Phenotypic and Genotypic Characteristics of Rhizobia Isolated from Meknes-tafilalet Soils and Study of Their Ability to Nodulate *Bituminaria bituminosa*

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**Authors’ contributions**

This work is a part of the PhD of the first author and was carried out in collaboration between all authors. Author BBM designed the study, authors BBM and IA performed the experiment and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JI and LN supervised the study and managed the literature searches, author EEF did the genotypic analysis. All authors read and approved the final manuscript.

**ABSTRACT**

**Aims:** The objectives were to isolate and characterize phenotypically and genotypically the rhizobial strains from the soils belonging to the Meknes-Tafilalet region in order to select strains that are able to nodulate *Bituminaria bituminosa*.

**Study Design:** An experimental study.

**Place and Duration of Study:** Department of biology (Soil & Environment Microbiology Unit) Faculty of Sciences, Moulay Ismail University and Technical Support Unit for Scientific Research, CNRST in Rabat; between January and August 2010.

**Methodology:** Samples from 23 different sites belonging to the Meknes-Tafilalet region were collected in order to select rhizobial strains that are able to nodulate *Bituminaria bituminosa*. The morphological, cultural and phenotypic parameters of isolated strains were studied. The phenotypic characteristics include colony morphology, growth speed, tolerances to temperature, salt and pH. To assess the genotypic diversity among the

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isolates, molecular characteristics based on 16S rDNA gene sequencing were performed. **Results:** The majority of the isolated strains showed fast-growing capacity (75%). Most strains tolerate neutral to alkaline pH, however some strains (18%) showed weak growth capacity at pH 4. All isolates were tolerant to high salt stress ([NaCl] = 3%). The genotypic characterization based on 16S rDNA gene sequencing of the twelve strains showed a high diversity between the isolates. **Conclusion:** Taken together, our results highlight the important biodiversity of the isolated rhizobial strains and open opportunities for the development of new bio-fertilizer.

**Keywords:** Bituminaria bituminosa; Rhizobium; 16S rDNA; symbiosis.

### 1. INTRODUCTION

Bacteria are an important part of the soil microflora because of their abundance (up to 10^9 cells per gram of soil) [1], their species diversity (a minimum of 4000–7000 different bacterial genomes per gram of soil [2]) and the multiplicity of their metabolic activities. They play a key role in the biogeochemical cycles of the main elements (carbon, sulphur, nitrogen, etc.). Nitrogen is an essential nutrient for plant growth and in order to reduce the abusive use of chemical fertilizers in agriculture and subsequently to protect environment and customer health, a high interest was provided for environmental friendly sustainable agricultural practices and organic farming systems [3]. Development and implementation of sustainable agriculture techniques, such as bio-fertilization, is of major importance to alleviate environmental pollution and to minimize the deterioration of nature [4].

Rhizobia are a community of bacteria that have the capacity to establish symbiotic associations with many legumes and a few non-legumes, which form root nodules in which the fixation of atmospheric nitrogen (N₂) takes place. Annually, ~ 44–66 million tons of N₂ are fixed by this symbiosis, providing practically half of all nitrogen used in agriculture. Rhizobia, so far described, are very diverse, and currently belong to about 40 species and seven different genera–Sinorhizobium, Mesorhizobium, Allorhizobium, Rhizobium, Bradyrhizobium, Azorhizobium [5] and *Methylobacterium* [6]. In the last decade, novel plant containing nitrogen-fixing legumes symbionts were described. The alpha-Proteobacteria include *Ochrobactrum* [7], *Devosia* [8], *Blastobacter* [9]; the beta-Proteobacteria include *Methylobacterium* [10], *Burkholderia* [11], *Cupriavidus* [12] and some unclassified strains are included in the gamma-Proteobacteria [13].

Despite the achievement of all these studies, there is still no common design of rhizobia diversity due to the large number of legume species and their wide geographical distribution [5].

In spite of the taxonomic diversity [14], all rhizobia establish symbiotic interactions using a similar molecular mechanism, linking signal molecule exchange between rhizobia strains and their host [15]. The host plants excrete inducing compounds that activate regulatory nod genes. Protein products of these genes are involved in the synthesis of Nod factors, which are secreted by bacteria and required for host-specific infection and nodulation of the legume hosts [16].

*Bituminaria bituminosa* is a *Psoralea* (Leguminosae) subspecie. It is a self-pollinating legume shrub [17] and is widely distributed in the Mediterranean region [18]. This perennial plant provides very nutritious feed to animals with its fruits and seeds [19]. It also supplies
nitrogen to the soil and protects the soil against erosion and therefore, it significantly enhances soil productivity. This shrub is not only used as fodder but also as medicinal plant and in the phyto-stabilization process due to its tolerance to many heavy metals [20].

The aim of this study was to isolate and characterize bacteria from soils originating from different localities in the Meknes-Tafilalet region of Morocco. Phenotypic and genotypic characteristics were performed. We have also investigated their capacity to nodulate *Bituminaria bituminosa* with the aim of reintroducing this shrub in regions from where it has disappeared.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection

Soil samples were collected from 23 sites of 10 different locations in the Meknes-Tafilalet region – of Morocco. From each site 1000 grams of soil to a depth of 10 to 30 cm were randomly sampled. Isolation of bacteria was performed after sample collection.

2.2 Isolation of Strains from Soil Samples

10 grams from each soil sample were suspended in 90 ml of sterile distilled water. 2ml of the soil sample suspension was flooded on Yeast Mannitol Agar (YMA) plates and incubated at 28°C [21]. Single colonies typical of rhizobia were subjected to the Gram staining. Purity of the cultures was ensured by repeatedly streaking the bacteria on YMA and verifying a single type of colony morphology.

2.3 Inoculate Preparation

After their purification, the isolates were grown, separately, for 72 h in Yeast Mannitol Broth (YMB) at 28°C on a rotary shaker at 200 rpm. Growth was monitored by optical density measurement of the cell suspension at 600nm. Cell densities were related to viable cell numbers measured as cfu.ml\(^{-1}\) by standard plate count and the number of bacterial cells was adjusted to \(10^9\) cfu.ml\(^{-1}\) [21].

2.4 Plant Nodulation Test

All the isolates were tested for nodulation of *B. bituminosa* in plastic bags and carried out in a greenhouse at the Faculty of Sciences, of Moulay Ismail University. Seeds were hand-sorted for size uniformity and freedom from damage, surface-disinfected by soaking in 1 \(\%\) mercuric chloride for 3 min followed by rinsing four times with sterile distilled water for 3.5, 10 and 15 min as previously described [22]. Surface-disinfected seeds were placed on water agar plates and incubated at 28°C for 7 days to ensure their pre-germination. The experiment was conducted in plastic bags filled with 500g of sterilized peat. Treatments were arranged in a randomized complete block with three replicate for each strain inoculated. The plants were watered twice a week. The treatments were maintained in a greenhouse under natural lighting and day/night temperatures of 25°C/12,3°C. Ten days after planting, we applied the inoculations. Each inoculum was applied at the bottom of the shoot at the rate of 15ml \(10^5\) cfu.ml\(^{-1}\) three times in order to multiply the infection chance.
2.5 Phenotypic Characteristics

2.5.1 Colony morphology

The isolates morphology was evaluated on Yeast Mannitol Agar (YMA) plates which were incubated from 3 to 7 days at 28°C. On the basis of the size, color, shape, transparency, borders and elevation the colonies were characterized [23].

2.5.2 Congo Red test

The isolates were tested on YMA agar containing 1% of Congo red and incubated from 3 to 7 days at 28°C. The colonies were characterized based on the absorbance of the red coloration [22].

2.5.3 Bromothymol blue test

To test if a reacting strain is acidic or alkaline, all isolates were cultured on YMA agar plates containing 1,5% Bromothymol blue for 3 to 7 days. The change of coloration in the plates records the type of reaction [22].

2.5.4 Use of carbohydrate as carbon source

The use of different carbon sources was tested on YMA medium by replacing the mannitol with the carbohydrate to be tested. The carbohydrates tested were: sucrose, starch and glucose.

2.5.5 Carbohydrate metabolism

2.5.5.1 Kligler-Hajna test medium

The Kligler-Hajna Agar was used to differentiate the strains by identifying their ability to ferment glucose, with or without producing gas, to screen for lactose fermenting and/or hydrogen sulfide (H₂S) production.

2.5.5.2 Simmons citrate test medium

This medium was used to select the strains able to utilize Ammonium Dihydrogen Phosphate and Sodium Citrate as their sole sources of nitrogen and carbon.

2.5.5.3 Mannitol motility test medium

This semisolid medium already prepared by Bio-Rad at 4% of agar was used to detect the strain’s motility.

2.5.6 Temperature tolerance

The tolerance of the isolates to the temperature was tested by incubating the inoculated YMA agar plates at 4, 28, 40 and 53°C.
2.5.7 pH tolerance test

The test of the capacity of the isolated strains to grow in acidic or alkaline media was determined on YMA agar plates whose pH had been adjusted and buffered to 4.0, 5.0, 7.0, 9.0 and 11.0 [24].

2.5.8 Salt tolerance test

All isolates were examined for their tolerance to salt on YMA supplemented with 0,1,2,3,4, and 5% of NaCl (w/v).

2.6 Genotypic characteristics

The molecular study involved 12 selected bacteria and was carried out at functional genomics platform, of the Technical Support Unit for Scientific Research, CNRST in Rabat-Morocco.

2.6.1 Genomic DNA extraction of isolates

DNA extraction from bacterial strains on liquid culture using the kit "Gen Elute Bacterial Genomic DNA kit" from SIGMA, Aldrich according to the protocol provided.

2.6.2 16S ribosomal DNA gene amplification

To amplify the 16S rDNA gene two primers were used FD1(5′ AGAGTTTGATCCTGGCTCAG 3′) and rp2 (5′ ACGGCTACCTTGTTACGACTT 3′) [25]. PCR amplification was carried out in 25 μl of the reaction volume containing template DNA (30 ng), Taq buffer (10x), MgCl2 (50 mM), dNTP mixture (10mM), fd1 primer (100 μM), rp2 primer (100μM), and (5 U/μl) of Taq DNA polymerase. PCR amplification was performed with a «Verity» thermal cycler model from ABI ((Applied Bio systems, Foster City, USA). The PCR temperature profile used was 96°C for 4 min followed by 35 cycles consisting of 96°C for 10 s, 52°C for 40 s, 72°C for 2 min, with a final extension step at 72°C for 4 min. Reaction efficiency was estimated by horizontal agarose gel electrophoresis (1% w/v) using a molecular weight marker of 100 bp and photographed. The photos were displayed by the "G Box" photo documentation system.

2.6.3 16S rRNA gene sequencing

Sequencing was performed on the 515 bp to 907 bp region of the 16S rRNA gene using the 3130XL Dye Terminator Cycle Sequencing (DTCS) Quick Start kit (Applied Biosystems) according to manufacturer instructions with 25–100fmol template DNA and 0.2 μM 515F and 907R primers (515F:GTGCCAGCMGCCGCGGTAA, 907R:CCGTCAATTCCCTTTRAGTTT) [25]. For the purposes of this study, both strands of the 16S rDNA gene were sequenced for 12 samples, only the forwards strand was sequenced for the remainder. The optimal thermocycling conditions for the cycle sequencing reaction were as follows: 25 cycles of 96°C for 1 min, 96°C for 10s, 50°C for 5s, and 60°C for 4 min, followed by a 4°C infinite hold. The Sephadex G50 superfine (Sigma Aldrich) was used to remove unincorporated dye terminators from the cycle sequencing reaction, according to manufacturer's instructions with an additional 300 μl wash of the column with distilled H2O and centrifugation at 1500×g for 3 min prior to applying the sample to the column.
DNA sequencing was performed on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) using the POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software. Preliminary identification was performed by FASTA search of the Ezbiocloud database and a more precise identification was performed by phylogenetic analysis with type strains of the nearest neighbourhoods. Isolates were regarded as belonging to a species when sequence similarity with the species type strain was at least 99% and to a genus when sequence similarity with a type strain was at least 97%.

3. RESULTS

3.1 Isolation of Strains from Soil Samples

In this study, 27 presumptive rhizobial strains were isolated from soils collected from different rhizospheres in a total of 23 sites which were surveyed in order to determine the distribution of rhizobia in the soils of Meknes-Tafilalet region of Morocco. The Gram staining and a microscopic observation of the isolates showed that all isolates were Gram negative.

Generally, the greater part of the presumptive rhizobial isolates had the same colony morphology over and above a fast (mean generation times of 2 to 4 h and high growth rate on YMA medium. However, the others strains have shown an intermediate growth rate with a mean generation time of 4 to 6h. 89% of the studied isolates, formed transparent to creamy mucoid colonies, 3 to 5 mm in diameter, within 3 days of incubation on YMA medium.

3.2 Authentication of Isolates

The ability of the presumptive rhizobial strains to nodulate *Bituminaria bituminosa* roots and their symbiotic effectiveness is very important [18]. This symbiosis may enhance the chance to reintroduce this plant on degraded soils. The 27 isolates were tested for their ability to nodulate roots (authentication as rhizobia). Table 1 showed that only 12 isolates were able to nodulate *B. butiminosa* roots. This authentication was repeated to verify their ability to nodulate. It was noted that, for both authentications, the nodules were pinkish; indicating the presence of leghemoglobin content which may affect the growth of *Bituminaria bituminosa*.

| Strain       | Control | ALR | FM | SV | TFr1 | TFr2 | ASA1 | ASA2 | FCV | EF2 | BB | SA | GOS2 |
|--------------|---------|-----|----|----|------|------|------|------|-----|-----|----|----|------|
| Nodule number| 0       | 24  | 9  | 7  | 29   | 8    | 11   | 15   | 7   | 12  | 52 | 8  | 15   |
| Wet shoot weight(g)| 13.2 | 16.7| 16.7| 15.5| 20.9| 17.7| 16.9| 12.2| 20.0| 7.4 | 10.8| 10.6| 8.6 |
| Dry shoot weight(g)| 6.9 | 8.9 | 7.0 | 7.6 | 9.5 | 8.0 | 7.9 | 7.5 | 7.8 | 5.4 | 6.4 | 5.6 | 6.2 |
| Wet root weight(g)| 7.1 | 8.7 | 8.0 | 7.9 | 9.8 | 8.6 | 5.7 | 7.7 | 6.2 | 4.7 | 7.3 | 5.2 | 8.5 |
| Dry root weight(g)| 3.5 | 4.1 | 4.0 | 3.8 | 4.3 | 4.0 | 4.0 | 4.5 | 3.9 | 3.5 | 4.0 | 3.6 | 4.1 |
We noticed that the means of all the treatment were calculated using "t-Student test" provide by "R Software". We observed that all the physiological traits of the divers treatment differ significantly comparing to the control. On the basis of the results of the symbiotic effectiveness tests, twelve isolates were chosen for further phenotypic and genotypic characterization.

### 3.3 Phenotypic Characteristics

On the basis of morphological parameters, we have confirmed differences between the isolates. The majority of isolates were fast-growing (75%) with a mean generation time of 2 to 4h, and the remaining 25% were characterized by an intermediate growth rate, showing mean generation times between 4 to 6h.

Culturing the strains on Congo Red-YMA revealed that almost all strains are whitish or pinkish colour; while their reaction on the Bromothymol Blue medium plates showed that 63% made the medium yellow, whereas 37% made it green. Regarding the growth of the isolates on different test media, we noticed that 91% of the strains were able to ferment glucose and 83% able to ferment mannitol while just 34% of them had the capacity to ferment lactose. No strain had the capacity to produce ferric sulfide. Half of the isolates produce the gas during their fermentation reaction and 66% used citrate as carbon source. All the isolates strongly prefer to use starch (91%) or sucrose (100%) as carbon source compared to glucose (low growth of all the strains) (Table 2).

Mainly, all the strains tolerate mostly neutral to alkaline pH. An average growth, of all isolates, was shown at neutral pH and the optimal development was verified at pH 9 and 11(100%). However, few strains were able to tolerate acidity (17% were able to growth at pH 4) and we have observed lower pH correlated with reduced growth. We noticed that above pH=5 all the strains showed a great growth till pH=11. All isolates were greatly tolerant to salinity. We remarked that 100% of the isolates have grown till 3% of NaCl. However, above 4% of NaCl, the strains become more sensitive to the high concentration of salinity. We have observed an interesting result which showed that 58% of all the isolates were able to show a slow growth at 5% NaCl. The growth response of the presumptive rhizobia to the temperature was varying from 4°C (66% tolerate weakly) to 28°C (the optimal temperature of growth) but not above 53°C. The different physiological characteristics of the 12 presumptive rhizobial strains are summarized in the Table 3.

### 3.4 Genotypic Characteristics

According to the expected size of the 16S rRNA gene [18] and for each presumptive rhizobial strain nodulating *B. bituminosa*, the 16S rDNA was amplified and a band of about 1500 bp was obtained. The 12 isolates were subjected to 16S rDNA gene sequence analysis. For our isolated strains, the 16S rDNA sequences determined in this study comprised 600 to 1500 nucleotides (Table 4). There are widely accepted criteria for delineating species in current bacteriology, stating that strains with a sequence similarity greater than or equal to 97% may be considered a genus level match. A species level match is based on a similarity greater than or equal to 99% [26].
Table 2. Biochemical characteristics of the strains isolated from *Bituminaria bituminosa* nodules

| Parameters tested                      | ALR | FM | SV | TFr1 | TFr2 | ASA1 | ASA2 | SFCV | EF2 | BB | SA | GOS2 |
|----------------------------------------|-----|----|----|------|------|------|------|------|-----|----|----|------|
| Kligler Hajna test                     |     |    |    |      |      |      |      |      |     |    |    |      |
| Glucose                                | +   | +  | +  | +    | +    | +    | +    | +    | +   | +  | -  | +    |
| Lactose                                | +   | +  | +  | -    | -    | -    | -    | -    | -   | -  | -  | -    |
| H2S gas production                     | -   | -  | -  | -    | -    | -    | -    | -    | -   | -  | -  | -    |
| Mannitol motility test                 | +   | +  | +  | +    | -    | +    | +    | +    | +   | +  | +  | -    |
| Simmon citrate test                   | -   | -  | -  | +    | -    | +    | +    | +    | -   | +  | +  | -    |
| Carbohydrate utilisation              |     |    |    |      |      |      |      |      |     |    |    |      |
| Starch                                 | ++  | ++ | ++ | ++   | ±    | ++   | ++   | ±    | +   | ±  | -  | ++   |
| Glucose                                | ±   | ±  | ±  | ±    | ±    | ±    | ±    | ±    | ±   | ±  | -  | ±    |
| Mannitol                               | +   | ++ | ++ | ++   | -    | ++   | ++   | +    | +   | +  | +  | -    |
| Sucrose                                | ++  | ++ | ++ | ++   | ++   | ++   | ++   | ++   | +   | +  | +  | ++   |
| (−: no growth; ±: Low growth; +: average growth; ++: good growth) |

Table 3. Phenotypic characteristics of the strains isolated from *Bituminaria bituminosa* nodules under environmental stresses

| Parameters tested                      | ALR | FM | SV | TFr1 | TFr2 | ASA1 | ASA2 | SFCV | EF2 | BB | SA | GOS2 |
|----------------------------------------|-----|----|----|------|------|------|------|------|-----|----|----|------|
| Growth                                 | 4   | -  | -  | ±    | -    | ±    | -    | -    | ±   | -  | +  | -    |
| Temperature (°C)                       | 28  | +  | ++ | ++   | ++   | ++   | ++   | ++   | ++  | +  | +  | ++   |
|                                        | 40  | +  | ++ | ++   | ++   | ++   | ++   | ++   | ++  | ++ | -  | -    |
|                                        | 53  | ±  | -  | -    | -    | -    | -    | -    | -   | -  | -  | -    |
| Growth Salinity (%)                    | 0   | ++ | ++ | ++   | ++   | ++   | ++   | ++   | ++  | ++ | +  | ++   |
|                                        | 1   | +  | +  | +    | +    | +    | +    | +    | +   | +  | +  | +    |
|                                        | 2   | +  | +  | +    | +    | +    | -    | +    | +   | +  | +  | +    |
|                                        | 3   | +  | +  | +    | ±    | +    | +    | +    | +   | +  | +  | +    |
|                                        | 4   | +  | ±  | ±    | ±    | +    | ±    | ±    | ±   | ±  | ±  | ±    |
|                                        | 5   | ±  | -  | -    | ±    | -    | ±    | -    | -   | -  | -  | -    |
| Growth pH                              | 4   | -  | -  | -    | -    | -    | -    | -    | ±   | ±  | -  | -    |
|                                        | 5   | +  | +  | +    | +    | +    | +    | +    | +   | ±  | +  | +    |
|                                        | 7   | +  | +  | +    | +    | +    | +    | +    | +   | +  | +  | +    |
|                                        | 9   | ++ | ++ | ++   | ++   | ++   | ++   | +    | +   | +  | +  | ++   |
|                                        | 11  | ++ | ++ | ++   | ++   | ++   | ++   | ++   | +   | +  | +  | ++   |
| (−: no growth; ±: Low growth; +: average growth; ++: good growth) |
The 16S rDNA gene sequence analysis showed that there was a high similarity (≥ 99%) between the test strains and their closest phylogenetic relative, which may indicate that 16S rDNA gene sequence data are helpful for identification of rhizobia at the species level (Table 4). The isolates ALR, FM and SV had 99.83% of similarity with the *Rhizobium nepotum* (accession number FR870231). The isolates Tfr1, Tfr2, ASA1, ASA2, SFCV, EF2, BB showed a percentage of similarity varying between 99.58 and 100% with *Rhizobium radiobacter* (AJ389904). The isolate SA had 99.84% sequence identity with *Pantoea agglomerans* (AJ233423). The isolate GOS2 showed only moderate 16S rDNA sequence similarity (98.64%) to their Ezbiocloud best match sequences from taxonomically well determined bacteria species, indicating that it may be potential new species. Based on the mentioned criteria and phylogenetic analysis, our results revealed that the isolated bacteria were dominated by the genus *Rhizobium* while one strain was assigned to the genus *Pantoea* (Table 4).

Table 4. Isolates identification according to Ez biocloud database

| Isolates | Type strain       | Sequence length(bp) | Similarity | Accession number |
|----------|-------------------|---------------------|------------|------------------|
| SA       | *Pantoea agglomerans* | 1233                | 99.84      | AJ233423         |
| ALR      | *Rhizobium nepotum*   | 1166                | 99.83      | FR870231         |
| FM       |                   | 1168                |            |                  |
| SV       |                   | 1175                |            |                  |
| Tfr1     | *Rhizobium radiobacter* | 694                | 100        | AJ389904         |
| Tfr2     |                   | 1000                | 99.70      |                  |
| ASA1     |                   | 1180                | 99.70      |                  |
| ASA2     |                   | 1182                | 99.58      |                  |
| SFCV     |                   | 1182                | 99.58      |                  |
| EF 2     |                   | 1180                | 99.92      |                  |
| BB       |                   | 1175                | 99.66      |                  |
| GOS2     | *Rhizobium tibeticum* | 1176                | 98.64      | EU256404         |

4. DISCUSSION

The present study provides the first characterization of strains sampled from different sites of the Meknes-Tafilalet region of Morocco with the ability to nodulate *Bituminaria bituminosa*. This important legume shrub is known for its main role in increasing soil fertility and also for its high nutritive value as a fodder. *B. bituminosa* is widely distributed in the Mediterranean Basin and Macaronesia [18].

The Rhizobium legume symbiosis is very important not just in agriculture for sustainable production, but also in basic biology as a model for symbiosis, evolution and differentiation [27]. Given the global interest in a balanced environment, there is a debate about the naming of rhizobial species which has been ongoing for the past few years [27]. The high interest of taxonomists, molecular biologists and agronomists has lead the research towards inoculants development [27].

However, no correlation was found between the phylogeny of bacterial isolates and their geographical origin, as reported for several legume-Rhizobium associations [28]. This lack of geographical correlation was confirmed by our results which showed that even though that many strains were sampled from different regions, they all matched to the same
species. Besides that, the geographic distribution of the rhizobial species is highly influenced by many ecological factors such as soil pH [29] which can influence many other soil bacterial and fungal communities [30]. Most rhizobia grow optimally at a pH between 6 and 7 [31]. However, a few rhizobia grow well at pH values of less than 5 [32], some strains of *Rhizobium tropici*, *Mesorhizobium loti*, *Bradyrhizobium* species and *Sinorhizobium meliloti* are very acid-sensitive [33]. And some rhizobial isolates from tree legumes (Acacia farnesiana, Dalbergia sissoo and Sesbania formosa) grow at pH12 [34]. The data of this study reported that the isolates studied, even though they belong to different species (*R. radiobacter*, *R. nepotum*, *R. tibeticum* and *Pantoea agglomerans*) are generally tolerant of alkalinity and neutrality. 16% of isolates were weakly acid tolerant, and were able to grow at alkaline pH. On the contrary, all the remaining isolates were sensitive to acid pH, and showed best growth at alkaline pH. Since extremes of pH can be a major factor limiting microorganisms in soil [35], the isolates capable to survive on a wide pH range and to withstand diverse stress conditions will become an important candidate for further strain improvement to highly acidic or alkaline conditions and also for the future applications as inoculant.

Like several previous studies, this study showed heterogeneity in the population of strains nodulating *B. bituminosa* based on phenotypic and genotypic characteristics [36]. In accordance with the result obtained by Küçük et al. [37], 75% of the strains were fast-growing with a mean generation time of 2 to 4h. The majority of our strains showed a good temperature tolerance with an optimum growth at 28°C (100%), in concordance with previous studies [38] which showed that Rhizobium bacteria are mesophytic, and can grow at temperatures between 10 and 37°C with an optimum temperature for growth of most isolates at 28°C. We have observed that, although weakly, some strains were able to grow at 4°C (25%). As many other limiting factors, salinity has a significant impact on both the growth of rhizobia and their metabolism as well as their symbiotic association. In the present study, we report that almost all of the isolated strains were able to tolerate salt concentrations above 3% (91% of isolates). Also, we noticed that fast growing isolates were more tolerant to high NaCl concentrations. Our results are in concordance with those of Küçük et al. [37] and Berrada et al. [39].

In fact, the studied strains showed a variable tolerance to different limiting factors, such as, temperature, pH, and salinity. This allows the selection of efficient isolates for further genetic studies. The genetic diversity of *Rhizobium* was widely studied by many investigators from the entire world [39].

In the present study we reported the genetic biodiversity of 12 strains isolated from the nodules of *Bituminaria bituminosa*: ALR, FM, SV, TFr1, TFr2, ASA1, ASA2, FSCV, EF2, BB, SA and GOS2 based on 16S rDNA sequencing in order to determine the taxonomic position of our isolates. The results showed that there was a genetic diversity between the 12 nodulating strains. In fact, the sequence analysis of 16S rDNA showed that three strains had 99.83% of similarity with *Rhizobium nepotum*, one strain showed 98.64% sequence identity with *Rhizobium tibeticum*, and one isolate had 99.84% sequence identity with *Pantoea agglomerans* which was reported as a non rhizobial bacteria able to nodulate legumes like *Hedysarum sp* [40]. Finally, seven strains had a percentage of similarity varying between 99.58 and 100% with *Rhizobium radiobacter*. This species (also referred to as *Agrobacterium radiobacter*) as the other species in the *Agrobacterium* genus (including the type species, *Agrobacterium tumefaciens*) have been placed into synonymy with the *Rhizobium* genus [41]. Several authors have reported that the *Rhizobium radiobacter* strains have been isolated from nodules of different legume hosts and different geographical
distribution [42]. According to its plasmid content, the *Rhizobium radiobacter* behaves either as a pathogen or a symbiont due to its unstable symbiotic state which strongly depends on the environmental conditions [43]. Most interestingly, *Rhizobium radiobacter* has an effect on promoting plant growth and increasing pathogen resistance against powdery mildew [43]. Finally, on the basis of the phenotypic and genotypic characteristics, the 12 strains have shown a significant tolerance to many limiting factors.

5. CONCLUSION

Our phenotypic and genotypic characterizations show that there is diversity among isolates studied which were all mostly fast growing and tolerant to temperatures from 4 to 53°C. The isolates showed a significant potential to grow under salinity stress conditions. These strains may be great candidates for their utilization both under extreme temperature conditions as well as in saline soils. The sequencing data of the 16S rDNA indicated that there is a genetic variability and thus the existence of a diversity among different rhizobial strains.

COMPETING INTERESTS

Authors have declared that no competing interests exit.

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