



## Diversity of Bacteria Nodulating *Medicago arborea* in the Northeast Area of Morocco

Kamal Guerrouj\* [a], Mustapha Bouterfas [b], Hanaa Abdelmoumen [c] and Mustapha Missbah El Idrissi [c]

[a] Institut Supérieur des Professions Infirmières et des Techniques de Santé Al Hoceima, Morocco - 32000

[b] Laboratoire des sciences de l'eau, de l'environnement et de l'écologie, Faculté des Sciences, Université Mohamed Premier, Oujda, Morocco.

[c] Laboratoire d'amélioration de la productivité des sols et environnement, Ecole Normale Supérieure, Université Mohamed V-Agdal, Av. Mohamed Belhassan El Ouazzani-Takaddoum, Rabat, Morocco.

\*Author for correspondence; e-mail: kamaledine.guerrouj@yahoo.fr

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### ABSTRACT

A Polyphasic characterization of 30 bacteria isolated from root nodules of *Medicago arborea* (Medic tree) plants growing in 3 arid soils of the arid northeast region of Morocco was performed. The phenotypic, symbiotic, and cultural characteristics analyzed allowed the description of a broad physiological diversity among the isolates. The phenogram obtained showed 3 main clusters at a similarity coefficient of 78%. The results obtained suggest that the phenotype of these rhizobia might have evolved for adaptation to the local conditions.

The genetic characterization which consisted in a rep-PCR using ERIC (Enterobacterial Repetitive Intergenic Consensus) primers showed a great diversity and 3 groups and 8 clusters were delimited at a similarity level of 80%. However, the 16S rDNA sequences showed a perfect identity of all the isolates with *Sinorhizobium meliloti* published sequences.

The results showed a relationship between rep-PCR fingerprinting and phenotypic techniques, which are complementary in diversity investigation and allowed revelation of relationships between *M. arborea* close-related bacterial isolates originated from different geographic location in the northeast area of Morocco.

**Keywords:** *Medicago arborea*, rhizobium, 16S rDNA, bacterial diversity, ERIC-PCR

### 1. INTRODUCTION

Nitrogen constitutes almost 80% of the atmosphere, but is metabolically inaccessible to plants due to the exceptional stability of the triple covalent bond (N<sub>2</sub>). The ability to catalyze enzymatic reduction of N<sub>2</sub> to NH<sub>3</sub> is limited to a variety of prokaryotes defined as nitrogen-fixing or diazotrophic microorganisms, which are widely distributed

in all ecosystems as either free-living organisms or in symbiotic association with a number of different plants. These N<sub>2</sub>-fixing prokaryotes can be anaerobic, facultative aerobic, aerobic, photosynthetic, or nonphotosynthetic. All carry out N<sub>2</sub> reduction by an enzymatic complex termed nitrogenase.

Rhizobia are soil bacteria capable of

eliciting nodules on leguminous plant roots and/or stems in which the bacteria fix nitrogen. Rhizobial genera include *Agrobacterium*, *Allorhizobium*, *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Cupriavidus*, *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Neorhizobium*, *Pararhizobium*, *Shinella*, *Ciceribacter* and *Ensifer* (syn. *Sinorhizobium*) [1].

*Medicago arborea* (Tree Medic) is a perennial woody leguminous shrub native to the Mediterranean region where it is currently found growing in rocky dry calcareous soils from the Canary Islands and Balearic Islands along southern Europe to Asia Minor [2]. *M. arborea* is very drought tolerant (<250 mm annual rainfall) and will also grow well in sandy soils. Therefore it has the appeal of being able to provide highly nutritious fodder for its wealth content on digestible proteins, while at the same time protecting fragile soils [3].

The northeast Moroccan area is known by its arid climate with huge fields entirely affected by erosion and soil degradation. Harsh edapho-climatic conditions and entropic factors contribute on desertification's installation and proliferation.

The characterization of nitrogen-fixing soil bacteria in symbiosis with *Medicago arborea* is envisaged to foster knowledge in the ecological behavior of semi-arid soil species and will provide the potential to promote plant growth in the harsh conditions prevailing in Mediterranean region. The aim of this work was to analyze the phenotypic and genetic diversity of bacterial isolates from root nodules of *M. arborea* grown in 3 different soils of the northeast region of Morocco.

## 2. MATERIALS AND METHODS

### 2.1 Soil Analysis

Three soils were selected for this study, all from the arid area of northeast Morocco; two from agricultural technique centre of

El Aioun and Bouarg and one from Harraza (Oujda region).

Chemical analysis are focused on pH, conductivity, soils organic matter cotenants, Nitrogen and Carbon proportions.

Samples analysis was performed in soil tests laboratory, INRA, Oujda, and in the "Centre Oriental des Sciences et Techniques de l'Eau" (COSTE), Faculty of Sciences, Mohammed Premier University, Oujda, Morocco.

### 2.2 Bacterial Strains

Bacteria were isolated from the root nodules of *Medicago arborea* plants grown in Oujda, El Aioun and Bouarg soils from the northeast area of Morocco. From young plants of each soil, 10 nodules were sampled, washed under running water, then encaged in a fine-mesh steel holder and surface sterilized by immersion in 5% Sodium hypochlorite for 3 min: and finally washed seven times with sterile distilled water. Each nodule was crushed on plates containing yeast extract mannitol agar (YMA) with 0.0025% (w/v) Congo red [4]. After incubation for 20 days at 28°C single colonies were selected and transferred several times on the YMA plates to ascertain purity. A total of 30 pure isolates were conserved on YEM slants at 4°C for routine analysis and in glycerol at -80°C in El Zaidin Experimental Station, CSIC, Granada, Spain.

### 2.3 Plant Nodulation Tests

Seeds of *M. arborea* were surface sterilized with 96% ethanol for 30 s followed by immersion in 15% (v/v) H<sub>2</sub>O<sub>2</sub> for 8 min, thoroughly rinsed with sterile distilled water, and allowed to germinate at 30°C in the dark. Isolates were tested for their ability to reinfect *M. arborea* seedlings. Inoculation and seed treatment were performed using the method of Vincent [4]. The plants were cultured in a growth chamber under a constant

temperature of 23°C and a photoperiod of 16 h (light) : 8 h (dark) and were watered with nitrogen-free nutrient solution. Indirect effectiveness of the nodules for nitrogen fixation was estimated by visual assay of red leghemoglobin presence in cross-sections and by the dark green intensity of the leaves in comparison with uninoculated control plants.

#### 2.4 Phenotypic Characterization

A total of 69 phenotypic assays were tested on the 30 strains isolated from root nodules of *M. arborea*. Inoculations were made with exponentially growing liquid cultures. Tolerance of the isolates to temperature was tested on liquid TY medium [5] at 30°C, 40°C and 45°C, respectively. Ability to grow in acid and basic media was determined in YEM medium whose pH had been adjusted and buffered to 5.0; 6.0; 7.0; 8.0 and 9.0, respectively, as described earlier [6]. For salt tolerance determination they were grown in 0, 171, 350, 513, 690, 861, and 1000 mM NaCl.

Utilization of 18 carbohydrates as sole carbon source was investigated on modified solid YEM medium as previously reported [7]. The monosaccharides glucose, galactose, fructose, D-arabinose, ribose, mannose and rhamnose, the disaccharides saccharose, lactose, salicine and maltose, the trisaccharides trehalose and raffinose, the polysaccharides starch and dextrin, the organic acids sodium acetate and D-gluconic acid, and the polyalcohols mannitol and inositol were used. Each compound (1% wDv) was filter-sterilized (0.22 µm) before use. To test the intrinsic heavy metal resistance, the isolates were grown on solid TY medium supplemented with (µg /ml) CuSO<sub>4</sub> × 5H<sub>2</sub>O (500), AlCl<sub>3</sub> (450), HgCl<sub>2</sub> (5), CdSO<sub>4</sub> (50), ZnSO<sub>4</sub> (250), Lead acetate (1000), FeCl<sub>3</sub> × 6H<sub>2</sub>O (750), MnCl<sub>2</sub> × H<sub>2</sub>O (1000), MnSO<sub>4</sub>

(1000), MgCl<sub>2</sub> (1000), MgSO<sub>4</sub> (1000), NiCl<sub>2</sub> × 6H<sub>2</sub>O (250), BaCl<sub>2</sub> × 2H<sub>2</sub>O (1000), and CoSO<sub>4</sub> (150). Antibiotic resistance of the isolates was tested on solid TY medium containing the following filter-sterilized antibiotics (µg/ml): ampicillin (20), carbanicilline (20), chloramphenicol (30), gentamycin (10), geneticin (20), neomycin (20), rifampicin (20), spectinomycin (20), nalidixic acid (20), and tetracyclin (50).

Hydrolysis of urea by the isolates was investigated on solid YEM medium amended with 2% (wDv) urea and 0,0012% (wDv) phenol red as previously described [8]. Gelatinase activity, reduction of nitrate and catalase activity were determined as indicated by Missbah El Idrissi [9], Lindstrom and Lehtomaki [8], and Graham [10], respectively. For evaluation of oxidase activity, the isolates were spread out over a piece of filter paper soaked in a solution containing 1% N, N-dimethyl-p-phenylendiamin oxalate 98%. The appearance of violet colonies indicated the presence of oxidase activity. Ability of the isolates to produce melanin was studied after growth on solid TY medium supplemented with CuSO<sub>4</sub> (40 µg/ml) and L-tyrosine (600 µg/ml) as previously described [11].

Computer cluster analysis of the 69 phenotypic traits tested was carried out for the bacterial isolates. The resemblance between pairs of isolates was calculated using the Pearson correlation coefficient and plotted as a dendrogram with the unweighted pair-group method with arithmetic average (UPGMA) [12].

#### 2.5 DNA Extraction and PCR Amplifications

Bacteria were grown in tryptone-yeast extract (TY) agar medium tubes [5] for 24-48 h at 28°C, and colonies were suspended in 2 mL of distilled water. The suspensions were then centrifuged twice at 3000 r/min

for 15 min. The resulting pellet was treated with proteinase K (20 mg/mL), and total DNA was extracted as previously described by Brenner et al. [13]. For rep-PCR (repetitive extragenic palindromic polymerase chain reaction), ERIC primers [14] ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') was used for amplification of the DNA. The DNA template was denatured for 5 min at 95°C, and PCR was carried out for 35 cycles (94°C for 30 s, 52°C for 1 min and 72°C for 1 min), with a final elongation step at 72°C for 7 min. PCR products were analyzed by horizontal electrophoresis in 2% agarose gels in TAE buffer at 55 V for 4 h. Gels were stained with ethidium bromide, visualized under UV radiation, and photographed with a digital camera. Cluster analysis of the electropherograms was performed with STATISTICA version 6 software (Statsoft). Comparative analysis of electrophoretic ERIC-PCR (enterobacterial repetitive intergenic consensus PCR) patterns was performed with Fingerprint software package (Bio-Rad).

PCR amplifications of 16S rRNA gene fragments were done using the two opposing primers fD1 (7-25) and rD1 (1524-1540) previously described [15]. The sequences of the *M. arborea* isolates and those of several *Sinorhizobium* species validly published were aligned using the ClustalW software [16]. Phylogenetic analyses were performed and visualized with MEGA 6 program [17], using the neighbor-joining algorithm [18]. Bootstrap analysis was based on 1000 resamplings.

### 3. RESULTS AND DISCUSSION

#### 3.1 Soil Analysis and Bacterial Strains

Thirty bacteria were isolated from *M. arborea* plants grown in 3 different soils of the northeast area of Morocco. All the 30

isolates are true symbionts of *M. arborea*, as they were able to establish effective symbiosis with the host plant in axenic conditions.

Soil salinization affects heavily the northeast region of Morocco. Root capacity to absorb water and nutrients decreases, results in degradation of vegetation cover and accentuation of desertification. In all the three chosen stations (Table 1), high soils mineralization degree referred by conductivity and critic nitrogen deficiency explains vegetation cover and agricultural production degradation in the northeast area of Morocco.

Although rhizobia are capable of survival under severe climates, such as low water potential, high temperatures, and high salinity [19], population densities tend to be lowest in the dry season and to increase as the water stress is relieved [20]. Bioclimate, soil characteristics and interaction of rhizobia with other soil microorganisms may play a key role in their survival in semiarid and arid environments [19].

#### 3.2 Phenotypic Characterization

Physiological and biochemical studies are considered as a basis for detailed polyphasic taxonomy. Although they cannot be used alone in taxonomic analysis, they are still necessary for the characterization and selection of rhizobial isolates adapted to marginal edapho-climatic conditions, and to provide information about their metabolic diversity. Phenotypic properties of *M. arborea* isolates are presented in table 2.

In this work, all the 30 isolates were unable to grow below pH 6, showing they are all neutro-basophilic. All the isolates grew well between 30 and 40°C and 53% of isolates were able to grow at 45°C. The tolerance to high temperatures exhibited by the isolates in this study may be related to their geographic origin. Similar results were found with rhizobia isolated from hot and

dry regions in Africa [21]. It is considered that high Tmax values represent a very important characteristic for bacterial survival under the hot and dry conditions prevailing in the areas under study, although the strains that persist at 45°C lose their infectiveness [22].

All the strains produce catalase and oxidase; 86% produce urease, more than 36% produce melanin and approximately 40% degrade gelatin. All the strains grew

vigorously in presence of carbanicillin and chloramphenicol conversely gentamycin, geneticin and tetracycline were the most potent antibiotics as they inhibited the growth of most of *M. arborea* rhizobia. Most of strains were tolerant to heavy metals such as manganese, aluminum, and magnesium, but they could not tolerate cadmium, nickel, mercury and copper.

**Table 1.** Soil stations used in this study and strains isolated.

	Soil characteristics						Climate		Isolates
	Texture	pH	Conductivity µs/cm	Total Nitrogen (%)	Total Carbone (%)	C/N rate	Bioclimate	Rainfall (mm)	
Haraza (Oujda) (34° 41' 12" N 1° 54' 41" W)	Clayey-slimy	7,88	199	0,09	4,4	48.8	Semi-arid with cool winter 0°C<m< 3°C	300-400	H1 to H10
Bouarg (35° 05' 45" N, 2° 51' 25" W)	Slimy	7,94	256	0,066	5,5	83.3	Semi-arid with cool winter 0°C<m< 3°C	300-400	B1 to B10
El Aioun (34° 35' 07" N, 2° 30' 09" W)	Clayey	7,26	223	0,054	4	74.0	Semi-arid with cool winter 0°C<m< 3°C	200-300	L1 to L10

**Table 2.** Results of biochemical and physiological tests for clusters formed by computer program analysis.

Test	Cluster I (23 strains)	Cluster II (6 strains)	Phenotype III (1 strain)
<b>Growth at:</b>			
30°C	+	+	+
40°C	22	+	+
45°C	12	3	+
<b>Tolerance to NaCl at:</b>			
0 mM	+	+	+
171 mM	+	+	+
342 mM	+	+	+

**Table 2.** Continued.

Test	Cluster I (23 strains)	Cluster II (6 strains)	Phenotype III (1 strain)
513 mM	21	+	+
690 mM	1	1	+
861 mM	1	1	+
1 M	1	1	+
<b>Growth at pH of:</b>			
5.0	-	-	+
6.0	20	+	+
7.0	+	+	+
8.0	+	+	+
9.0	+	+	+
<b>Heavy metal resistance (<math>\mu\text{g/mL}</math>)</b>			
MnCl <sub>2</sub> (500)	+	+	+
MgSO <sub>4</sub> (1000)	17	4	-
HgCl <sub>2</sub> (5)	9	-	-
Lead acetate (1000)	2	3	+
NiCl <sub>2</sub> (250)	-	2	+
<b>Intrinsic antibiotic resistance (<math>\mu\text{g/mL}</math>)</b>			+
Chloramphenicol (30)	+	+	
Tetracycline (5)	-	5	+
Neomycin (20)	5	+	-
<b>Carbohydrate utilization</b>			+
D-Galactose	+	+	
Sucrose	+	+	+
Trehalose	21	+	+
Maltose	21	+	+
Dextrine	5	+	+
Starch	-	-	-
Sorbitol	+	+	+
Mannitol	+	+	+

All the isolates were also able to use mannose, mannitol, D-galactose, D-sorbitol, rhamnose, D-glucose, D-sucrose. However, none of them used dextrin or starch. We observed a great diversity in the catabolism of 5 carbohydrates, salicine, trehalose, maltose, lactose and raffinose.

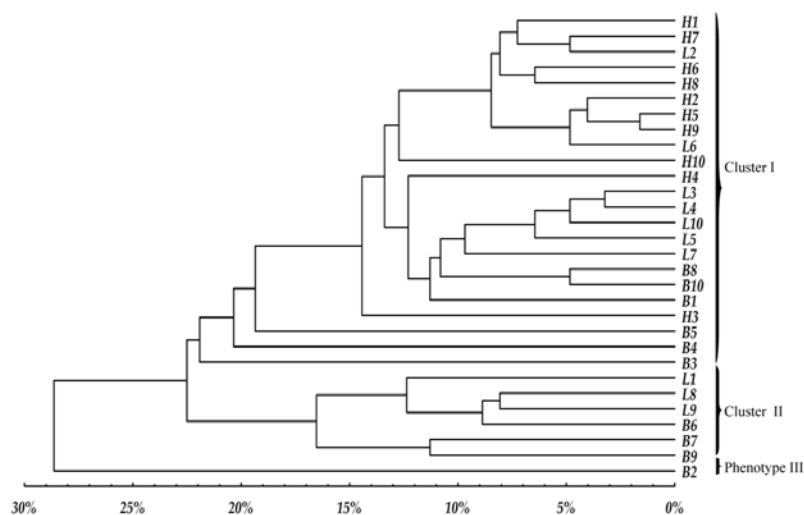
All the isolates grew on 171 mM NaCl and 10 % tolerated 690 mM. The negative effect of salinity on the growth of rhizobia

observed in our results has also been reported by several authors [21, 23, 24, 25, 26]. Moawad and Beck [27] showed that *R. leguminosarum* strains tolerated NaCl concentrations of 1,000 mM. Theoretically, osmotolerant rhizobia would survive and persist in zones presenting problems of salinity and could be useful for inoculation of legumes growing in such soils [28].

The numerical analysis of the 69

phenotypic characteristics of 30 rhizobial isolates shows a wide diversity among *M. arborea* isolates. The phenogram obtained illustrates the level of dissimilarities between the strains and the diversity. At a similarity

coefficient of 78%, 3 main clusters were formed (Figure 1). Phenotype III is represented by the single isolate B2 showing strong tolerance to acidity, salinity and to high temperature.



**Figure 1.** Dendrogram generated by UPGMA clustering representing phenotypic relationships between *Medicago arborea* isolates.

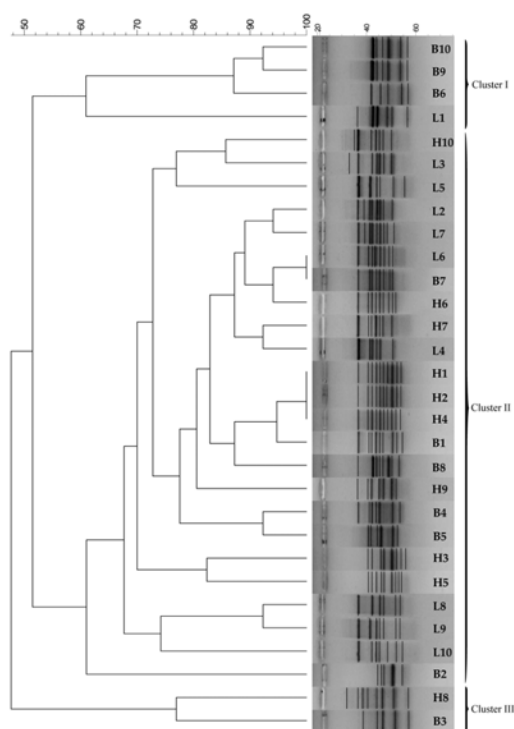
Cluster 1 contains 23 strains all of them are rifampicine resistant and tetracycline sensible.  $\text{FeCl}_3$ ,  $\text{CdSO}_4$ ,  $\text{NiCl}_2$  and lead acetate affects strains growth. All cluster isolates are enabling to growth on 8%  $\text{KNO}_3$ . In cluster II, six strains are grouped L1, L8, L9, B6, B7 and B9, they assimilate some bi-saccharine (raffinose, trehalose, maltose, lactose and sucrose). They are inhibited by gentamycin and the heavy metals  $\text{HgCl}_2$ ,  $\text{CuSO}_4$ ; but shows high resistance to neomycin,  $\text{CdSO}_4$  and  $\text{ZnSO}_4$ .

### 3.3 rep-PCR Fingerprinting and 16S rDNA Sequencing

The rep-PCR genomic fingerprinting is an adequate technique to differentiate rhizobial strains [29, 30], and many other closely related sub-species or strains and/or determining phylogenetic relationship [31, 32]. The ERIC-

PCR is a powerful tool to analyze microbial ecology and evolution [33]. Therefore, the genetic diversity of the 30 isolates was determined by ERIC-PCR DNA fingerprinting. This method is highly discriminating and enables identification of genetic diversity at the intraspecies level [34].

As shown in the dendrogram (Figure 2), at similarity coefficient of 80%, 3 cluster and 8 groups can be distinguished. ERIC-PCR technique reveals a huge diversity among the isolates. However, there was a great similarity between some isolates and some have even identical fingerprints although isolation sites are very far, over 100 km away. This may be due to wind transportation of soil particles or anthropic activities (mobility, agricultural work tools...etc.).



**Figure 2.** Dendrogram generated by fingerprint software package (Bio-Rad) from ERIC-PCR fingerprinting of the *Medicago arborea* isolates.

The results obtained supported the possibility of important lineage differences within the isolates from *M. arborea*. Similar results were found by Ogutcu et al. [35], showing that all rep-PCR fingerprints performed with ERIC, REP, and BOX primers are sensitive and reliable for identification and characterization of *Rhizobium leguminosarum* subsp. *ciceri* strains isolated from the chickpea plant species.

Several reasons could be advanced to explain this huge diversity of rhizobia nodulating *M. arborea*. Edaphic factors including soil moisture, salinization, temperature and climate have been shown to differentially modify the nodulating success of strains of various *Rhizobium* species in the field [36]. Although temporal induced changes in the field population could also

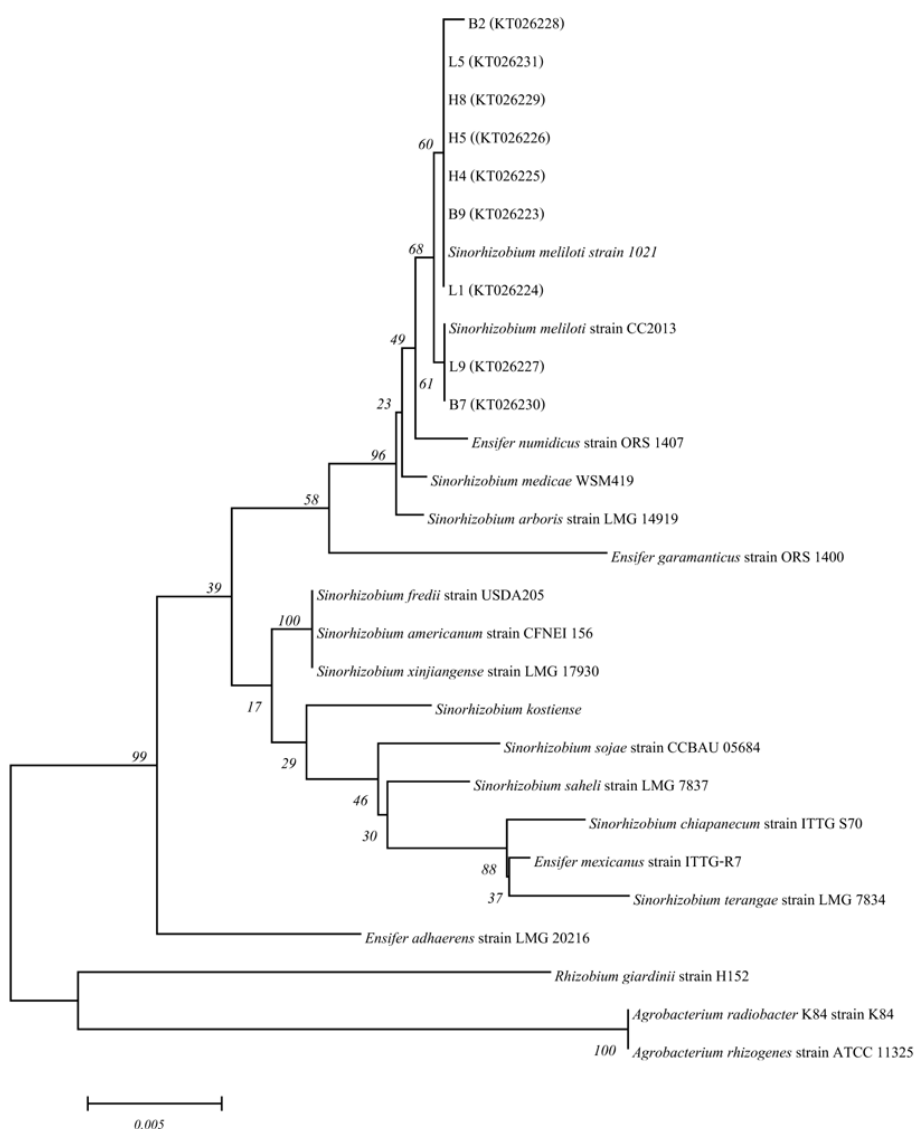
generate differences in the isolates recovered [37].

The analysis of results showed that the ERIC-PCR fingerprinting and phenotypic approaches revealed a high polymorphism degree among the 30 bacterial strains isolated from *Medicago arborea* roots nodules. Therefore, in table 3 we showed the correlation in discrimination given by ERIC-PCR and phenotypic approaches. Indeed, 73.3% of isolates were grouped by same way using ERIC-PCR and phenotypic techniques. Molecular methods coupled with phenotypic analysis methods (polyphasic taxonomy) have always been considered as main basis for identification of novel species within rhizobia clad and as a criterion for competent strains selection, which can survive under various environmental stress [38, 39, 40, 41, 42, 43]. Results obtained in this work support the efficiency of using rep-PCR and phenotypic approaches to analyze the diversity of rhizobia despite of their origins and especially in case of close related strains, in a level where other techniques show low discrimination.

The nearly complete sequence of the 16S rRNA gene (1450 bp) from the strains B2 (KT026228), H8 (KT026229), H5 (KT026226), H4 (KT026225), L1 (KT026224), B9 (KT026223), L5 (KT026231), L9 (KT026227) and B7 (KT026230), representative of each one of the three clusters, showed they were members of the genus *Sinorhizobium* of the family *Rhizobiaceae* within the Alphaproteobacteria. The sequences of the strains are almost identical. A phylogenetic tree inferred from 16S rRNA gene sequences (Figure 3) indicated that strains clustered with the type strains *Sinorhizobium meliloti* strain 1021. Pairwise alignments between globally aligned sequences of strains with various *Sinorhizobium* type species indicated that strains B2 (KT026228), H8 (KT026229), H5 (KT026226), H4 (KT026225), L1 (KT

026224), B9 (KT026223) and L5 (KT026231) presented identity values of 100 % with that of *S. meliloti* strain 102. While, strains L9 (KT026227) and B7 (KT026230) showed identity values of 100 % with that of *S. meliloti* strain CC2013. The sequencing

and phylogeny of the 16S rDNA confirm the affiliation of the *M. arborea* isolates within the species *Sinorhizobium meliloti*. Same results were reported for bacteria nodulating *Medicago sativa* and *Medicago truncatula* [44].



**Figure 3.** Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of strains from nodules of *Medicago arborea* and phylogenetically related species within the genus *Sinorhizobium*. Bootstrap values are indicated as percentages derived from 1000 replications. Bar, 5 nucleotides substitutions per 1000 nucleotides. The tree is rooted on *Rhizobium giardinii* strain H152.

**Table 3.** Isolates classification by ERIC-PCR and phenotypic approaches.

ERIC-PCR fingerprinting		Phenotypic tests		Correlation between ERIC-PCR and phenotypic test	Correlation percentage
Cluster II	H1, H7, L2, H6, H2, H5, H9, L6, H10, H4, L3, L10, L5, L7, B8, B1, H3, B5, B4, L4, L8, L9 and B2	Cluster I	H1, H7, L2, H6, H2, H5, H9, L6, H10, H4, L3, L10, L5, L7, B8, B1, H3, B5, B4, B10, H8 and B3	H1, H7, L2, H6, H2, H5, H9, L6, H10, H4, L3, L10, L5, L7, B8, B1, H3, B5 and B4	73,3%
Cluster I	B9, B6, L1 and B10	Cluster II	B9, B6, L1, L8, L9 and B7	B9, B6 and L1	
Cluster III	H8 and B3	Phenotype III	B2		

The 16S rRNA gene is useful to classify the bacteria from domain, phylum, class, subclass, order, family, genera and species, but it can fail in the differentiation of phylogenetically close species that can be delineated by sequencing of other genes such as *nodC* gene and housekeeping genes (*atpD*, *glnII*, *dnaK* and *recA*), giving better relative performance when used as molecular markers, either individually or in combination [42, 43, 45, 46]. However, as all the sequences analyzed in this work, were similar (100% similarity) with *Sinorhizobium meliloti* strains previously published. We consider that there is no need to go further with other discriminating methods such as multilocus sequence analysis (MLSA).

#### 4. CONCLUSION

The analysis of diversity of 30 rhizobia strains isolated from root nodules of *Medicago arborea*, cultivated in 3 different ecoregions of the northeaster area of Morocco, allowed the description of high polymorphism degree as revealed by their different enzymatic activities and their

adaptation modalities to different abiotic factors in arid and semi-arid climates. ERIC-PCR fingerprinting and phenotypic techniques were used to analyze diversity of the isolates and to reveal physiological and metabolic relationships between strains originated from different geographic locations. There is a high level of phenotypic diversity confirmed by the high genetic diversity as revealed by the rep-PCR technique used. The phenotypic diversity of the isolates that nodulate *M. arborea* can be envisaged as a trait of particular ecological importance, as it permits the establishment of a functional symbiosis even if the soil conditions change and become severe, such as pH variations, salinity gradients, extreme temperatures, and low carbon. The isolates adapted to these variations will continue to grow in the rhizosphere, and their population will be more effective. However, the 16 rDNA sequences analysis showed that *Sinorhizobium meliloti* was the only rhizobial species isolated from *M. arborea* root nodules, which suggests restricted host specificity between the plant and its microsymbiont.

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