Eco. Env. & Cons. 27 (4) : 2021; pp. (1702-1718)
Copyright@ EM International
ISSN 0971–765X

Characterization of rhizobia nodulating *Acacia* isolated from Moroccan Desert

Jamal Eddine El Jamali[1], Loubna Temsamani[1], Ibtissam El Hilali[2], Fatiha Brhada[1], Imane Chaoui[3], Mohammed El Mzibri[3] and Abdelkarim Filali-Maltouf[1]***

[1]Laboratory of Microbiology and Molecular Biology, Faculty of Sciences, University Mohammed V, Rabat, Morocco
[2]National School of Applied Sciences, Agadir, Morocco
[3]Unit of Medical and Biological Research, National Center of Energy, Nuclear Sciences and Techniques, Rabat, Morocco

(Received 17 May, 2021; Accepted 11 June, 2021)

ABSTRACT

Fifty seven rhizobia nodulating *Acacia* were isolated from the soil by trapping at laboratory with seedling of *A.gummifera* and *A.raddiana*. The target sites for soil collection were dunes of the Moroccan desert where acacia has disappeared from decades. The bacteria isolated from the collected nodules displayed a wide tolerance to salinity (44% of strains tolerated up to 1190 mM Nacl) to acidity and alkalinity (80% of strains grew well at pH 4.5) and to extreme temperatures ranging from 4 °C to 50 °C (44% grew at temperature of 45 °C). The strains were resistant to different antibiotics but were sensitive to tetracycline. They were also highly resistant to heavy metals like Aluminium, Zinc and Manganese (400 µg/ml). The study of genetic variability of these isolates was assessed by DNA fingerprinting using REP-PCR. Patterns obtained on agarose gel were strain specific and indicated a considerable diversity. Restriction Fragment Length Polymorphism (RFLP) analysis of 16 S rRNA genes (rDNA) indicated a large diversity of strains and showed a relatedness of some isolates to *Mesorhizobium huakii* and to *Sinorhizobium fredii for others*.

***Key words**: Rhizobia, Acacia, Desert, Phenotypic analysis, Diversity, Rep-PCR, ARDRA.*

Introduction

One of the major harmful issues that agriculture is confronting is the impact of abiotic stresses. In arid and Saharan regions of Morocco, drought and salinity are a serious threat to agriculture and they often occur together.

Drought may have serious implications on soil microbial community structure and activity (Clark *et al*., 2012; Bell *et al.,* 2008) and lead to a slowing down of N and C mineralization (Hueso *et al.,* 2013). Yan and Marschner showed that soil microbial activity and biomass are negatively affected by salinity (Yan and Marschner, 2012).

Water scarcity and high salinity dominate the impact on legumes productivity by affecting symbiotic nitrogen fixation (Davey and Simpson, 1990; Graham, 1993; Katerji *et al*., 2011).

Isolated desert rhizobia that are adapted to extreme conditions of Saharan environments, such as drought, high salt concentration and thermal variations, can live in symbiosis with *Acacia* plants (Duponnois *et al*., 2007). The latter play an important role for their ability to fix sand dunes and limit their progression in different regions of southern Morocco (Zerhari and Filali-Maltouf, 1998). They also

contribute to the restoration of degraded soils (Teixeira and Rodríguez, 2016). These pioneering plants, are widespread in Africa and the Middle East (Zahran, 1999). In Morocco, they covers different areas such as Atlantic Plain (Mediterranean climate), Middle Atlas (relatively humid), Souss region, pre-Saharan and arid Saharan regions (Lebrazi *et al*., 2018). These leguminous trees are well adapted to desert environments and fight actively against desertification in the majority of the south Mediterranean area and sub Saharan countries. They have the ability to fix sandy soils thanks to their very deep root system, and have the ability to fix atmospheric nitrogen following the symbiotic association with bacteria of the *Rhizobiaceae* family. This fertilization of desert lands allows the accumulation of mineral and organic nitrogen compounds assimilated by other more demanding plant species, involved in maintaining balances and functioning within desert ecosystems (Zahran, 200; Rabia *et al*., 2020). Therefore, efficient inoculants rhizobia must have an interesting benefit for these trees. Strains isolated from a wide range of Saharan and arid areas, will be interesting in this point of view. The evaluation of their diversity, their symbiotic performances and their nodulation spectrum, must be the best approach allowing a good selection of the strains to use as inoculum. In addition, in recent years, the use of plant growth-promoting rhizobacteria (PGPR) has shown great promise for treating crops and improving their yield and tolerance to biotic or abiotic stresses (Saravanakumar *et al.,* 2007; Rojas-Tapias *et al*., 2012; Sena *et al.,* 2020, Sijilmassi *et al.,* 2020).

Studies have investigated the rhizobia-legume tree association (Zhang *et al*., 1991; De Lajudie *et al*., 1994; Lindström and Mousavi, 2010; Wang *et al.,* 2018) and a number of different phenotypic and genotypic methods used to identify and describe strains. Each of these methods permits a certain level of phylogenetic classification, from the genus to the strain specific level. The availability of several sensitive and accurate PCR–based genotyping methods (Judd *et al.,* 1993; Selenska-Pobell *et al.,* 1995; Das *et al.,* 2014) has enabled the differentiation among closely related bacterial strains and the detection of higher rhizobial diversity than previously considered (Vinuesa *et al.,* 2008; Rabia *et al.,* 2020).

Consequently, the taxonomy of root-and stem-nodulating bacteria deeply changed in the recent years. Most of these bacterial species are in the *Rhizobiaceae* family in the alpha-proteobacteria and are in either the *Rhizobium*, *Allorhizobium*, *Mesorhizobium*, *Ensifer* (*Sinorhizobium*), *Bradyrhizobium* or *Azorhizobium* genera (Berrada and Fikri-Benbrahim, 2014). However, recent studies have reported the presence of other nonclassical rhizobia belonging to the Betaproteobacteria (De Lajudie *et al.,* 2019; Hassen *et al.,* 2020), Gammaproteo bacteria (Mahdhi *et al.,* 2019), and Actinobacteria (Boukhatem *et al.,* 2016). In some cases, new rhizobia species arise through lateral transfer of symbiotic genes (Velázquez *et al.,* 2005).

Repetitive sequence-based polymerase chain reaction (rep-PCR) genomic fingerprinting and restriction fragment length polymorphism (RFLP) analysis of PCR amplified 16S rRNA gene have been used successfully as genotypic screenings methods (Versalovic *et al.,* 1995; Ben Amor *et al.,* 2007; Das *et al.,* 2014). PCR-RFLP analysis of the 16 S rRNA provides an estimate of the phylogenetic relationships between bacteria (Laguerre *et al.,* 1996; Han *et al.,* 2008) and is especially applicable for the determination of inter and intra-genetic relationships (Janda and Abbot, 2007; Moura *et al.,* 2020). However, 16S rRNA analysis is based on the features of only one gene, where-as rep-PCR generates fingerprints of the entire genome.

In this work we aimed to: (i) isolate rhizobia strains from Moroccan desert by soil trapping using two species of acacia: *A.gummifera* and *A.raddiana*. (ii) Identify and characterize the different strains by PCR based methods (Rep-PCR: Repetitive extragenic palindromic sequences) and ARDRA (Amplified 16S ribosomal DNA restriction analysis) and phenotypic features.

Materials and Methods

Bacterial strains isolation

Three arid regions of the South East of Morocco were prospected: Merzouga, Erg Lihoudi and Mhamid El Ghizlane (Figure 1) and soil samples were collected from different sites in each one.

A. gummifera and *A. raddiana* fresh nodules were obtained and used to isolate bacteria by trapping on young *Acaccia* grown on dunes soil samples or after inoculation of seedlings with suspensions of dunes soils samples (Figure 2). Plants were grown in a growth chamber at 28 °C according to a regular cycle of illumination: 16 hours of light and 8 hours of darkness.

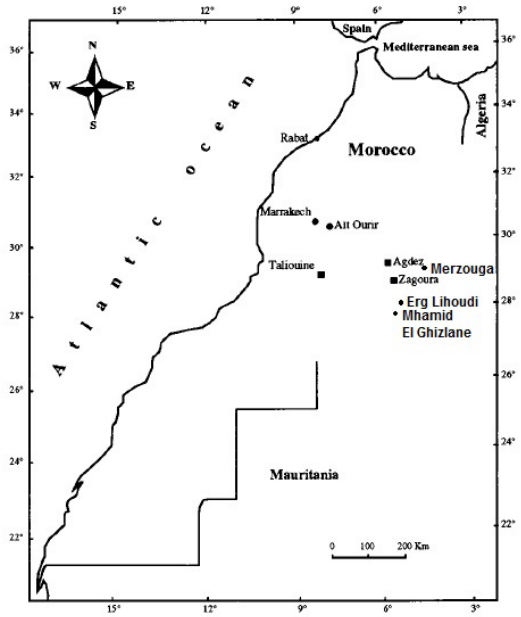

Fig. 1. Locations of the tree principal regions prospected in the desert of Morocco.

Nodules were washed several times with tap water and surface sterilized by immersion for 5 min in 0.1% $HgCl_2$ solution and washed with sterile distilled water. Then, every single nodule was crushed separately in 1 ml sterile solution of 0.5 ml of NaCl and a loopful of the mixture was streaked on yeast extract mannitol agar plates (YMA) according to Vincent (1970). Bacterial colonies obtained after 3-5 days, were routinely checked for their purity by repetitive streaking.

Authentication of rhizobia

The obtained isolates were checked for the ability to renodulate their host plants *A. gummifera* and *A.raddiana.* Three to Four days-old seedlings were planted at the rate of four seedlings in plastic pots containing sterilized sand. Plants tests were inoculated with the bacterial culture and the controls ones were kept uninoculated. Four replications were carried for the plants tests and negative controls. The plants were then cultivated in growth chamber.

Reference strains

We used ten reference strains belonging to Rhizobium (*Rhizobium tropici* CIAT 899, *Rhizobium leguminosarum biovar phaseoli ORS 663, Rhizobium leguminosarum biovar viciae* ORS 639), Mesorhizobium (*Mesorhizobium loti* NZP 2213, *Mesorhizobium plurifarium* ORS 1032, *Mesorhizobium huakuii* ORS1752), Sinorhizobium (*Sinorhizobium fredii* USDA 205, *Sinorhizobium meliloti* ORS 665, *Sinorhizobium arboris* ORS 1755) and *Bradyrhizobium (Bradyrhizobium japonicum* USDA 110).

Physiological characterization

Bacterial strains were tested for their ability to grow under various conditions. Tolerance to NaCl was evaluated by determining growth on agar medium supplemented with 340 mM, 510 mM, 680 mM, 850 mM, 1020 mM, 1190 mM, 1360 mM and 1530 mM NaCl.

All tests were performed in triplicate on YEM agar plates and incubated at 28°C for seven days. Bacterial growth was compared to the controls.

The tests of the growth temperature were performed by incubating the isolates at the following temperatures: 4°C, 10 °C, 15 °C, 35 °C, 40 °C, 45 °C and 50°C.

A B

Fig. 2. Nodules obtained on roots of both *Acacia raddiana* (A) *and Acacia gummifera* (B)after direct inoculation with suspensions of dunes soil samples collected in Merzouga region.

Tolerance to extreme pH was tested by the capacity of strains to grow on agar plates adjusted to pH 4.0, 4.5, 5.0, 6.0, 8.0, 8.5 and 9.0. Bacterial suspensions were used to inoculate different media. The pH was adjusted with NaOH or HCl and YEM agar media were buffered with 25 mM HOMOPIPES (pH 4-5) and 20 mM MES (pH 5-7). Bromothymol blue (BTB) was added as an indicator at the concentration of 25µg.ml^{-1} to reveal changes in the pH after 24 hours.

Intrinsic antibiotic resistance was examinated by using 9 filter-sterilized antibiotics: Streptomycin, spectinomycin, rifampicin, kanamycin, chloramphenicol, erythromycin, ampicilin, tetracycline and nalidixic acid at three concentrations: 10 µg.ml^{-1}, 50 µg.ml^{-1}and 100 µg.ml^{-1}.

The bacterial tolerance to heavy metals was analysis in YEM medium supplemented with Aluminium, Manganese and Zinc at the concentration of 400 µg.ml^{-1} and with Cadmium at the concentrations of 10 µg.ml^{-1}, 30 µg.ml^{-1} and 50 µg.ml^{-1}. The different metals were added at their chloride forms.

Carbohydrate utilization was studied by using API 50 CH test (Biomerieux, France) as described by the manufacturer. Inocula of strains to be tested were obtained from YEM agar plate cultures; bacteria were washed and resuspended in the following medium: (NH4)2SO4, 2 g l^{-1} ; K2HPO4, 0.2 g l^{-1}; $MgSO_4$.7H2O, 0.2 g l^{-1} and 2 g l^{-1} of yeast nitrogen base. Results were scored every day for 7 days as described by Kerstersetal, 1984.

The efficiency of the symbiosis

Symbiosis efficiency was determined by the vigor of the plants, the green color of their leaves and by the pink/red color in the cross sections of nodules. This color results from the accumulation of hemoprotein and leghemoglobin in nitrogen–fixing nodules (Ott *et al.,* 2005).

DNA purification

For REP-PCR, the genomic bacterial DNA was isolated from liquid cultures collected at the late exponential phase using Phenol-Chloroform-Isoamylic alcohol according to Sambrook and Russell (2006). Purified DNA was dissolved in 10 mM Tris-Hcl buffer containing 1mM EDTA (PH: 7.8).

For ARDRA, the total DNA was extracted using alkaline lysis method. Bacteria cells were grown on agar slopes of half concentrated tryptone yeast extract medium (TY) for 48h at 28 °C (Beringer, 1974). They were re-suspended in a lysis buffer (SDS 10%, NaOH 5N), and boiled for 15 min. Then, 200 µl of sterile distilled water was added and cells were pelleted by centrifugation for 10 min at 13000 g. The aqueous phase (200 µl) containing lysed cell suspension was collected and stored at +4°C for use.

REP-PCR genomic fingerprinting

PCR reaction was carried out in a final volume of 28 µl. The reaction mixture contained 50 ng of template DNA, 2.5 U Taq polymerase (promega), 1 X PCR-buffer, 3.3 mM $MgCl_2$, 0.54 Mm dNTP (desoxyribonucleotide triphosphate), 30 pmol of each primers (REP I and REP II), 1% Dimethylsulfoxide (DMS0) and Bovine Serum Albumine (10%). The primers used were REP I (5′-IIIICGICGICATCIGGC-3′) and REP II (5′-ICGICTTATCIGGCCTAC-3′) (Promega) (Versalovic *et al.,* 1991). PCR amplification was carried out according to the following program: 95 °C for 6 min followed by 30 cycles of 94 °C for 1min, 40 °C for 1min, 65 °C for 8 min and final extension step at 65 °C for 16 min. Amplifications were performed with DNA thermal cycler (GENE AMP). Reaction efficiency was estimated by separating 15 µl of the REP-PCR products on 1.2% (W/V) agarose gels containing 1 mg/ml ethidium bromide and scanned under UV illumination. A 1KB ladder (Promega) and 100 pb ladder (Promega) were included as a size reference markers at both sides of each gel.

16S rRNA gene amplification

The 16s rRNA gene was amplified using the primers 41f (5′-GCTCAGATTGAACGCTGGCG-3′) and 1488 (5′CGGTTACCTTGTTACGACTTCACC-3′) (Herrera-Cervera *et al.,* 1999) in 70 µl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 50 µM of dNTP, 4 U Taq DNA polymerase (Promega), 5 pmol of each primer, and 4 µl of lysed cell suspension. PCR amplification was performed in Gene Amp Thermocycler using the following PCR program: 2 min at 94 °C for initial denaturation, 10 cycles of (40 sec at 94 °C, 60 sec at 60 °C and 2 min at 72 °C) and 25 cycles of (40 sec at 94 °C at 60 sec at 50°C and 60 sec at 72 °C) followed by a final extension of 30 min at 72 °C. Amplification was ascertained by agarose (1% w/v) gel electrophoresis. Gel staining was performed as described for the REP-PCR.

For ARDRA analysis,10 µl of each PCR-amplified product were separately digested with the restric-

tion endonucleases *MSP*I (Promega), *Hha*I (New England), *Hinf*I (Promega), *Taq*I (Biolabs). The digestion products were separated on a horizontal 1.8% (w/v) agarose gel in TBE buffer (Sigma). The gels were run at 75 volts for 3 h. Gels were then stained and visualized as for the REP-PCR.

Numerical analysis

For each gel, the bands of the same lane were assigned a band number and scored by the presence (1) or absence (0) and then recorded in a binary matrix. Pairwise comparison of the bands patterns was made by unweighed pair group method with the average (UPGMA) clustering method and the dendrograms were constructed by STATISTICA clustering analysis.

Results

Trapping and authentication of the strains

Fifty-seven isolates were obtained from the various sites of the South east Moroccan Sahara, by trapping on two Moroccan acacia species, *A. gummifera* and *A. raddiana*. The isolates were infective and induced nodule formation on the two Acacia species tested.

All isolates produce a circular and mucoid colonies of 3 mm diameter on yeast extract mannitol medium (YEM) after 48 h of incubation.

The efficiency of the symbiosis

In this study, nodules and strains were obtained in the 3 regions; in Merzouga, nodules and strains collected from *A. gummifera* prevail however in the 2 other regions (Erg Lihoudi and Mhamid El Ghizlane) most nodules and strains were obtained from *A. raddiana* (Figure 3).

Physiological characterization

Salt tolerance

The isolates were analyzed for their ability to grow on different salt concentrations ranging from 340 mM to 1530 mM. More than 90% of bacteria grew well at 2% Nacl (340mM). There was a gradual decrease in percentage of isolates growing at higher salt concentrations.

However, more than 60% of strains tolerated 5% Nacl (850 mM) and about 16% could even grow at 8% Nacl (1360mM)(Figure 4).

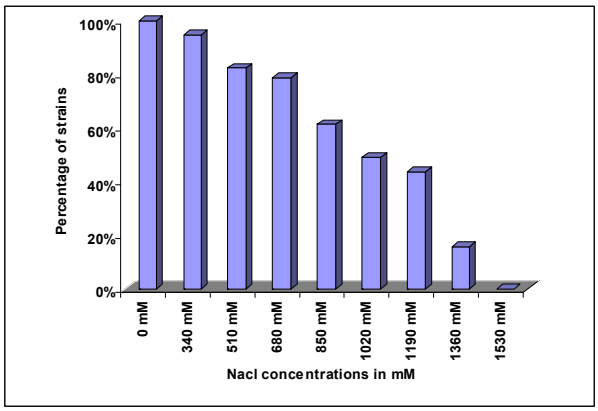

Fig. 4. Tolerance of Rhizobia isolates to different concentration of NaCl.

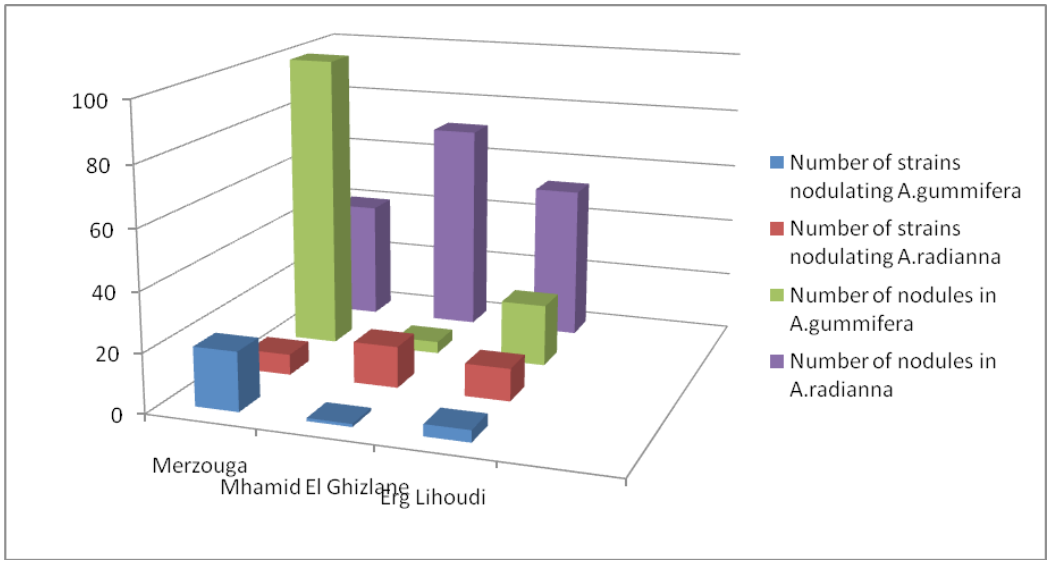

Fig. 3. Efficiency of the symbiosis in consideration with the Sampling soil region and the plant species.

Growth temperature

The isolates were tested for growth on temperature range from 4 °C to 50 °C and show the ability to grow under extreme temperatures. In fact, 68% of strains grew at 4 °C and 44% of them grew at 45 °C. Meanwhile, 9% of the strains could grow at the temperature of 50 °C (Figure 5).

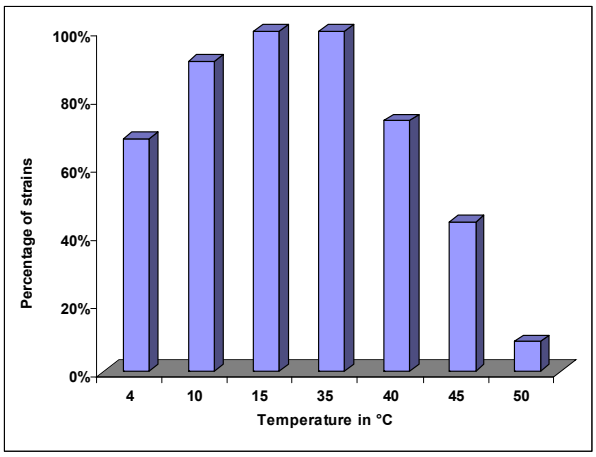

Fig. 5. Tolerance of rhizobia isolates totemperature.

pH tolerance

Al the tested isolates grew well on a pH range from 6 to 9. However, strains varied in their sensitivity to acid pH. 81% of strains were acid tolerant and exhibit a tolerance at pH 4.5. Whereas 44% of the strains could grow at pH 4 (Figure 6).

Resistance to antibiotics

Intrinsic resistance to antibiotics showed a general resistance to Nalidixic acid, and more than 60% of strains exhibited a high resistance to the concentration of 100 µg.ml^{-1} of streptomycin, spectinomycin, chloramphenicol, erythromycin and ampicillin. Also, 58% and 40% were resistant to 100 µg.ml^{-1} of rifampicin and kanamycin respectively. More resistance was observed with other tested concentrations (50 and 100 µg.ml^{-1}). Nevertheless, the isolates were highly sensitive to tetracycline, 31% of strains grow at 10 µg.ml^{-1}but none at 50 or100µg.ml^{-1} (Figure 7).

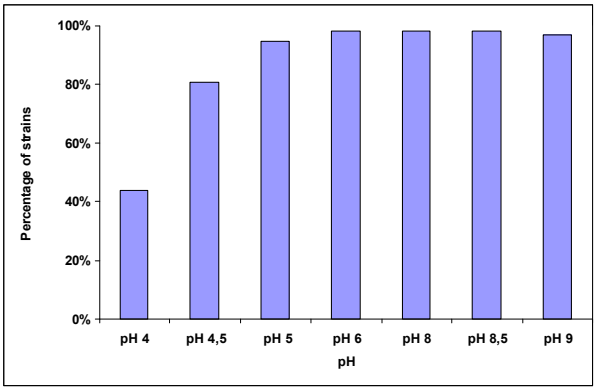

Fig. 6. Tolerance of Rhizobia isolates to pH.

Heavy metal resistance

The Rhizobia isolates showed a high tolerance to Aluminum, Zinc and Manganese (400 µg.ml^{-1}), whereas responses to other metals were variable. Nevertheless, we noted that strains were sensitive to Mercury for which only 9% of strains grew at concentration of 50µg.ml^{-1} (Figure 8).

Carbohydrate utilization

The isolates displayed a large variability in their ability to use different carbohydrates as carbon source. All strains were able to assimilate esculine and raffinose. A total of 60% of strains have assimilated 32 different carbohydrates. Carbon substrates consists in large part of monosaccharaides (galactose, glucose, xylose, fructose, N-acétylglucosamine, arabinose, ribose, mannose, rhamnose, arbutine, xylose), polyols (glycerol, mannitol, inositol, xylitol, arabitol, sorbitol) and disaccharides (maltose, saccharose, lactose, melibiose, trehalose, gentiobiose, turanose, cellobiose). However, three of the used carbohydrates: sorbose, inuline, and starch were not assimilated by more than 10% of strains. The remaining carbohydrates such as dulcitol, glycogène, fucose, tagatose, arabitol, amygdaline, erythritol, salicine showed variable percentage of assimilation depending on the strains (Figure 9). The results showed that Rhizobia isolates seemed to use preferentially monosaccharides carbohydrates as carbon source.

REP-PCR analysis and clustering

The rep-PCR technique generated different molecular profiles which showed to be specific to each isolate (Figure 10). The readable loci obtained were polymorphic with bands weight ranging between 250 pb and 5000 pb.

To evaluate the isolates diversity, dendrogram of rep patterns were constructed using an UPGMA tree building. The Pairwise comparison of rep profiles of the 57 *Acacia* isolates revealed a clustering of five groups and many independents lineages (nine) at 87% of similarity (Figure 11). However, strains

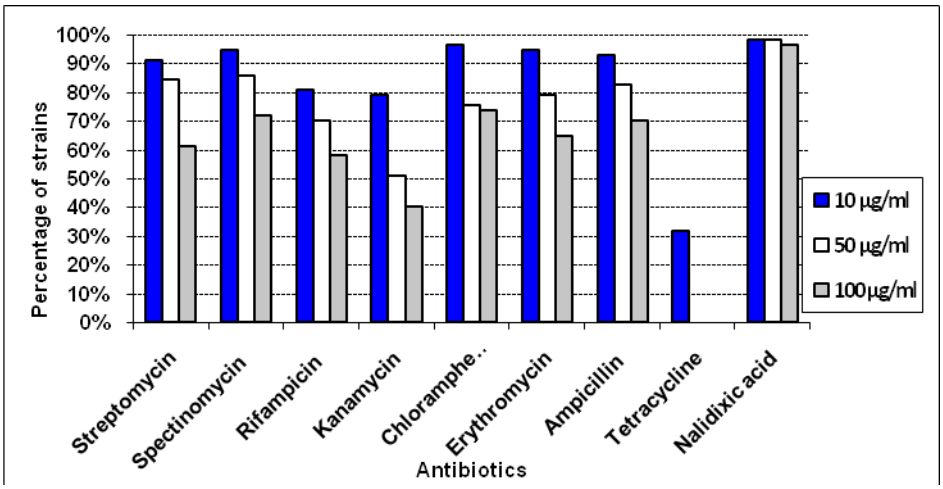

Fig. 7. Resistance of Rhizobia isolates to antibiotics

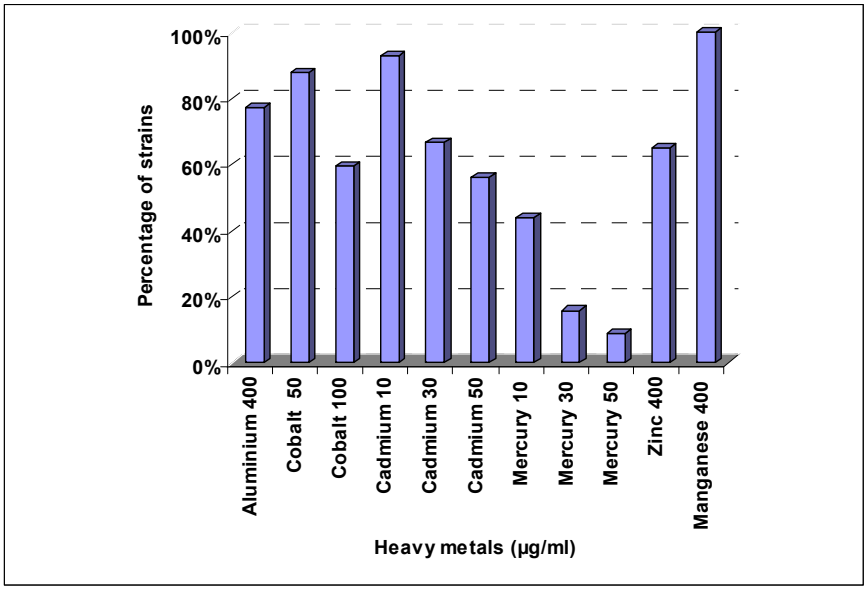

Fig. 8. Resistance of Rhizobia isolates toheavy metals.

were clustered with reference strains used into eight major groups linked at a genetic similarity level of 77 %. They contain three separate groups of reference strains: *Mesorhizobium* group, *Rhizobium leguminosarum* group with *Rhizobium tropici* and *Sinorhizobium* group. In addition, five groups of Rhizobia isolates; were identified: group1 and group 2 were divided into two majors groups and each one was divided into several branches indicating a high potential of diversity.

The clustering of the different reference strains used is logically accepted. Two strains showed some relatedness: MSMC 61-12 closed to *Bradyrhizobium japonicum* with 90% of similarity; and MSMC 61-11 related to *Rhizobium leguminosarum* with more than 75% of similarity. The other strains seemed to be heterogeneous.

ARDRA analysis

The 16S rDNAs of forty five isolated strains were amplified. The PCR allowed the amplification of a single band of about 1500 pb (Data not shown).

The PCR product derived from each strain was digested separately by four restrictions endonucleases *MspI* (Figure 12), *TaqI*, *HhaI*, ***and*** *HinfI*, and the resulting fragments were separated by electro-

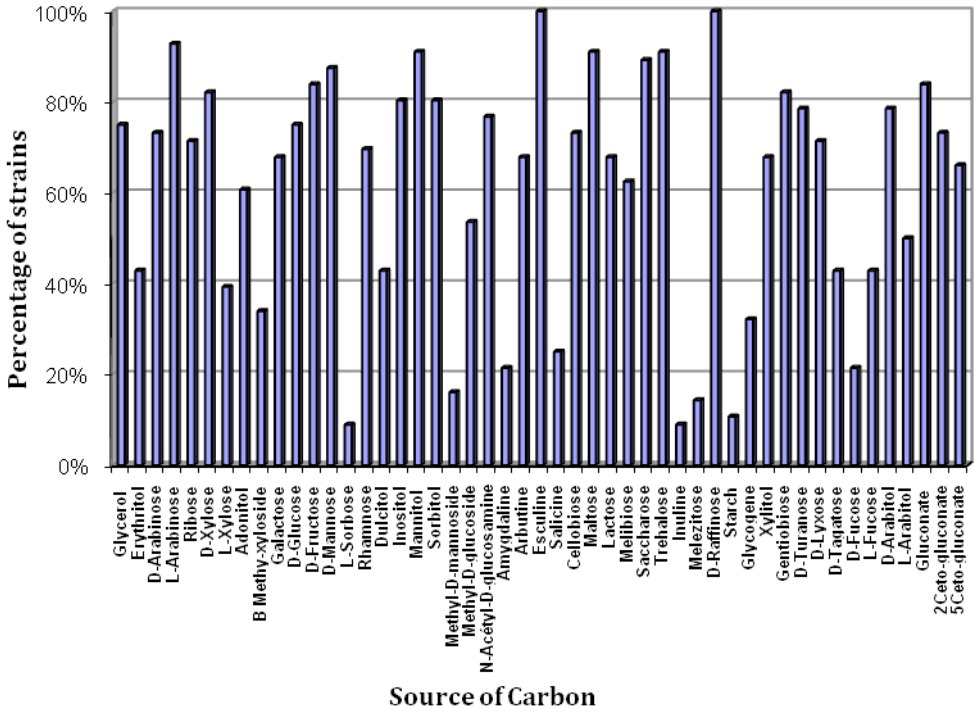

Fig. 9. Assimilation of different carbon substrates by Rhizobia isolates.

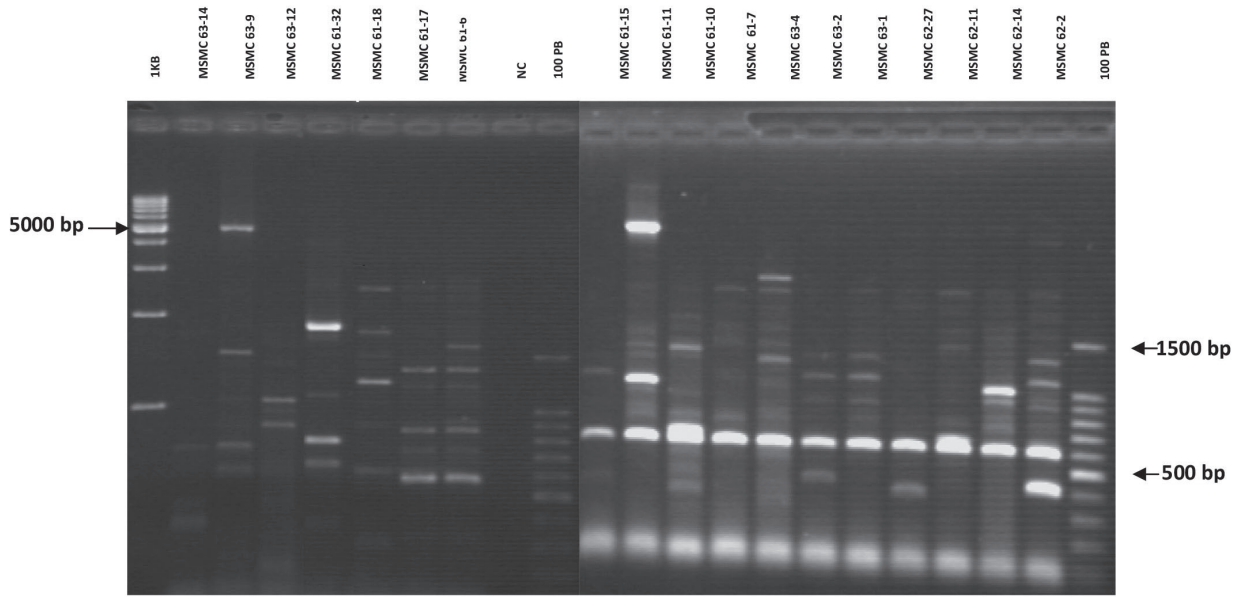

Fig. 10. Electrophoretic patterns of REP/PCR amplification products. NC: Negative control, 100 pb and 1 kb molecular weight markers.

phoresis. One to eight or nine restriction fragments were obtained with each enzyme.

From five to eleven distinct restriction patterns were detected with each of the four endonucleases and the strains used. More than forty restriction combinations were obtained, and RFLP fingerprintings generated were very distinct. Restriction fragments smaller than 100 pb were excluded from the analysis because they were poorly resolved.

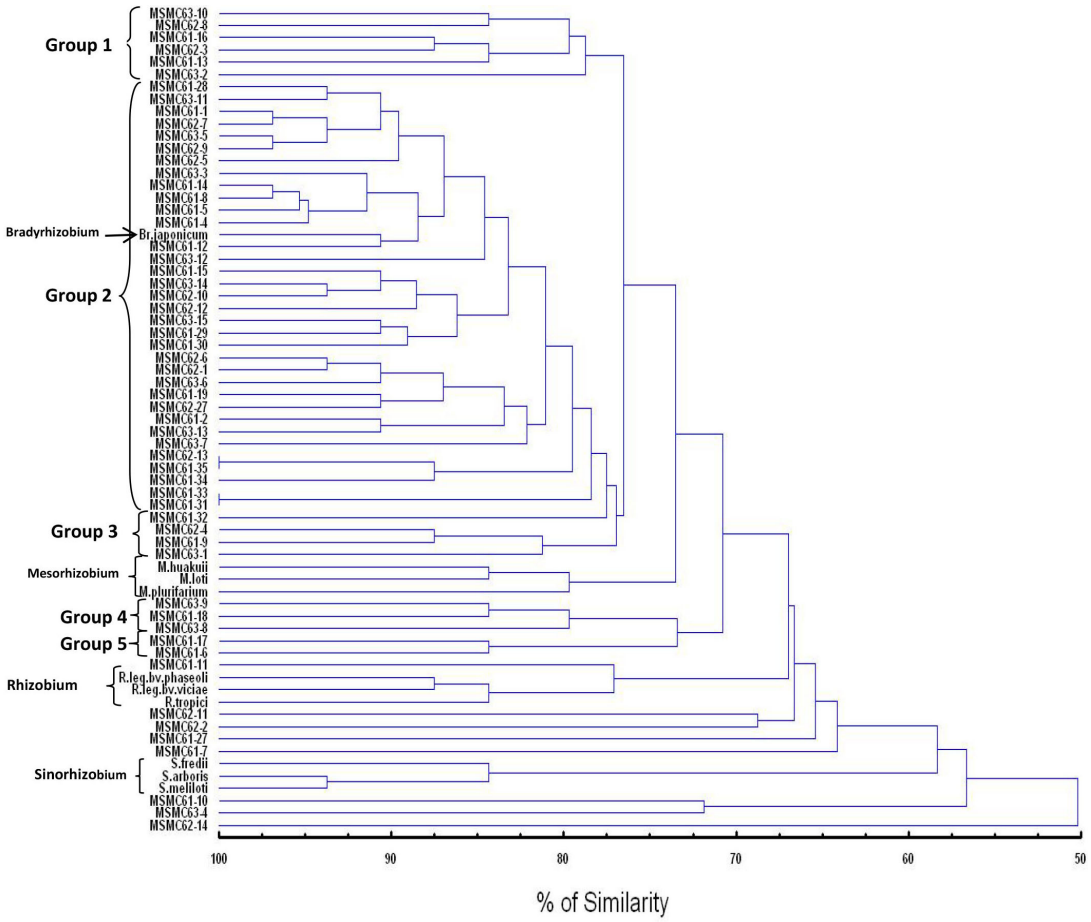

Fig. 11. Dendrogram showing the genetic relationships among strains based on REP-PCR amplification results.

A high polymorphism was revealed especially with MspI followed by Taq I. Paire-wise comparison of restriction profiles obtained of both rhizobial and reference strains were used to generate a phylogenetic tree.

The clustering analysis revealed a clear distinction between strains (Figure 13). Six clusters were delimited at the genetic similarity level of 70%. Cluster 1 correspond to *Bradyrhizobium japonicum* which form an independent lineage; Cluster 2 was formed only by the two reference strains : *Sinorhizobium arboris* and *Sinorhizobium meliloti*; Cluster 3 was formed by *Sinorhizobium fredii* and thirty three strains which nodulate *Acacia*; Cluster 4 was formed by *Mesorhizobium huakii*, *Mesorhizobium plurifarium, Mesorhizobium loti* and seven *rhizobium* isolates; Cluster 5 which contain no reference strain is divided into two subgroups above 75% of similarity and seemed to be more heterogeneous, and cluster 6 was particularly formed by the three reference strains: *Rhizobium leguminosarumbiovar viceae, Rhizobium leguminosarum biovar phaseoli* and *Rhizobium tropici*.

Phylogenetic clustering of the reference strains was in a perfect agreement with other well established results based on ARDRA analysis (Laguerre *et al.*, 1994; Laguerre *et al.*, 1997).

In cluster 3, four *Acacia* strains showed close lineages to *Sinorhizobium fredii* for more than 85% of similarity: MSMC 63-14, MSMC 63-15, MSMC63-8 and MSMC 62-3. The latest was very close for more than 90% of similarity.

At 15% of divergence level, the cluster 4 was subdivided into three subgroups and an independent lineage formed by MSMC 61-30; SubgroupI showed six interesting strains which were very close to *Mesorhizobium huakii*: MSMC 62-13, MSMC 61-14, MSMC 61-29, MSMC 61-31, MSMC 61-33 and MSMC 61-34; and subgroup II which contained two references strains *Mesorhizobium plurifarium* and

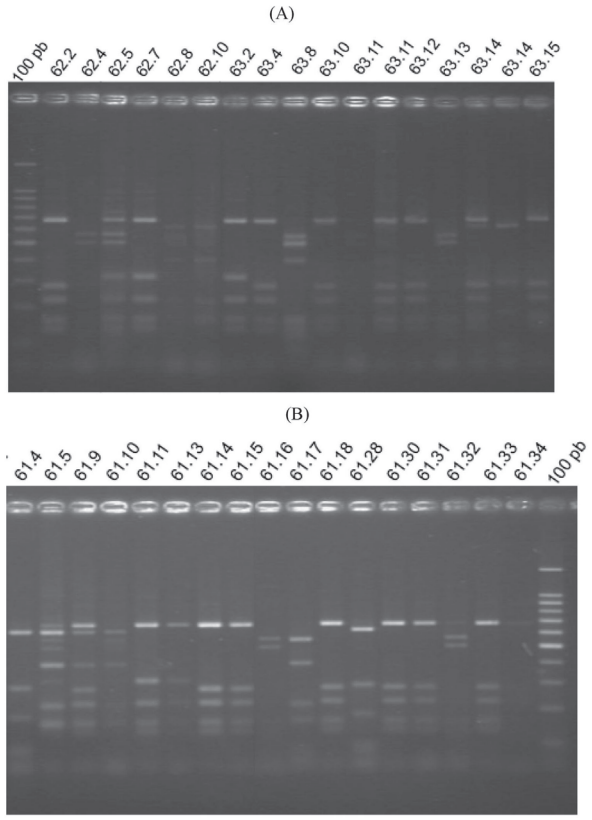

Fig. 12. Restriction patterns of PCR-amplified 16S rDNA region digested with MspI (A & B)

Mesorhizobium loti.

Discussion

The molecular and physiological approaches used in this study allowed us to detect and isolate free-living bacteria from soil samples. Using trapping technique, isolates were collected from root nodules after nodulation of the *Acacia* host plant. Even though, the host plant has disappeared from sampled soil regions from a decade. Boukhatem *et al.* (2012) and Zahran (1999) reported that *Acacia spp*. and tree legumes in general could be nodulated by either *rhizobium spp*. and/or *Bradyrhizobium spp.*

The isolated strains were well adapted to the arid climate, all of them being able to grow at 35 °C, but having a different behaviour above 35 °C; 44% of the isolates showed to be tolerant at 45 °C. In this case, the isolates showed behaviour similar to that of their counterparts isolated from the root of nodules of *Acacia Senegal* and *Prosopis chilensis*, growing in hot dry regions of Sudan (Zhang *et al.,* 1993; Zahran *et al.,* 1994; Räsänen *et al.,* 2001). Our findings are in agreement with previous studies reporting that rhizobia isolated from acacia species tolerated elevated temperature (Boukhatem *et al.,* 2012; Lebrazi *et al.,* 2018; Mahdhi *et al.,* 2019). The existence of highly tolerant *Rhizobium sp* strains was also observed in Kenya, in areas where soils are exposed to high temperatures for long periods of the year (Karanja and Wood, 1988; Pinto *et al.,* 1998).

Strains were uniformly tolerant to high pH. Jordan (1984) showed that the majority of rhizobia could tolerate pH up to 9. The same result was recorded for strains nodulating *Acacia* (Brigido *et al.,* 2007; Zerhari *et al.,* 2000) and Mahdhi *et al.,* (2019) found that the majority of Acacia isolates showed a neutral and baso-tolerant tendency.

At low pH, 44% of strains exhibited an acid-tolerant character since they grew well at pH 4. However, Lebrazi *et al.* (2018) found a high tolerance to acid soil pH for rhizobial bacteria isolated from Acacia species from Morocco.

Some rhizobia strains could grow at low pH up to 3.5 (Appunu *et al.,* 2009). Various rhizobial strains such as *R.tropicii, R.leguminosarum bv.trifoli* and *Mesorhizobium loti,* were described as acid-tolerant and presented a good growth at pH 4(Brigido, 2012; Gauri *et al.,* 2011; Maâtallah *et al.,* 2002).

Strains exhibited a wide tolerance to salinity (NaCl). A total of 16% of strains showed a great osmotolerance since they were able to grow at 8% NaCl. High salt tolerance ranging between 0.5 and 1M was reported for *Sinorhizobium meliloti* (Brigido, 2012; Trivedi and Arora, 2005). Rhizobia isolated from woody legumes like *Acacia*, *Hedysarum, Prosopis* and *Leucaenacan* were also reported to tolerate high NaCl concentrations up to 500 and 800 mM (Tilak *et al.,* 2005; Räsänen, 2002). Our findings are in agreement with previous studies reporting that rhizobia isolated fromacacia species tolerated high soil salinity (Boukhatem *et al.,* 2012; Lebrazi *et al.,* 2018). Shetta *et al.* (2014) and Sakrouhi *et al.* (2016) showed that rhizobia nodulating acacia species collected from dry zones are tolerantto high soil salinity.

High salt tolerance showed to aid in the tolerance to high pH and temperature (Shamseldin and Werner, 2005; Kulkarni and Nautiyal, 2000). In fact, many species of rhizobia were reported to develop adaptation to salinity stress by intracellular accumulation of compatible solute (Vriezen *et al.,* 2007). Salt tolerance can give strains advantages to survive, to

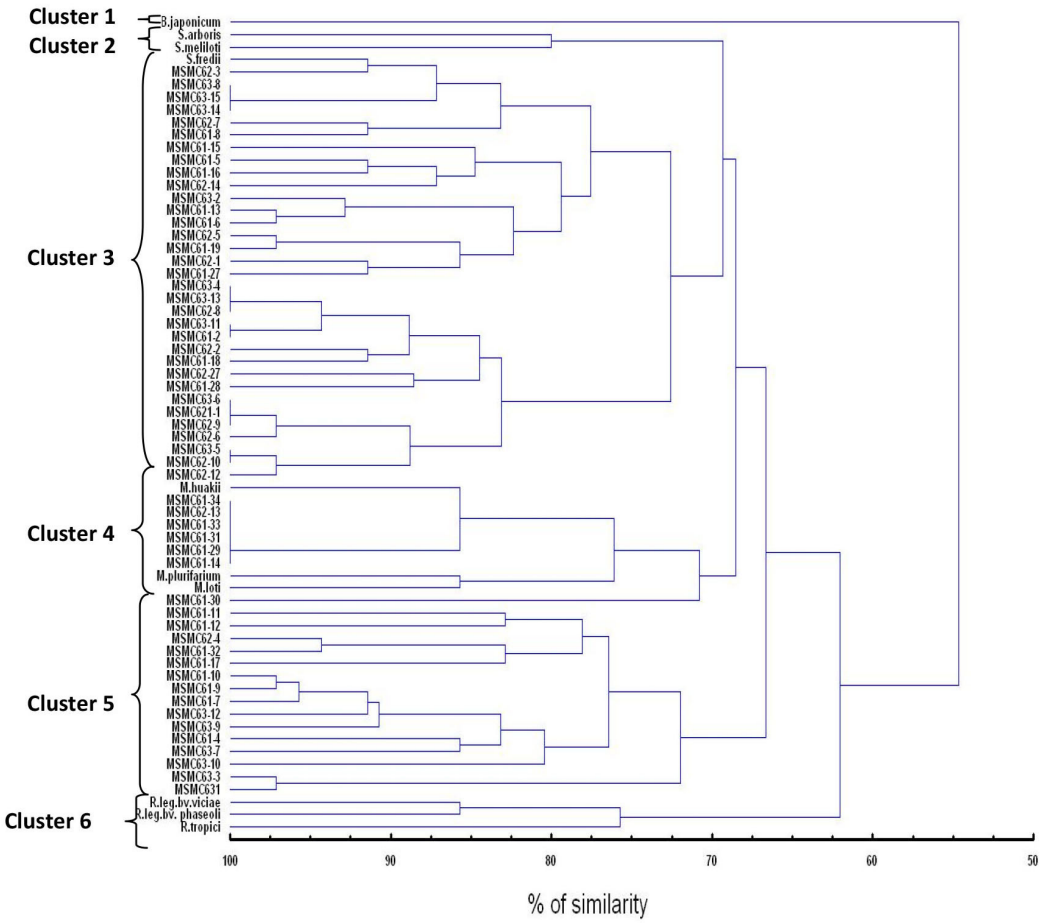

Fig. 13. Dendrogram inferred from the analysis of the restriction patterns of the 16S rDNA obtained for collected strains and reference strains used in this study.

multiply in stress conditions, such as saline soil, and to efficiently infect their host plants. Our results showed a wide tolerance of strains to abiotic tresses such as NaCl, pH and extreme temperatures. These characteristics may be related to the environment conditions prevailing in the original sampling sites, which mean that strains can survive under the influence of the environmental factors variations.

Strains seemed to be differently affected by antibiotics. However, they were highly sensitive, to tetracycline and at a lower degree to kanamycin but they were mostly resistant to nalidixic acid. Although, they showed a similar resistance to different concentrations tested of streptomycin, spectinomycin, rifampicin, kanamycin, chloramphenicol, erythromycin and ampicillin. This result, indicate that strains exhibited a multiple antibiotic resistance. Schwinghamer (1967); Cole and Elkan (1979) described several strains of *rhizobium sp.,* resistant to several antibiotics like chloramphenicol, erythromycin, penicillin, neomycin. It was reported that the same *rhizobium* strain could exhibit a multiple resistance to different antibiotics (Lebrazi *et al.,* 2018; Milicic *et al.,* 2006). The antibiotics resistance degree varies between strains even of the same species (Rabia, 2020; Odee *et al.,* 1997; Shishido and Pepper, 1990; Vincent, 1970).

Isolates were in general resistant to the heavy metals tested (Alu, Co, Cd, Zn, Mn) but they were less resistant to Hg. They displayed different response to the heavy metals tested according to their concentrations (Lebrazi *et al.,* 2018; Milicic *et al.,* 2006). Resistance to heavy metals may confer an advantage to the tolerant isolates by making them more competitive in soil (Chaintreuil *et al.,* 2007; Lesueur *et al.,* 1993).

Strains assimilated a large range of carbohydrate source like (Monosacharides, polyols, disaccharides)

but a large percentage could not use sorbose, inulin and starch. In general Rhizobia displayed different profiles in carbohydrate utilization (Sharma *et al.,* 2010; Graham *et al.,* 1991; Razika *et al.*, 2012), our results were consistent with those of Fall *et al.* (2008), Ourarhi *et al.* (2011) and Rabia *et al.* (2020).

Phenotypic data are necessary for the characterization and the selection of adapted strains to marginal edapho-climatic conditions and to provide information about their diversity.

Besides the morphological features, Rep-PCR was chosen as a first screening technique to assess the genetic diversity of strains. This method constitutes a rapid, efficient and less laborious mean to fingerprint a large number of bacterial genomes, and to distinguish between closely related strains (Trindade *et al.,* 2003; Rademaker *et al.,* 2000; de Bruijn, 1992). The electrophoretic patterns obtained showed a high polymorphism and were found to be specific to each strain. Based on genetic similarity, strains were divided into five major groups. Groups presented no relation to their site of origin and to their level of tolerance showed by the phenotypic tests.

The ARDRA analysis is used to characterize bacterial isolates (Laguerre *et al.,* 1994) and to classify species into a genus (Yang *et al.,* 2007). In our case, the technique was used principally to evaluate the relatedness between the isolates and ten reference strains. The restriction analysis generated distinct patterns with the four enzymes used and especially with *MspI*. Some of our isolates were grouped in the same clade with rhizobial reference strains used. Strains MSMC 62-3, MSMC 63-8, MSMC 63-15 and MSMC 63-14 were related to *S. fredii*, and MSMC 61-34, MSMC 62-13, MSMC 61-33, MSMC 61-31, MSMC 61-29, MSMC 61-14 were related to *M.huakii*. The other strains were clustered distinctly from those of the reference strains used and were revealed to be heterogeneous.

A slight relationship appeared between the clustering patterns of some strains and their site origine. In fact, strains from cluster 3 were from Erg Lihoudi region, and those from cluster 4 were mainly from Merzouga region.

Our results corroborated with several studies, which revealed a high heterogeneity in the populations of rhizobia nodulating *Acacia spp*. (Ndiaye. 1996; Khbaya *et al.,* 1998; Mc Inroy *et al.,* 1999; Mohammed *et al.,* 2000; Odee *et al.,* 2002; Woldemeskel *et al.,* 2005; Ben Romdhane *et al.,* 2005; Vargas *et al.,* 2007). This proved that the strains were genetically different.

In Kenya, *Acacia sp* was nodulated by rhizobia belonging to four genera, *Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium*; whereas, in Moroccan soils, *Acacia sp.*was reported to be nodulated by *Sinorhizobium* species (Khbaya *et al.,* 1998, Sakrouhi *et al.,* 2016). This high diversity of rhizobia nodulating *Acacia* may be in relation with edaphic conditions.

Several strains nodulating wild legumes are being not identified and show characters which are totally different from the existing ones, a finding which points out that new species and genera of root-nodule bacteria may emerge (Zahran, 2001; Chen *et al.,* 2003). Moreover, new bacteria species belonging to alpha-beta and gamma proteobacteria were found in root nodules of leguminous plants (Chen *et al.,* 2005; Shiraishi *et al.,* 2010). In symbiotic rhizobacteria species, the evolution of the population structure may be influenced by many environmental conditions like the type of soil, the genotype of the host plant (Ben Romdhane *et al.,* 2005; Demezas *et al.,* 1995), or the geographical isolation. One additional element that can also play a critical role in the evolution of rhizobial populations is the occurrence of large plasmids that can have an evolutional history different from the *evolutional history* of the *strains* leaving in (Schofield *et al.,* 1987; Young and Wexler, 1988; Suominen *et al.,* 2001). Several papers reported that soils contain a large diversity of non-symbiotic bacteria, which can acquire symbiotic properties by lateral gene transfer between bacteria in the soil (Segovia *et al.*, 1991; Sullivan *et al.,* 1995; Sullivan *et al.,* 1996; Sullivan and Ronson. 1998; Sy *et al.*, 2001; MacLean *et al.,* 2007).

Conclusion

We have isolated free-living rhizobia from soil samples collected from Moroccan desert and noticed a hitherto hidden diversity of rhizobia capable of nodulating *A.gummifera* and *A.raddiana*. The genetic heterogeneity observed by using molecular approach leads us to make a comparative analysis between strains on the basis of their phenotypic characteristics. The large number of different genotypes obtained suggests that Moroccan desert regions may hide a wide rhizobial diversity, still largely unexplored and with an important potential for improving the growth of *Acacia* in arid soils. Results of the

physiological tests indicated that strains exhibited a marked tolerance with respect to salinity, acidity and alkalinity, and to temperatures tested. However, strains were able to grow on a large variety of carbon substrates. Molecular characterization was not sufficient to precise the rhizobia genus of these bacteria, and sequencing of the 16S rRNA gene should be undertaken.

Acknowledgement

These studies were supported by University Mohammed V, Rabat (Programme d'Urgence 2009-2014).

References

Appunu, C., Reddy, L. M. L., Reddy, C. V. C. M, Sen, D. and Dhar, B. 2009. Symbiotic diversity among acid-tolerant bradyrhizobial isolates with cowpea. *The Journal of Agricultural Sciences.* 4 : 126-131

Bell, C. W., McIntyre, N. E., Cox, S. B., Tissue, D. T. and Zak, J. C. 2008. Soil microbial responses to temporal variations of moisture and temperature in a Chihuahuan desert grassland. *Microbial Ecology.* 56: 153-167.

Ben Amor, K., Vaughan, E.E. and de Vos, W.M. 2007. Advanced Molecular Tools for the Identification of Lactic Acid Bacteria. *J. Nutr.* 137 : 741S-747S

Ben Romdhane, S., Nasr, H., Samba-Mbaye, R., Neyra, M. and Ghorbal, M. H. 2005. Diversity of *Acacia tortilis* rhizobia revealed by PCR/RFLP on crushed root nodules in Tunisia. *Annals of Microbiology.* 55 : 249-254.

Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 84 : 188-198

Berrada, H. and Fikri-Benbrahim, K. 2014. Taxonomy of the Rhizobia: Current Perspectives. *Br. Microbiol. Res. J.* 4 : 616-639.

Boukhatem, Z.F., Domergue, O., Bekki, A., Merabet, C., Sekkour, S., Bouzza, F., Duponnois, R., de Lajudie, P. and Galiana, A. 2012. Symbiotic characterization and diversity of rhizobia associated with native and introduced acacias in arid and semi-arid regions in Algeria. *FEMS Microbiol Ecol.* 80 : 534-547.

Boukhatem, Z.F., Merabet, C., Bekki, A., Sekkour, S., Domergue, S., Dupponois, O. and Galiana, A. 2016. Nodular bacterial endophyte diversity associated with native *Acacia* spp. in desert region of Algeria. *Afr J Microbiol Res.* 10 : 634-645

Brigido, C. 2012. *Tolerance of chickpea mesorhizobia to acid and salt stress.* 2012. Thesis of PhD degree in Biology. University of Évora Portugal.

Brigido, C., Alexandre, A., Laranjo, M. and Oliveira, S. 2007. Moderately acidophilic mesorhizobia isolated from chickpea. *Letters in Applied Microbiology.* 44 : 168-174.

Chaintreuil, C., Rigault, F., Moulin, L., Jaffré, T., Fardoux, J., Giraud, E., Dreyfus, B. and Bailly, X. 2007. Nickel Resistance Determinants in Bradyrhizobium Strains from Nodules of the Endemic New Caledonia Legume Serianthes calycina. *Appl. Environ. Microbiol.* 73 : 8018-8022.

Chen, W.M., de Faria, S.M., Straliotto, R., Pitard, R.M., Simoes-Araùjo, J.L., Chou, J.H., Chou, Y.J., Barrios, E., Prescott, A.R., Elliott, G.N., Sprent, J.I.,Young, J.P.W. and James, E.K. 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from SouthAmerica. *Appl. Environ. Microbiol.* 71 : 7461-7471.

Chen, W.M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G. and Boivin-Masson, C. 2003. Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. *Journal of Bacteriology.* 185 : 7266–7272.

Clark, I.M., Buchkina, N., Jhurreea, D., Goulding, K.W.T. and Hirsch, P.R. 2012. Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. *Philosophical Transactions of the Royal Society.* 367: 1235-1244.

Cole, M.A. and Elkan, G.H. 1979. Multiple antibiotic resistance in *Rhizobium japonicum*. *Appl. Environ. Microbiol.* 37 : 867-870.

Das, S., Dash, H.R., Mangwani, N., Chakraborty, J. and Kumari, S. 2014. Understanding molecular identification and polyphasic taxonomic approaches for genetic relatedness and phylogenetic relationships of microorganisms. *J. Microbiol. Methods.* 103 : 80-100

Davey, A.G. and Simpson, R.J. 1990. Nitrogen fixation by subterranean clover (*Trifolium subterraneum L.*) in an acid soil in response to moisture deficits. *Soil Biology and Biochemistry.* 21 : 9-14

de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 58 : 2180-2187.

De Lajudie, P. M., Andrews, M., Ardley, J., Eardly, B., Jumas-Bilak, E. and Kuzmanovi´c, N. 2019. Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *Int. J. Syst. Evol. Microbiol.* 69 : 1852-1863.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M.D., Dreyfus, B., Kersters, K. and Gillis, M. 1994. Polyphasic taxonomy of rhizobia: emendation of the genus Sinorhizobium and description of *Sinorhizobium*

meliloti comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. Nov. *Int J Syst Bacteriol.* 44 : 715-733

Demezas, D.H., Reardon, T.B., Strains, S.R., Watson, J.M. and Gibson, A.H. 1995. Diversity and genetic structure of a natural population of *Rhizobium leguminosarum* bv. *Trifolii subterraneum, L. Mol. Ecol.* 4 : 209-220.

Duponnois, R., Plenchette, C., Prin, Y., Ducousso, M., Kisa, M., Bâ, A.M. and Galiana, A. 2007. Use of mycorrhizal inoculation to improve reafforestation processwith Australian Acacia in Sahelian ecozones. *Ecol. Eng.* 29 : 105–112

Fall, D., Diouf, D., Ourarhi, M., Faye, A., Abdelmounen, H., Neyra, M., Sylla, S.N. and Missbah El Idrissi, M. 2008. Phenotypic and genotypic characteristics of *Acacia senegal* (L.) Willd. root-nodulating bacteria isolated from soils in the dryland part of Senegal. *Lett Appl Microbiol.* 47 : 85-97.

Gauri, Singh, A.K., Bhatt, R.P, Pant, S., Bedi, M.K. and Naglot, A. 2011. Characterization of *Rhizobium* isolated from root nodules of *Trifolium alexandrinum. Journal of Agricultural Technology.* 7 : 1705-1723.

Graham, G. 1993. An interview with Gordon Graham. In: LOGOS Library Acquisitions (eds). *Practice & Theory.* 17 : 413-416.

Graham, P. H., Sadowsky, M.J., Keiser, H. H., Barnet, Y. M., Bradley, R. S., Cooper, J.E., De Lay, J., Jarvis, B. D. W., Roslycky, E. B., Strijdom, B.W. and Young, J. P.W. 1991. Proposed minimal standards for the description of new genera and species of root and stem nodulating bacteria. *Int J Syst. Bacteriol.* 41 : 582-587

Han, T.X., Wang, E.T., Wu, L.J., Chen, W.F., Gu, J.G., Gu, C.T., FuTian, C. and Chen, W.X. 2008. Rhizobium multihospitium sp. nov., isolated from multiple legume species native of Xinjiang, China. *International Journal of Systematic and Evolutionary Microbiology.* 5: 1693-1699

Hassen, A. I., Lamprecht, S. C. and Bopape, F. L. 2020. Emergence of b-rhizobia as new root nodulating bacteria in legumes and current status of the legume rhizobium host specificity dogma. *World J. Microbiol. Biotechnol.* 36 : 1-13.

Herrera-Cervera, J. A., Caballero-Mellado, J., Laguerre, G., Hans-Volker, T., Requena, N., Amarger, N., Martinez-Romero, E., Olivares, J. and Sanjuan, J. 1999. At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil. *FEMS Microbiol. Ecol.* 30 : 87-97.

Hueso, R.O., Maestre, F.T., de los Ríos, A., Valea, S., Theobald, M.R., Vivanco, M.G., Manrique, E. and Bowker, M.A. 2013. Nitrogen deposition alters nitrogen cycling and reduces soil carbon content in low-productivity semiarid Mediterranean ecosystems. *Environmental Pollution.* 179 : 185-193.

Janda, J.M. and Abbot, S.L. 2007. 16S rRNA Gene sequencing for bacterial identification in the diagnostic laboratory : Pulses, Perils, and Pitfalls. *J. Clin. Microbiol.* 45 : 2761-2764.

Jordan, D.C. 1984. Family III. Rhizobiaceae. In: N. R. Krieg, J. G. Holt (ed.). *Bergey's Manual of Systematic Bacteriology*, vol.1, The Williams and Wilkins Co., Baltimore, pp. 234-242.

Judd, A.K., Schneider, M., Sadowsky, M.J. and de Bruijn, F.J. 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* 59 : 1702-1708.

Karanja, N.K. and Wood, M. 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: Tolerance of a high temperature and antibiotic resistance. *Plant and Soil.* 112 : 15-22.

Katerji, N., Mastrorilli, M., Lahmer, F.Z., Maalouf, F. and Oweis, T. 2011. Faba bean productivity in saline–drought conditions. *European Journal of Agronomy.* 35: 2-12.

Kersters, K., Hinz, K. H., Hertle, A., Sergers, P., Lievens, A., Siegmann, O., and De Ley, J. 1984. *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int. J. Syst. Bacteriol.* 34: 56-70.

Khbaya, B., Neyra, M., Normand, P., Zerhari, K. and Filali-Maltouf, A. 1998. Genetic diversity and Phyllogeny of rhizobia that nodulate Acacia spp. In morocco assessed by analysis of rRNA genes. *Appl Environ Microbiol.* 64 : 4912-4917.

Kulkarni, S. and Nautiyal, C. S. 2000. Effect of salt and pH stress on temperature-tolerant *Rhizobium* sp. NBRI330 nodulating *Prosopis juliflora. Curr. Microbiol.* 40 : 221-226.

Laguerre, G., Allard, M. R., Revoy, F. and Amarger, N. 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl. Environ. Microbiol.* 60 : 56-63.

Laguerre, G., Mavingui, P., Allard, M.R., Charnay, M.P., Louvrier, P., Mazurier, S.I. Rigottier-Gois, L. and Amarger, N. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology.* 62 : 2029-2036.

Laguerre, G., Van Berkum, P., Amarger, N. and Prévost, D. 1997. Genetic diversity of rhizobial symbionts isolated from legume species within the genera Astragalus, Oxytropis, and Onobrychis. *Appl. Environ. Microbiol.* 63 : 4748-4758.

Lebrazi, S., Chraibi, M., Fadil, M., Barkai, H. and Fikri-Benbrahim, K. 2018. Phenotypic, genotypic and symbiotic characterization of rhizobial isolates nodulating *Acacia* sp. in Morocco. *J Pure Appl Microbiol.* 12 : 249-263.

Lesueur, D., Diem, H.G., Dianda, M. and Leroux, C. 1993. Selection of Bradyrhizobium strains and provenances of *Acacia mangium* and *Faidherbia albida*: relationship with their tolerance to acidity and aluminium. *Plant Soil.* 149 : 159–166

Lindström, K. and Mousavi, S.A. 2010. Rhizobium and Other N-fixing Symbioses. In: *Encyclopedia of Life Sciences* (ELS). John Wiley & Sons, Ltd: Chichester.

Maâtallah, J., Berraho, E. B., Sanjuan, J. and Lluch, C. 2002. Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Moroccan soils. *Agronomie.* 22 : 321-329.

MacLean, A.M., Finan, T.M. and Sadowsky, M.J. 2007. Genomes of the symbiotic nitrogen-fixing bacteria of legumes. *Plant Physiol.* 144 : 615–622

Mahdhi, M., Tounekti, T. and Khemira, H. 2019. Phenotypic and Genotypic Characterization of Microsymbionts of Acacia Species Plants Grown in South-Western Saudi Arabia. *Journal of Soil Science and Plant Nutrition.* 19 : 631-638.

Mc Inroy, S.G., Campbell, C.D., Haukka, K., Odee, D.W., Sprent, J.I., Wang, W.J., Young, J.P.W. and Sutherland, J.M. 1999. Characterization of rhizobia from African acacias and other tropical woody legumes using biolog and partial 16S rRNA sequencing. *FEMS Microbiol Lett.* 170 : 111-117

Milicic, B., Delic, D., Kuzmanovic, D., Stajkovic, O. and Josic, D. 2006. Intrinsic antibiotic resistance of different *Bradyrhizobium Japonicum* and *Rhizobium Galegae* strains. *Roumanian Bitechnological Letters.* 11 : 2723-2731.

Milicic, B., Delic, D., Stajkovic, O., Rasulic, N., Kuzmanovic, D. and Josic, D. 2006. Effects of heavy metals on rhizobial growth. *Roumanian Biotechnological Letters.* 11 : 2995-3003.

Mohammed, S.H., Smouni, A., Neyra, M., Kharchaf, D. and Filali-Maltouf, A. 2000. Phenotypic characteristics of root-nodulating bacteria isolated from *Acacia* spp. Grown in Libya. *Plant and Soil.* 224 : 171-183

Moura, E.G., Carvalho, C.S., Bucher, C.P. C., Souza, J.L.B., Aguiar, A.C.F., Ferraz Junior, A.S.L., Bucher, C.A. and Coelho, K.P. 2020. Diversity of Rhizobia and Importance of Their Interactions with Legume Trees for Feasibility and Sustainability of the Tropical Agrosystems. *Diversity.* 12 : 206.

Ndiaye, A.A. 1996. Diversité et fixation d'azote des rhizobiums d'Acacia. Mémoire de DEA. Dakar : Université Cheikh Anta Diop.

Odee, D.W., Haukka, K., McInroy, S.G. Sprent, J.I., Sutherland, J.M. and Young, J.P.W. 2002. Genetic and symbiotic characterization of rhizobia isolated from tree and herbaceous legumes grown in soils from ecologically divers diverse sites in Kenya. *Soil Biol Biochem.* 34 : 804-811.

Odee, D.W., Sutherland, J.M., Makatiani, E.T., Mc Inroy, S.G. and Sprent, J.I. 1997. Phenotypic characteristics and composition of the rhizobia associate with woody legumes growing in diverse kanyen conditions. *Plant and Soil.* 188 : 65-75.

Ott, T., Van Dongen, J.T., Günther, C., Krusell, L., Desbrosses, G., Vigeolas, H., Bock, V., Czechowski, T., Geigenberger, P. and Udvardi, M.K. 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Curr Biol.* 29 : 531-535.

Ourarhi, M., Abdelmoumen, H., Guerrouj, K., Benata, H., Muresu, R., Squartini, A. and Missbah El Idrissi, M. 2011. Colutea arborescens is nodulated by diverse rhizobia in Eastern Morocco. *Arch Microbiol.* 193 : 115-124.

Pinto, P.P., Raposeiras, R., Macedo, A.M., Seldin, L., Paiva, E. and Sa, N.M.H. 1998. Effects of high temperature on survival, symbiotic performance and genomic modifications of bean nodulating Rhizobium strains. *Rev. Microbiol.* 29 : 295-300

Rabia, K., Xiao Xia, Z., Rifat, H. and Mukhtar, A. 2020. Molecular Characteristics of Rhizobia Isolated from *Arachis hypogaea* Grown under Stress Environment. *Sustainability.* 12 : 6259

Rademaker, J. L. W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L. and Vauterin, P. and de Bruijn, F.J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology.* 50 : 665–677

Räsänen, L. A. 2002. Biotic and abiotic factors infl uencing the development of N2-fixing symbioses between rhizobia and the woody legumes *Acacia* and *Prosopis.* Academic Dissertation in Microbiology. University of Helsinki Finland

Räsänen, L.A., Sprent, J.I. and Lindström, K. 2001. Symbiotic properties of sinorhizobia isolated from Acacia and Prosopis nodules in Sudan and Senegal. *Plant and Soil.* 235 : 193-210.

Razika, G., Amira, B., Yacine, B. and Ammar, B. 2012. Influence of carbon source on the production of exopolysacharides by Rhizobium sullae and on the nodulation of *Hedysarum coronarium* L. legume. *Afr. J. Microbiol. Res.* 6 : 5940-5946.

Rojas-Tapias, D., Moreno-Galván, A., Pardo-Díaz, S., Obando, M., Rivera, D. and Bonilla, R. 2012. Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (Zea mays). *Applied Soil Ecology.* 61 : 264-272.

Sakrouhi, I., Belfquih, M., Sbabou, L., Moulina, P., Benaa, G., Filali-Maltouf, A. and Le Quéré, A. 2016. Recovery of symbiotic nitrogen fixing acacia rhizobia from Merzouga Desert sand dunes in South East Morocco -Identification of a probable new species of Ensifer adapted to stressed environments. *Syst. Appl. Microbiol.* 39 : 122-31

Sambrook, J. and Russell, D. W. 2006. Purification of Nucleic Acids by extraction with Phenol: Chloroform. Cold Spring Harb Protocols.

Saravanakumar, D., Vijayakumar, Kumar, C. N. and Samiyappan, R. 2007. PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Protection.* 26 : 556-565.

Schofield, P.R., Gibson, A.H., Dudman, W.F. and Watson, J.M. 1987. Evidence for genetic exchange and recombination of rhizobium symbiotic plasmids in a soil population. *Appl. Environ. Microbiol.* 53 : 2942-2947

Schwinghamer, E.A. 1967. Effectiveness of rhizobium as modified by mutation for resistance to antibiotics, Antonie van Leeuwenhoek. *J. Microbiol. Serol.* 33 : 121-136.

Segovia, L., Pinero, D., Palacios, R. and Martinez-Romero, E. 1991. Genetic structure of a soil population of nonsymbiotic Rhizobium leguminosarum. *Appl Environ Microbiol.* 57 : 426-433.

Selenska-Pobell, S., Gigova, L. and Petrova, N. 1995. Strain-specific fingerprints of Rhizobium galegae generated by PCR with arbitrary and repetitive primers. *J. Appl. Bacteriol.* 79 : 425-431.

Sena, V.G.L., Moura, E.G., Macedo, V.R.A., Aguiar, A.C.F., Price, A.H., Mooney, S.J. and Calonego, J.C. 2020. Ecosystem services for intensification of agriculture, with emphasis on increased nitrogen ecological use efficiency. *Ecosphere.* 11 : 1-14.

Shamseldin, A. and Werner, D. 2005. High salt and high pH tolerance of new isolated Rhizobium etli strains from Egyptian soils. *Curr Microbiol.* 50 : 11-16.

Sharma, M.P., Srivastava, K. and Sharma, S.K. 2010. Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of Central India. *Plant Soil Environ.* 56 : 375–383

Shetta, D., EL-Sayed, AWB., Nasr, T.A. and Shaarawy, N.M. 2014. Influences of mineral fertilization with NPK, inoculation and methods of inoculation on seedling growth of two woody legume trees. *World Appl Sci J.* 29 : 825-834.

Shiraishi, A., Matsushita, N. and Hougetsu, T. 2010. Nodulation in black locust by the Gammaproteobacteria *Pseudomonas* sp. and the Betaproteobacteria *Burkholderia* sp. Syst. *Appl. Microbiol.* 33 : 269–274

Shishido, M. and Pepper, I. L. 1990. Identification of dominant indigenous *Rhizobium meliloti* by plasmid profiles and intrinsic antibiotic resistance. *Soil Biol. Biochem.* 22 : 11-16.

Sullivan, J.T. and Ronson, C. W. 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proc Natl Acad Sci USA.* 95 : 5145-5149.

Sullivan, J.T., Bertrand, D.E., Van Berkum, P. and Ronson, C.W. 1996. Four unnamed species of non symbiotic rhizobia isolated from the rhizosphere of Lotus corniculatus. *Appl Environ Microbiol.* 62 : 2818-2825.

Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B. and Ronson, C.W. 1995. Nodulating strains of rhizobium loti arise through chromosomal symbiotic gene transfer in the environnement. *Porc Natl Acad Sci (USA).* 92 : 8985-8989.

Suominen, L., Lortet, G., Roos, C., Paulin, L. and Lindström, K. 2001. Identification and structure of the Rhizobium galegae common nodulation genes: evidence for horizontal gene transfer. *Mol Biol Evol.* 18 : 906–916.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., De Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C. and Dreyfus, B. 2001. Methylotrophic Methylobacterium Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes. *J Bacteriol.* 183 : 214-220.

Teixeira, H. and Rodríguez-Echeverría, S. 2016. Identification of symbiotic nitrogen-fixing bacteria from three African leguminous trees in Gorongosa National Park. *Syst. Appl. Microbiol.* 39 : 350-358.

Tilak, K.V., Ranganayaki, N., Pal, K.K., De, R., Saxena, A.K., Nautiyal, C.S., Mittal, S., Tripathi, A.K. and Johri, B.N. 2005. Diversity of plant growth and soil health supporting bacteria. *Curr. Sci.* 89 : 136-150.

Trindade, P. A., Mcculloch, J. A., Oliveira, G. A. and Mamizuka, E.M. 2003. Molecular techniques for MRSA typing: current issues and perspectives. *Braz J Infect Dis.* 7 : 32-43.

Trivedi, R. and Arora, S. 2005. Characterization of acid and salt tolerance *Rhizobium* spp. isolated from saline soils of Gujarat. *Int Res J Chem.* 3 : 8-13.

Trujillo, M.E, Alonso-Vega, P., Rodríguez, R., Carro, L., Cerda, E., Alonso, P. and Martínez-Molina, E. 2010. The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. *ISME J.* 4 : 1265-1281.

Vargas, L., Lisboa, B., Scholles, D., Pfeifer Silveira, J., Jung, G., Granada, C., Neves, A., Braga, M. and Negreiros, T. 2007. Genetic diversity and symbiotic efficiency of black wattle-nodulating rhizobia in soils of Rio Grande do Sul State, Brazil. Revista Brasileira de *Ciencia do Solo.* 31 : 647–654.

Velázquez, E., Peix, A., Zurdo-Piñiro, J.L., Palomo, J.L., Mateos, P.F., Rivas, R., Muñoz-Adelantado, E., Toro, N., García-Benavides, P. and Martínez-Molina, E. 2005. The Coexistence of Symbiosis and Pathogenicity-Determining Genes in Rhizobium rhizogenes Strains Enables Them to Induce Nodules and Tumors or Hairy Roots in Plants. *Molecular Plant-Microbe Interactions.* 18 : 1325-1332.

Versalovic, J., Kapur, V., Koeuth, T., Mazurek, G.H., Whittam, T.S., Musser, J.M. and Lupski, J.R. 1995. DNA fingerprinting of pathogenic bacteria by fluorophore-enhanced repetitive sequence-based polymerase chain reaction. *Arch Pathol Lab Med.* 119: 23-29.

Versalovic, J., Koeuth, T. and Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acids Res.* 19 : 6823- 6831.

Vincent, J. M. 1970. *A Manual for the Practical Study of Root Nodule Bacteria*, IBP Handbook, No. 15, Blackwell Scientific Publications, Ltd., Oxford, England

Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Mahna, S. K., Prasad, B. N., Moe, H., Selvaraju, S. B., Thierfelder, H. and Werner, D. 2008. Multilocus Sequence Analysis for Assessment of the Biogeography and Evolutionary Genetics of Four *Bradyrhizobium* Species That Nodulate Soybeans on the Asiatic Continent. *Appl. Environ. Microbiol.* 74: 6987-6996.

Vriezen, J.A.C., de Bruijn, F. J. and Nüsslein, K. 2007. Responses of Rhizobia to Desiccation in Relation to Osmotic Stress, Oxygen, and Temperature. *Appl Environ Microbiol.* 73 : 3451-3459.

Wang, Q., Liu, J. and Zhu, H. 2018. Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* 9 : 313.

Wolde-meskel, E., Terefework, Z., Frostegard, A. and Lindstrom, K. 2005. Genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in southern Ethiopia. *International Journal of Systematic and Evolutionary Microbiology.* 55 : 1439-1452.

Yan, N. and Marschner, P. 2012. Response of microbial activity and biomass to increasing salinity depends on the final salinity, not the original salinity. *Soil Biology and Biochemistry.* 53 : 50-55.

Yang, J.H., Liu, H.X., Zhu, G.M., Pan, Y.L., Xu, L.D. and Guo, J.H. 2007. Diversity analysis of antagonists from rice-associated bacteria and their application in biocontrol of rice diseases. *J Appl Microbiol.* 104 : 91-104.

Young, J. P. W. and Wexler, M. 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 134 : 2731-2739.

Zahran, H. H., Rasanen, L. A., Karsisto, M. and Lindstrom, K. 1994. Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. *World J Microbiol Biotechnol.* 10 : 100-105.

Zahran, H.H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in arid climate. *Microbiol Mol Biol Rev.* 63 : 968-989.

Zahran, H.H. 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *J. Biotechnol.* 91 : 143-153.

Zerhari K., Aurag J. and Filali-Maltouf A. 1998. Biodiversity of *Rhizobium* sp. strains isolated from *Acacia* spp. species in south of Morocco. In: *Biological Nitrogen Fixation for the 21st Century.* Elmerich C. et al. (eds). Kluwer Academic Publishers, Dordrecht, The Netherlands.

Zerhari, K., Aurag, J., Khbaya, B., Kharchaf, D. and Filali-Maltouf, A. 2000. Phenotypic characteristics of rhizobia isolates nodulating *Acacia* species in the arid and Saharan regions of Morocco. *Lett. Appl. Microbiol.* 30 : 351-357.