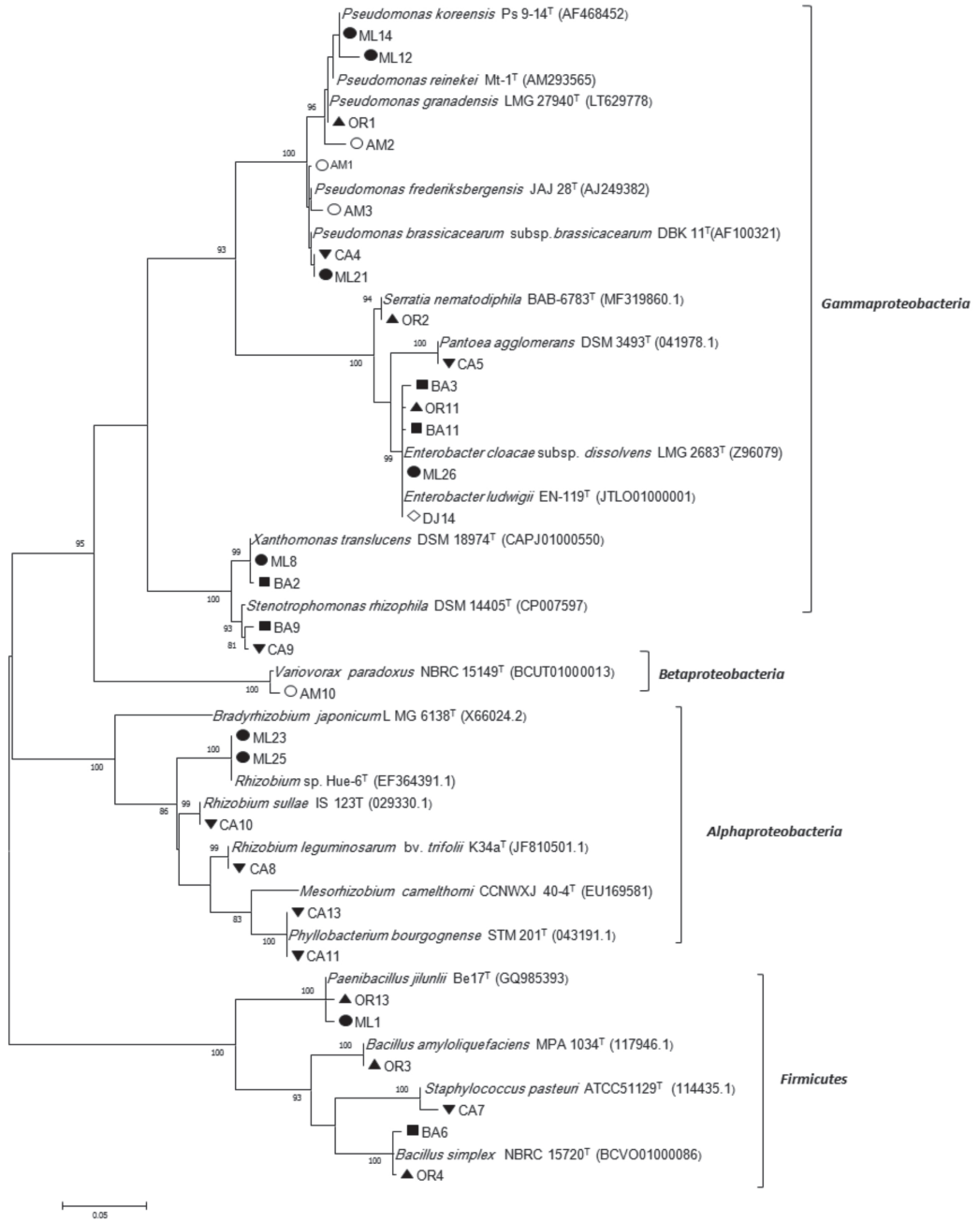

Fig. 4. Maximum Likelihood phylogenetic tree based on partial 16S rRNA sequences of representative strains isolated from nodules of *Hedysarum pallidum* growing in six different geo-climatic areas of Algeria: AM (Amoucha site), BA (■Aïn Babouche site), DJ (Djbel Boutaleb site), CA (▼Constantine site), ML (●Aïn M'lila site), OR(▲Oran site). Bootstrap values are indicated as percentages derived from 1,000 replications. The tree is rooted with *Bradyrhizobium japonicum* LMG 6138^T (X66024.2) and *Mesorhizobium camelthorni* CCNWXJ 40-4^T (EU169581). T: type strain.

climatic factors in shaping the bacterial diversity of *Hedysarum pallidum* (Fig. 5).

The results of biochemical tests showed that the majority of strains were positive for nitrate reductase (93%), urease (98%) and endoglucanase (88%). Moreover, tested strains were able to withstand a wide range of pH from 4 to 11 and they were able to tolerate up to 1.5M of NaCl.

Nodulation test

After two months of plant culture, in the jars inoculated with the strains affiliated to

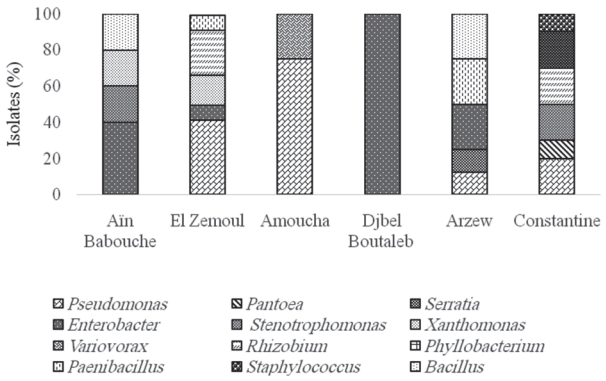

Fig. 5. Diversity percentage at the genus level of culturable bacteria associated with root nodules of *Hedysarum pallidum.*

Alphaproteobacteria represented by (CA10, CA11, ML25, ML23, CA8) the formation of bumps like small nodules was observed.

Among the remaining isolates, strains belonging to the class of *Gamma* and *Beta-proteobacteria* were not able to reinfect their original host in axenic conditions. Nonetheless, we noted the formation of shovel-like branchings or bends in lateral roots that acquire a curved and flattened shape inoculated with strains belonging to different genera: BA2, BA11, ML21, ML8, AM10, CA9, OR13, further, the aerial part of inoculated plants with the most strains was better developed compared to the control plants (not inoculated).

DISCUSSION

This work was carried out to evaluate the phenetic and genetic diversity of isolated bacteria from *Hedysarum pallidum* root nodules grown in six eco climatic different zones in Algeria. Phylogenetic analysis of partial 16SrDNA sequences grouped isolated strains into *Alphaproteobacteria*, *Betaproteobacteria Gammaproteobacteria* and *Firmicutes*.

Gammaproteobacteria was the most abundant group from nodule bacteria isolates represented by *Pseudomonas, Enterobacter, Pantoea, Serratia, Stenotrophomonas* and *Xanthomonas* genera. These results are in agreement with previous studies which reported that these genera are commonly isolated from plant tissues including legume nodules as endophytes with rhizobia from several wild and culturable legumes (Benhizia *et al.,* 2004; Ourarhi *et al.,* 2011; Peix *et al.,* 2012, 2015; Ibañez *et al.,* 2017).

In fact, *Pseudomonas* was the dominant genera obtained inside nodules of our host plant, the selected strains were affiliated to different *Pseudomonas* species isolated from soil and legume roots. Indeed, this genus was widely found in nodules of different species of *Hedysarum* (Benhizia *el al.*, 2004) in Algeria; Later, from nodules of *Medicago truncaluta* (Zakhia *et al.,* 2006) in Tunisia and from *Sphaerophysa salsula* nodules in China (Deng *et al.,* 2011).

Seven representative strains presented higher similarity with clinical strains that does not cause disease in plants belonging to *Enterobacteriaceae* family [*Enterobacter cloacae E. ludwigii, Serratia nemathophila* and *Pantoea agglomerans*]. Besides, previous studies reporting the occurrence of *Enterobacteriaceae* strains in nodules of *Vicia faba* (Kan *et al.,* 2007); *Glycine max* (Li *et al.,* 2008). Similary, Indiguez *et al.* (2005) proved that plant defense response regulate enteric endophytic colonization, reducing the virulence of the strains by removing TTSS-SPI1 increased endophytic.

In line with our findings, a study conducted by De Meyer *et al.* (2015) reported the presence of *Stenotrophomonas* and *Xanthomonas* as NRE isolated from indigenous legumes in Flanders.

Within *Betaproteobacteria* class, AM10 strain was identified as *Variovorax paradoxus* with 100% of homology. To our knowledge, this specie was found for the first time in *Hedysarum pallidum* root nodules. Whereas, it has been reported as an endophyte on *Zea mays* and as plant growth-promoting agent in metal-degraded soils (Pereira *et al.,* 2014).

Alphaproteobacteria group was represented by two genera: *Rhizobium* and *Phyllobacterium.* As far as we know, these two groups were reported for the first time with *Hedysarum pallidum* root nodules. Despite, several studies revealed the unculturable character of rhizobial symbionts and the presence of bacterial endophytic within root nodules of *H. confertum* (*H. humile*), *H. naudinianum* and *H. perrauderianum* (Tondello *et al.,* 2011; Torche *et al.,* 2014). Indeed, Benhizia *et al.* (2004) reported *Gammaproteobacteria* as the sole culturable nodules occupants within three wild of *Hedysarum* species (*Hedysarum spinosissimum* ssp. *capitatum*, *H. pallidum* and *H. carnosum*) collected in Algeria. However, no reports confirm the ability of these bacteria to nodulate *Hedysarum* species in axenic conditions. Therefore, nearly complete 16Sr RNA sequencing was performed on the four representative's strains of the class *Alphaproteobacteria* (CA8, CA10, ML23, ML25) to ensure their identity. Indeed, blast results classified them as (*Rhizobium leguinosarum* bv. *trifolii* (100%),

Rhizobium sullae (99.38%), *Rhizobium* sp. (98.89%) respectively, indicating that culturability of the rhizobial occupant was possible.

Regarding *Firmicutes* phylum which includes *Bacillus*, *Paenibacillus* and *Staphylococcus* genera, diverse studies reported the ability of these genera to inhabit different plant roots for instance, the investigated plant species *Hedysarum pallidum,* from wild legume in Tunisia (Zakhia *et al.,* 2006) and from *Glycine max* (Soybean) in China (Li *et al.,* 2008)

DGGE approach could be represented an important strategy for revealing the unculturable bacterial community associated with *H.pallidum* roots. Nevertheless, these profiles allowed the detection of new specie comparing to the culture-dependent methods identified as *Mesorhizobium camelthorni* CCNWXJ 40-4^T (EU169581), first isolated by Chen *et al.* (2011) from *Alhagi sparsifolia* or camelthorn in China. The appearance of most abundant uncultivable *Mesorhizobium* by DGGE analysis in all tested sites was in line with previous results on two endemic species of *Hedysarum* in Algeria (*H. naudinianum* and *H. perrauderianum)* using another culture-independent method (16S amplicon cloning according by Torche *et al.* (2014)) which considered *Mesorhizobium* sp. as a true inducer of nodule organogenesis. However, *M. camelthorni* was isolated from nodules of forage legume plant *Astragalus glombiformis* growing in Eastern Morocco (Guerrouj *et al*., 2013) using culture dependent methods.

Non-culturability of this taxon from different endemic species tested so far of *Hedysarum* growing in Algeria by culture-dependent methods could be due to a genome mutation under the influence of environmental factors or an acquired unculturability status in vitro. This result was seemed to be a wide spread phenomenon in Mediterranean wild legume (Muresu *et al*., 2008, 2013; Tondello *et al*., 2011).

Chen *et al.* (2011) reported that the type strains of *M. camelthorni* can nodulate *Sophora alopecuroides*, *Glycyrrhiza inflata* and *Medicago sativa* in cross-infection tests. Therefore, it seems that a possible strategy that could be employed to isolate the rhizobial symbionts of *H. pallidum* is to inoculate *Medicago sativa* or *Astragalus glombiformis* with surface-sterilized crushed *H. pallidum* nodules, and see if nodules form on *M. sativa* or *A. glombiformis* roots.

About nodulation test, attempts at re-isolating of rhizobia from small nodules upon surface sterilization were inconclusive. It was not clear whether this could be due to an acquired unculturability status, otherwise, obtained structures were not true nodules and this effect of unculturability may be due to the lack of specificity between these strains and the plant. In fact, *R. sullae* and *R. leguminosarim* are the specific symbionts of *Trifolium* and *Hedysarum coronarium* legumes respectively and as our results *Mesorhizobium* is the most dominant taxon and the true inducer of nodulation in *H. pallidum* nodules. So, they may present other way of interactions in *H. pallidum* nodules.

According to previous reports, rhizobia can also behave as endophytes in nodules and it is frequent the isolation of rhizobial strains from nodules that despite their inability to reinfect their host plant, they often promote its growth (Peix *et al.,* 2012; Muresu *et al.,* 2008). For instance, *R.leguminosarum* bv phaseoli and *Mesorhizobium loti* were isolated from *Trifolium pretense*. Combinaison of endophytic *R. leguminosarum* bv phaseoli and *R. leguminosarum* bv trifolii resulted in the promotion of clover growth (Sturtz *et al.,* 1997). Further, several studies reported the differences in host plant preference for specific rhizobial within natural populations among legume genera and species (Evans *et al.,* 1996; Laguerre *et al.,* 1993).

Noting the formation of root shovels with some strains was previously described in *Hedysarum coronarium* by Tola *et al.* (2009) which these shovels act as efficient calcium-absorbing organs.

Inability of remaining strains to reinfect their host plant, it could be were suggested presumably endophytic or opportunistic in the root nodules. Indeed, diverse studies highlighted that the presence of different non rhizobial endophytes inside root nodules have beneficial effects including plant growth promotion (Tariq *et al.,* 2014; Ibañez *et al.,* 2017; Singh, 2018). As was previously mentioned, plant health, nodulation and yield were improved which co-inoculated with endophytic *gammaproteobacteria* belonging to *Enterobacter*, *Bacillus* and *Pseudomonas*, compared to inoculation with rhizobia alone (Sturz *et al.,* 1997; Rajendran *et al,.* 2008).

A principal component analysis (PCA) showed that sampled soils in the six regions were different, which may explain bacterial diversity from *H. pallidum* root nodules. Various factors may be involved in competitive growth in the rhizosphere and in competition for root colonization. In fact, structure of bacterial communities depends on soil,

biotic and abiotic environmental factors influencing their rhizospheric competence and nodule occupancy by individual genotypes, among these factors is competition for nutrient sources (Vlassak and Vanderleyden, 1997; Gaiero *et al.*, 2013).

Interestingly, nodules of *Hedysarum pallidum* appeared characterized by the constant presence of an unculturable *Mesorhizobium* using DGGE approach, but at the same time they harbor a wide range of taxa encompassing some that have high 16S similarities to legume nodulating bacteria detected for the first time in this legume and others that fall into the category of non-nodulating endophytes, proving an influence of the geo-climatic factors in bacterial distribution of *Hedysarum pallidum*. Further investigations targeting the culturability of *Mesorhizobium*, nodulation gene properties, nitrogen fixation abilities and competition for nodulation of rhizobia and endophytic strains would provide to understand the nature of bacterial interaction and to contribute to a better elucidation of the ecological plant-microbe interactions within the *Hedysarum* genus.

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