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Phenotypic and Genotypic Characterisation of Bacteria Associated with *Acacia gummifera* Wild

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study aimed to isolate and analyse the phenotypic and genotypic features of bacteria nodulating *Acacia gummifera* Wild.
Study Design: An experimental study.
Place and Duration of Study: The study was done at the Department of Biology (Soil & Environment Microbiology Unit), University of Sciences Meknes, Moulay Ismail University and National Center of Research Science and Technical Division Rabat-Morocco, during April 2015 to May 2016.
Methodology: Samples were collected from 15 sites of Skhour Rhamna region, Morocco, to isolate bacteria that can be able to nodulate *Acacia gummifera.* Phenotypic parameters as growth speed, mobility, tolerances to temperature, salt, and pH were studied. To evaluate the genotypic characteristics, a molecular characterisation based on the 16S rRNA gene was performed.
Results: The majority of the isolate is fast growing. All isolate tolerated high temperatures (40°C), and a NaCl concentration that exceeds 800 mM and the majority of them increased under pH

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ranging from 7 to 10. Furthermore, the molecular characterisation showed the bacterial diversity belonging to the genera *Ensifer, Rhizobium* and *Acinetobacter*.
Conclusion: The Moroccan gum tree recruits a diversified strain that can be developed as a new bio-fertiliser.

Keywords: Acacia gummifera; rhizobia; phenotypic characterization; molecular characterization.

1. INTRODUCTION

In Morocco as in Africa, a number of *Acacia* species have been introduced from Australia to stabilise soil, increase soil fertility and to produce fuelwood and fodder [1]. However, the introduction of exogenous species in some cases may have squeal on native ecosystems or even alteration of the hydrological regime and reduction of the biodiversity through the genetic pollution or genetic erosion. Several examples attest to the negative effects of introducing exotic *Acacia* trees. Likewise, in Algeria and Senegal, past experience has shown that the use of *A. holosericea* and *A. mearnssi* has changed the soil microflora and has had a negative impact on the early growth stages of indigenous forest species such as *Faidherdia albida* in Senegal and *Quercus suber* in Algeria [2].

As a result, renewed interest should be dedicated to the native species, and the management should consider the needs of the ecosystem to ensure the conservation of the desired species. In Morocco, there are four spontaneous species of *Acacia*, among them *Acacia gummifera* Wild. is the only endemic species. This Moroccan gum is a shrub that takes the form of a bush 1 to 2 m high [3], or that of a tree of 6m high with twisting branches [4]. The intrinsic qualities of this species are several, and like the others legumes, *A. gummifera* can alliance with the *Rhizobium* group- a symbiosis for fixing nitrogen. This property allows it to colonise poor or degraded soils, improve, maintain or restore soil fertilisation. However, the importance of *Acacia gummifera* and the rhizobia nature associated with it have been the subject of only a few studies [5,6,7,8] from which, it is therefore interesting to evaluate the prevalence of symbiosis in this species and to specify the nature of rhizobia associated with it. The interest of this work is to study an indigenous *Acacia* species and its rhizobial community.

2. MATERIALS AND METHODS

2.1 Isolation

The isolates were issued from the nodules of young *Acacia gummifera* seedlings obtained by the rhizobia trapping method, by sowing *Acacia gummifera* seeds on the rhizospheric soil collected after several surveys in the region of Mechrâa ben abbou (Skhour Rhamna Morocco).

2.2 Authentication

The ability to induce nodules has been evaluated using Leonard's jar [9]. For this, the first part of the jar was filled with a mixture of sand and vermiculite previously sterilised, while the second part was filled with the nutritive solution of Fahreus, 1957 [10]. A bead of compress connects the two Lenard's jar parts. The seeds of *A. gummifera* were scarificed via sulfuric acid (98% for 1 hour), and pre-germinated are sown in the sand-vermiculite mixture, then inoculated immediately with 2 mL of the bacterial suspension in exponential phase. The treatments were organised in a randomised complete block with triplicates for each inoculated strain, and they were kept in a greenhouse under natural light for 3 months before collecting the nodule.

2.3 Phenotypic Characterisation

The phenotypic characteristics studied include an identification of the colony size, colour, shape, transparency, and elevation, while the differentiation of the Gram-positive bacteria from the Gram-negative ones was done by the Gram's stain, the mannitol-mobility test, the ability of the isolates to alkalise or acidify the medium using YMA medium [11] supplemented with bromothymol blue at 0.0025% (w/v), different temperatures (4°C, 28°C, 37°C, 40°C at 50°C), pHs (3, 4, 5, 6, 7, 8, 9, 10 and 11) and salt (0, 172, 344, 517, 689, 862, 1190, 1200, 1360, 1500 mM). The use of different carbon sources has been tested on YMA medium by replacing the mannitol with the carbohydrate to be tested. The carbohydrates tested were: D-sucrose, D-glucose, D-arabinose, maltose, lactose, D-galactose, and starch.

2.4 Genotypic Characterisation

The genotypic characterisation was based on the 16s rRNA gene which was carried out within the laboratory of molecular biology and functional

genomics of the National Center of Research Science and Technical Division UATRS Rabat-Morocco.

2.4.1 DNA extraction

DNA extraction from the liquid medium of the isolates was performed via the BIOLINE ISOLATE II GENOMIC DNA KIT Platform Kit.

2.4.2 Real-time PCR amplification

DNA submitted to PCR obtained an amplicon of 1500 bp using the universal primers Fd1 and RP2 (AGAGTTTGATCCTGGCTCAG and ACGGCTACCTTGTTACGACTT, respectively) [12]. The PCR reactions were carried out in 25 µL reaction buffer at 1/10 of the final volume, 0.125 µL of each primer (100 µM), 0.2 µL of the Taq polymerase (5 µL / L) and 5 µL of the DNA sample. In the negative control, the 5 µL of DNA was replaced by 5 µL of sterile H_2O. The amplifications were performed according to the following conditions: a first denaturation at 95°C for 1 min and then cycle in each a denaturation at 95°C for 15 seconds, hybridisation at 52°C for 20 seconds and a 72°C elongation for 15 seconds and a final elongation at 72°C for 3 min.

2.4.3 Sequencing

The amplicons were sequenced using the BigDye v3.1 kit (Applied Biosystems) of the ABI 3130xl Genetic Analyzer Sequencer. The reaction consists of an introduction into a final volume of 10 µL, 0.75 to 1.5 µL of template DNA and 3.2 to 5 pmol / µL of each primer 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCCTTTRAGTTT) [12]. The optimal conditions of the sequencer are as follows: for 25 cycles 96°C for 1 min, 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min.

2.4.4 Sequence analysis

The sequences obtained were analysed with the program DNA Baser v 4.36.0 (http://www.dnabaser.com) corrected manually. The sequences were then compared to those available in the NCBI database using the BLAST program (Basic Local Alignment and Search Tool, NCBI) to determine their phylogenetic affiliation. The identification of the genus and the species was carried out as described by Drancourt et al. [13], where > 99% similarity of a strain to a species has already been described, between 97% – 99% similarity a strain to a genus and > 97% represents a new species. The phylogenetic tree was determined by the Mega v 6.06 software [14], and the 16S rRNA gene sequences of the selected isolates were deposited in the GenBank database under accession numbers (Table 3).

3. RESULTS

3.1 Isolation from Soil Samples

In this study, 100 bacterial isolates were isolated from the nodules taken after trapping *A. gummifera* plants in soil samples collected from 15 different sites of Mechraa ben abbou (Skhour rhamna region).

3.2 Phenotypic Characterisation

The isolates were purified, selected and tested for their infectivity against *Acacia gummifera* and their tolerance to different stresses induced by salinity, high temperatures, and pH. Moreover the results of the authentication showed that 20 isolates can nodulate *Acacia gummifera*, and all are gram-negative bacteria.

Tables 1 and 2 show the phenotypic identification of 8 isolates nodulating *A. gummifera*, the most tolerant ones have been the subjects of a molecular study. Their phenotypic profile revealed that the majority (61%) are mobile and have acidified the YMA medium containing bromothymol blue. 50% of the isolates grow in the YMA medium containing a NaCl concentration that exceeds 800 mM. The better growth of the strains was recorded at 28°C, although all isolates recorded average growth at 40°C, no multiplication was recorded in temperatures exceeding 45°C. Finally, all the isolates can grow on an alkaline medium at pH between 9 to 10. However, no growth was observed at acid pH except isolate A10 which was shown to be able to multiply at pH = 4 (Table 1). All strains used starch and sucrose as a source of carbon other than mannitol, so the degradation of other sugars differed from one isolate to another.

In summary, the phenotypic characterisation has made it possible to say that there is a great diversity in the strains obtained. This allowed the identification of 5 distinct groups from the dendrogram of Fig. 1.

Table 1. Phenotypic characteristics of nodulative *A. gummifera* isolates

Isolates	A24	A26	A32	A12	A10	A17	A4	A15
Ph								
3	-	-	-	-	-	-	-	-
4	-	-	-	-	+	-	-	-
5	-	-	-	+	+	-	-	-
6	+	+	+	+	+	-	-	+
7	+	+	+	+	+	-	-	+
8	+	+	+	+	+	+	-	-
9	+	-	+	+	+	-	+	-
10	+	-	+	+	+	-	-	-
11	-	-	-	-	-	-	-	-
Temperature (°C)								
4	-	+	+	-	+	+	-	-
28	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+
45	-	+	+	-	+	-	+	-
50	-	-	-	-	-	-	-	-
Salinity (mM)								
0	+	+	+	+	+	+	+	+
172	+	+	+	+	+	+	+	+
344	+	+	+	+	+	+	+	+
517	+	+	+	+	+	-	+	+
689	+	+	-	+	+	-	+	+
862	+	+	-	+	+	-	+	+
1190	-	+	-	+	+	-	-	+
1200	-	+	-	+	+	-	-	-
1360	-	-	-	+	-	-	-	-
1500	-	-	-	-	-	-	-	-

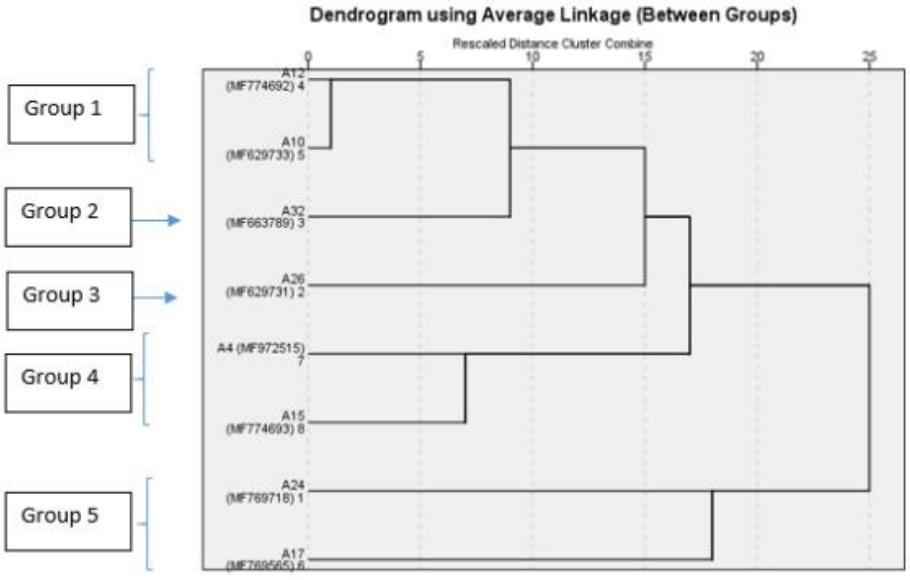

Fig. 1. Dendrogram made by Ward method and Euclidean distance using mean links (UPGMA) between the phenotypic and biochemical features of the 8 nodule isolates *Acacia gummifera* constructed by the software SPSS v. 17.0

Table 2. Biochemical characteristics of *A. gummifera* nodulating isolates

Isolates	A24	A26	A32	A12	A10	A17	A4	A15
Mobility	-	+	+	+	-	-	+	-
Use of carbon								
Sucrose	+	+	+	+	+	+	+	+
Glucose	-	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	-	+	+
Maltose	-	+	+	+	+	-	+	+
Galactose	-	+	+	+	+	+	+	+
Starch	+	+	+	+	+	-	+	+
Mannitol	+	+	+	+	+	+	+	+

-: no growth, +: growth.

3.3 Genotypic Characterisation

About 1500 bp bands were obtained after amplification of the 16S rDNA. By visually evaluating and comparing to the size marker, the size of the bands corresponded well to the 16S rRNA gene.

However, the sequence analysis of the ribosomal RNA 16S gene suggested two strains; A32 (MF663789) and A26 (MF629731) which have a percentage of similarity of 99% with *Rhizobium giardinii,* a strain A24 (MF769718) with 100% sequence identical to *Rhizobium azibense*, A10 (MF629733), A12 (MF774692) and A4 (MF972515) have 99% identical sequence respectively to *Rhizobium naphthalenivoran*s, *Rhizobium pusense* and *Rhizobium nepotum*. As for the isolate A17 (MF769565), it has 97% of sequence belonging to *Ensifer adharens*. Finally, the isolate A15 (MF774693) has 99% identical sequence to the non-rhizobial species *Acinetobacter schindleri* but capable of nodulating *A. gummifera* (Table 3).

The phylogenetic tree has shown that the isolate A32 (MF663789), and A10 (MF629733) are positioned on the same branch as the species *R. azibense, R. giardinii*, *and R. naphthalenivorans. R.pucsense.* Strain A15 is in the same phylogenic position as *Acinetobacter schindleri* (Fig. 2).

Fig. 2. Phylogenetic tree obtained by neighbor-joining method of ARN16S of isolates nodulating *A. gummifera*. Assigned accession numbers are shown in Table 3

Table 3. Genotypic character of isolates nodulating *A. gummifera*

Isolates	Species	% similarity	Accession number
A24	*Rhizobium azibense*	100%	MF769718
A26	*Rhizobium giardinii*	99%	MF629731
A32	*Rhizobium giardinii*	99%	MF663789
A12	*Rhizobium pusense*	99%	MF774692
A10	*Rhizobium naphthalenivorans*	99%	MF629733
A17	*Ensifer adharens*	97%	MF769565
A4	*Rhizobium nepotum*	99%	MF972515
A15	*Acinetobacter schindleri*	99%	MF774693

4. DISCUSSION

During the historical period, *A. gummifera* has suffered an intense degradation because of the intensification of crops and anthropogenic activities [15]. The natural regeneration of this endemic species is very rare, and the propagation of this species via seed-grown plants seems to be interesting. As a result, the nodulation and efficiency of the isolate are essential for the establishment of this species after transplanting in the fields and for maximum use of their atmospheric nitrogen fixation potential. The present study proposes a phenotypic and genotypic characterisation of isolates taken from nodules of *A. gummifera* obtained by trapping on rhizospheric soil from the Skhor Rhamna region. Eight isolates were able to re-nodulate *A. gummifera*. According to their growth on YMA medium supplemented with bromothymol blue, they acidified the medium. They are therefore fast-growing bacteria, and the majority (86%) has tolerated high concentrations of NaCl (517 mM), the same results were observed in bacteria associated with other species of *Acacia* [8]. All strains multiplied under a temperature of 40°C although 50% were able to grow under high temperatures (45°C), which is in agreement with the results of Zerhari et al. [5] and Fterich et al. [16] indicating that the majority of *A. raddiana* isolates from Tunisia and *A. gummifera* from Morocco, were able to multiply at 40°C. The isolates of the present study showed pH resistance ranging from 7 to 10, which corroborates with the results found by [17]. Indeed, according to Yan et al. [18], fast-growing rhizobia are generally tolerant of the alkaline medium [18,19]. It is important to test the ability of rhizobia to grow in extreme pH and salinity. The acidity of the soil can affect nodulation. Indeed, the rhizobia subjected to salt stress can have morphological alterations leading to the modification of the profile of the polysaccharides and the extracellular lipopolysaccharides. It may have an impact on symbiosis because of their involvement in the initial stages of symbiotic interactions [20].

Furthermore, 75% isolates have used the different carbon sources other than mannitol, so they have a wide range of assimilation towards carbon substrates, which is characteristic of the fast-growing bacteria [21].

This diversity of strains isolated from the same site with different phenotypic traits suggests that there are soil-level microsites with distinct nutrient availability, moisture content, and competitions that can induce different adaptations [22]. Although phenotypic identification is a crucial and necessary step for the characterisation and selection of isolates suitable for edapho-climatic conditions, it remains global. Molecular identification was required to assign adequate profiles to the isolates obtained. The phylogenetic tree confirmed that there is a great diversity between the strains that belong to the different genera: *Rhizobium*, *Ensifer*, and *Acinetobacter*. It appears that *Acacia gummifera* can be nodulated by different rhizobia, mainly associating with the genus *Rhizobium*, which has been confirmed by the study of Berkia et al. [6].

According to the phylogenetic tree (Fig. 2), the study found that strains A32 (MF663789) and A10 (MF629733) are positioned on the same branch as the species *R. azibense, R. giardinii*, and *R. naphthalenivorans*. *R. pucsense* is phylogenetically far from other species of the genus *Rhizobium*. Indeed it has been shown that this species is somewhat close to *R. radiobacter* [20]. Strain A17 has 97% homology with the 16S rDNA sequence with *Ensifer adharens*, which is known by its ability to nodulate several species of *Acacia* [16,8,23]. This species forms with other species of the genus *Sinorhizobium* a single monophyletic group [24]. Moreover, the A15 isolate is in the same phylogenic position as *Acinetobacter schindleri,* this species belongs to the genus *Acinetobacter,* which is considered as

non-rhizobial but it has been able to renodulate *A. gummifera* as in other leguminous plants [25,26].

5. CONCLUSION

This study showed that the Moroccan gum tree recruits competent and diversified strains, which have been able to support the extreme abiotic conditions (high temperature and salinity). The nodulation of *A. gummifera* is ensured by the strains belonging to the groups: *Rhizobium, Ensifer,* and *Acinetobacter*. In practical terms, nodulation of greenhouse grown *A. gummifera* plants indicates that it would be necessary to cover seeds and seedlings with inoculation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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