Phenotypic, Genotypic and Symbiotic Characterization of Rhizobial Isolates Nodulating *Acacia sp.* in Morocco

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Thirty eight isolates nodulating acacia were isolated from different Moroccan areas to determine their ability to survive under various stress conditions, in order to select resistant ones. The response of these isolates to different environmental stress was evaluated to show their preliminary variability based on phenotypical characteristics. Tolerance to increasing levels of NaCl (0.5-10% w/v), pH (4.8-11), temperature (4-55°C) and resistance response to seven heavy metals and five antibiotics were studied as phenotypic markers. The majority of isolates from different *Acacia* trees were fast growing. Numerical analysis of these phenotypic characteristics showed that the isolates were divided into three distinct clusters presenting intra and inter-cluster diversity. The symbiotic effectiveness of different isolates was studied and showed significant differences with respect to nodule number, nitrogen fixation and improving plants growth (shoot and root dry weight) compared to the uninoculated and non-fertilized control under greenhouse conditions. On the basis of phenotypic and symbiotic studies, six isolates (I1, I3, I5, I6, I16 and I28) were selected for genotypic characterization. The selection of such rhizobial isolates able to adapt to various environmental stress conditions and to improve plant’s growth is important for their future valorization to develop inoculants for Acacia especially in arid areas.

**Keywords:** Rhizobium, Acacia, phenotypic characterization, symbiotic effectiveness, Morocco.

Nitrogen, an essential nutrient for plant’s growth and development, is sometimes limiting because it is generally present as unusable form even though the atmosphere contains 78% of N₂ by its volume. Hence, N₂ fixing associations are very important to convert the most stable and abundant form of N₂ to a useful form (NH₃). The *Rhizobium*-legumes symbiosis is of particular agricultural importance, producing about 50% of 175 million tons of total nitrogen biologically fixed and providing nearly half of the nitrogen used in agriculture per year¹. In addition, the increasing use of organic / bio-fertilizers would reduce the need for chemical fertilizers and their adverse effects such as environmental pollution and nature degradation². Previous studies have been performed to investigate and to identify rhizobia associated with legumes³-⁴. These nitrogen fixing bacteria are classified into 13 genera containing about 98 species⁵.

*Acacia* is a legume tree qualified as anti-desert plant which plays a crucial role in the dynamic and structure of plant communities, provides several services by agro-ecosystems and contributes to the restoration of degraded soils⁶-⁷ due to its ability to form a nitrogen-fixing symbiosis with rhizobia. Acacia plantations cover an area of 1,128,000 ha of natural forests in Morocco and are
among the fast growing, N$_2$-fixing multipurpose woody legumes which are prominent among the exotic species planted in arid and semiarid lands$^8$. This tree covers different areas such as Atlantic Plain (Mediterranean climate), the Middle Atlas (relatively humid) and Souss, presahara and Sahara arid regions.

In this study, N$_2$-fixing bacteria were isolated from Acacia root nodules and the effects of some environmental stresses including temperature, alkalinity/acidity, heavy metals, antibiotics and salt on their growth were investigated to provide a phenotypic characterization.

Moreover, their symbiotic effectiveness was studied and a genotypic characterization was conducted for the most performant ones.

### Table 1. Geographic origin and geodesic coordinates of the sampling sites.

| Strain name | Acacia species | Geographic origin | Coordinates °N, °W | Altitude |
|-------------|----------------|-------------------|--------------------|----------|
| I1          | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I2          | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I3          | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I4          | Acacia saligna | Cap de l’eau (Nador) | 35°08'10'' N, 2°25'30'' W | 1 m      |
| I5          | Acacia saligna | Cap de l’eau (Nador) | 35°08'10'' N, 2°25'30'' W | 1 m      |
| I6          | Acacia saligna | Saidia             | 35°05'15'' N, 2°17'16'' W | 20 m     |
| I7          | Acacia saligna | Berkane            | 34°55'12'' N, 2°19'11'' W | 173 m    |
| I8          | Acacia saligna | Meknes             | 33°53'36'' N, 5°32'50'' W | 548 m    |
| I9          | Acacia saligna | Saidia             | 35°05'15'' N, 2°17'16'' W | 20 m     |
| I10         | Acacia saligna | Agadir             | 30°25'12'' N, 9°33'53'' W | 16 m     |
| I11         | Acacia saligna | Berkane            | 34°55'12'' N, 2°19'11'' W | 173 m    |
| I12         | Acacia saligna | Berkane            | 34°55'12'' N, 2°19'11'' W | 173 m    |
| I13         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I14         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I15         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I16         | Acacia saligna | Berkane            | 30°25'12'' N, 9°33'53'' W | 16 m     |
| I17         | Acacia saligna | Casablanca         | 33°35'17'' N, 7°36'40'' W | 27 m     |
| I18         | Acacia saligna | Casablanca         | 33°35'17'' N, 7°36'40'' W | 27 m     |
| I19         | Acacia saligna | Casablanca         | 33°35'17'' N, 7°36'40'' W | 27 m     |
| I20         | Acacia saligna | Rabat              | 34°01'31'' N, 6°50'10'' W | 79 m     |
| I21         | Acacia saligna | Rabat              | 34°01'31'' N, 6°50'10'' W | 79 m     |
| I22         | Acacia saligna | Rabat              | 34°01'31'' N, 6°50'10'' W | 79 m     |
| I23         | Acacia saligna | Rabat              | 34°01'31'' N, 6°50'10'' W | 79 m     |
| I24         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I25         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I26         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I27         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I28         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I29         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I30         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I31         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
| I32         | Acacia saligna | Agadir             | 30°25'12'' N, 9°33'53'' W | 16 m     |
| I33         | Acacia saligna | Meknes             | 33°53'36'' N, 5°32'50'' W | 548 m    |
| I34         | Acacia saligna | Meknes             | 33°53'36'' N, 5°32'50'' W | 548 m    |
| I35         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I36         | Acacia saligna | Fez                | 34°02'13'' N, 4°59'59'' W | 104 m    |
| I37         | Acacia saligna | Saidia             | 35°05'15'' N, 2°17'16'' W | 20 m     |
| I38         | Acacia saligna | Oujda              | 34°41'12'' N, 1°54'41'' W | 450 m    |
MATERIAL AND METHODS

Collection of nodules
Nodules were collected according to the method recommended by Vincent et al. from two Acacia species (A. saligna and A. horrida) growing in three Moroccan regions (Oriental, center and North-West) (table 1). A dug of 15-25 cm approximately was carried around the plant to a depth of at least 20-50 cm to extract a part of its root system. Then, roots were rinsed delicately with running water and nodules were detached to 1-2 cm of their attachment point.

Isolation of rhizobia from nodules and phenotypic characterization of isolates
The nodules, surface sterilized by ethanol 95% for 10 sec and mercuric chloride solution (HgCl₂) for 4 min according to Vincent et al., were aseptically crushed with a sterile glass rod in NaCl (9‰) drops. Rhizobia were isolated on Yeast-Mannitol-Agar medium (YMA) supplemented with 0.0025% (w/v) Congo red using a standard procedure. The single colonies were selected and checked for purity by repeated streaking on YMA. Individual colonies morphology was characterized based on size, color, mucosity, borders, transparency, elevation and Gram stain reaction.

Isolates authentification
Acacia cyanophylla’s seeds were surface sterilized by rinsing in ethanol 95% (v/v), soaking for 4 min in 0.2% HgCl₂ (w/v), and washed three times in sterile distilled water. They were subsequently scarified with H₂SO₄ 95% and germinated on 0.9% agar for 2 to 3 days in the dark. Well germinated seeds were aseptically transferred into pots filled with sterile sandy-loam soil at rate of three seeds per pot. The pots were then kept in a greenhouse. Each seedling was inoculated with 1 ml of a freshly prepared bacterial suspension (10⁸ UFC/ml). Three pots were used for each isolate. Non-inoculated plants were used as nitrogen-free control (C₀). Nitrogen control (NC) was considered as non-inoculated plants receiving weekly 0.5% KNO₃ (w/v) as nitrogen source. After six months, plants were harvested, nodules separated from roots then rhizobia reisolated from nodules.

Bromothymol blue test
The ability of isolates to acidify or basify the YMA medium was evaluated by adding bromothymol blue (BBT) at 0.0025% (w/v) as pH indicator (yellow for acid reaction and dark blue for basic reaction). The inoculated plates were incubated at 28°C for 3 to 10 days.

Effect of extrinsic factors
Determination of Salt tolerance
Salinity tolerance of the isolates was examined on YMA containing different NaCl concentrations (0.5%, 1.5%, 2%, 5%, 7% to 10% (w/v)), after an incubation period of 7 days at 28°C.

Determination of pH tolerance
The ability of rhizobial isolates to grow in basic or acidic media was evaluated by inoculating them on YMB medium which pH values were adjusted to 4.8, 5.8, 6.8, 8.8 and 11 with NaOH or HCl. The results were obtained after 7 days of incubation at 28°C.

Determination of temperature tolerance
Isolates were incubated at different temperatures (4°C, 14°C, 28°C, 37°C, 44°C, 54°C) to estimate the maximal and optimal growth temperatures of each isolate, and to evaluate their tolerance to different temperatures. The bacterial growth was evaluated after 7 days of incubation.

Effect of intrinsic factors
Determination of heavy metals resistance
Resistance of tested rhizobial isolates was assessed against seven heavy metals using different concentration levels on YMA medium. Heavy metals stock solutions were filter sterilized (0.22 µm) and added to sterile agar medium to obtain concentrations which vary from 0.025 to 3 mmol l⁻¹ of: AsNa₂HPO₄, CuSO₄·5H₂O, NiCl₂·6H₂O, K₂Cr₂O₇, ZnSO₄·7H₂O, Cd(NO₃)₂, 7H₂O and Pb(NO₃)₂. The plates were inoculated with about 10⁶ cell/ml (10µl) and the bacterial growth was evaluated after 7 days of incubation at 28°C. Isolates were considered resistant when growth occurred and sensitive when no growth was detected.

Determination of antibiotic resistance
Antibiotic resistance was tested for different concentrations (10-100 µg) of five antibiotics (Ampicillin, Kanamycin, Streptomycin, Tetracycline and Chloramphenicol) which were added to agar media as filter-sterilized aqueous stock solutions. 10µl (~10⁸cell/ml) of each tested
isolate were seeded, incubated at 28°C then their growth (Presence/absence) was scored after 7 days for resistance/sensitivity.17

**Evaluation of the symbiotic effectiveness of different rhizobial isolates**

Isolates were tested for their symbiotic effectiveness by inoculating them separately to young plantlets of *A. cyanophylla* and *A. horrida*. Those plantlets were grown in plastic pots maintained in the greenhouse under the same conditions described above for the test of isolates authentication. Three replications were carried out for inoculated and non-inoculated plants. Plants were harvested six months after inoculation. The root nodules formed on each plant were counted; plant’s shoot and root dry matters were measured after drying at 70°C for 48 h. The average plant’s dry weight from three replicates inoculated with the same isolate was used to evaluate the relative efficiency (RE) expressed as the percentage of

**Table 2. Effect of NaCl concentration, pH and temperature on rhizobial isolates**

| Isolates | NaCl % | pH   | Temperature (°C) |
|----------|--------|------|------------------|
|          | 0.5    | 1    | 2    | 5    | 7    | 10   | 4.8  | 5.8  | 6.8  | 8.8  | 11   | 4    | 14   | 28   | 37   | 44   | 55   |
| 11       | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 12       | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    |
| 13       | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 14       | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 15       | +      | +    | +    | +    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 16       | +      | +    | +    | +    | -    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 17       | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 18       | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 19       | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 110      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 111      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 112      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 113      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 114      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 115      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 116      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 117      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 118      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 119      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 120      | +      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 121      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 122      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 123      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 124      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 125      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 126      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 127      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 128      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 129      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 130      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 131      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 132      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 133      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 134      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 135      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 136      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 137      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |
| 138      | +      | +    | +    | +    | -    | -    | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    |

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gain in aerial parts dry matter of inoculated plants (PDPi) compared to non inoculated control plants receiving KNO₃ (PDCn):
RE = PDPi / PDCn x 100

Then aerial part’s nitrogen percentage and total nitrogen content were determined by using Kjedahl method⁸.

**PCR amplification and sequencing of 16S rRNA gene**

Total genomic DNA from bacterial isolates was extracted by a quick cell lysis in boiling water bath. The nucleotide sequences of the 16S rRNA gene (rDNA) were determined by direct sequencing of appropriate PCR products from each strain with the universal primers 8F (5'- AGA GTT TGA TCC TGG CTC AG -3’) and 1540R (5’- AAG GAG GTG ATC CAG CAG CC-3’). Each 50 µl reaction contained 1 µl of the cell lysate (approx. 20ng DNA), 1,25 U of GoTaq® G2 DNA polymerase (Promega), 1X reaction buffer, 0,2 mMdNTPs, 0,15 µM of each primer, as well as 5% DMSO. The PCR protocol was set to: 4 min 95°C initial denaturation, 35 cycles of amplification: 1 min 95°C for denaturation, 1 min 55°C for annealing, 2 min 72°C for elongation; the final elongation step was set to 7 min. The nucleotide sequence of the PCR products was determined for both strands by Sanger sequencing. DNA sequences were compared to the GenBank database by basic local alignment search tool (BLAST) requests using the blast-n algorithm and optimization for highly similar sequences (megablast).

**Numerical Analysis**

The numerical analysis includes comparison of phenotypic characteristics between studied isolates taken in pairs. This method of analysis was used as a primary method of characterization and grouping of unidentified isolates⁹. A dendrogram performed with Ward method and Euclidian distance on the numerical analysis of phenotypic traits was obtained with XLSTAT software.

**Statistical analysis**

Means were compared using analysis of variance at P = 0.05. The used Software was Statgraphics Centurion XVI.

**RESULTS**

**Morphologic characterization**

All isolates tested were gram negative bacilli and most of them were fast-growing bacteria (63%) as indicated by the observation of a color variation from blue to yellow in YEM agar plates containing BBT, whereas, 21% were intermediate growing and 16% were slow growing.

**Effect of extrinsic factors**

Results of isolate’s tolerance to some environmental stress factors are presented in Table 2.

All tested isolates grew in 5% NaCl, 87% were tolerant to 7% NaCl and 71% could persist to high salinity of 10% NaCl. Furthermore, the fast growing isolates showed higher salt tolerance than slow-growers.

All isolates tested were well adapted to high or low pH values: 100% were able to grow in variable pH values from 4.8 to 6.8, 97% survived at pH of 8.8 and 11.

It was also found that all isolates were able to survive at temperature ranging between 4 and 28°C. 95% of them presented a tolerance to a temperature of 37°C and 79% to 44°C. However, all isolates couldn’t tolerate high temperature (55°C).

| Metal’s concentration (mmol l⁻¹) | AS 100% | Cr 100% | Cu 65% | Cd 63% | Zn 100% | Ni 84% | Pb 100% |
|---------------------------------|---------|---------|--------|--------|---------|--------|--------|
| 0.250                           | 100%    | 100%    | 65%    | 63%    | 100%    | 84%    | 100%   |
| 0.500                           | 100%    | 100%    | 60%    | 60%    | 100%    | 71%    | 100%   |
| 1.0                             | 100%    | 94%     | 48%    | 23%    | 94%     | 55%    | 100%   |
| 2.0                             | 100%    | 94%     | 0%     | 0%     | 92%     | 29%    | 95%    |
| 3.0                             | 100%    | 68%     | 0%     | 0%     | 31%     | 0%     | 29%    |

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### Table 4. Results of phenotypic tests differentiating between isolates nodulating Acacia of different clusters formed by numerical analysis

| Heavy metals resistance (mmol l\(^{-1}\)) | Cluster 1 | Cluster 2 | I 26 |
|-----------------------------------------|-----------|-----------|------|
| As 0.25                                 | 10        | 27        | 1    |
| As 0.5                                  | 10        | 27        | 1    |
| As 1.0                                  | 10        | 27        | 1    |
| As 2.0                                  | 10        | 27        | 1    |
| As 3.0                                  | 10        | 27        | 1    |
| Cr 0.25                                 | 10        | 27        | 1    |
| Cr 0.5                                  | 9         | 26        | 1    |
| Cr 1.0                                  | 9         | 26        | 1    |
| Cr 2.0                                  | 4         | 6         | 1    |
| Cu 0.25                                 | 7         | 17        | 0    |
| Cu 0.5                                  | 7         | 15        | 0    |
| Cu 1.0                                  | 6         | 10        | 0    |
| Cu 2.0                                  | 0         | 0         | 0    |
| Cu 3.0                                  | 0         | 0         | 0    |
| Cd 0.25                                 | 7         | 16        | 1    |
| Cd 0.5                                  | 7         | 15        | 1    |
| Cd 1.0                                  | 4         | 5         | 0    |
| Cd 2.0                                  | 0         | 0         | 0    |
| Cd 3.0                                  | 0         | 0         | 0    |
| Zn 0.25                                 | 10        | 27        | 1    |
| Zn 0.5                                  | 10        | 27        | 1    |
| Zn 1.0                                  | 10        | 25        | 1    |
| Zn 2.0                                  | 10        | 24        | 1    |
| Zn 3.0                                  | 5         | 7         | 0    |
| Ni 0.25                                 | 9         | 21        | 1    |
| Ni 0.5                                  | 8         | 18        | 0    |
| Ni 1.0                                  | 7         | 13        | 0    |
| Ni 2.0                                  | 4         | 5         | 0    |
| Ni 3.0                                  | 0         | 0         | 0    |
| Pb 0.25                                 | 10        | 27        | 1    |
| Pb 0.5                                  | 10        | 27        | 1    |
| Pb 1.0                                  | 10        | 27        | 1    |
| Pb 2.0                                  | 10        | 26        | 0    |
| Pb 3.0                                  | 1         | 9         | 0    |
| Antibiotic resistance (µg ml\(^{-1}\))   |           |           |      |
| Amp 10                                  | 10        | 15        | 1    |
| Amp 25                                  | 10        | 10        | 1    |
| Amp 50                                  | 10        | 10        | 1    |
| Amp 100                                 | 10        | 9         | 1    |
| Chl 10                                  | 10        | 16        | 1    |
| Chl 25                                  | 8         | 7         | 1    |
| Chl 50                                  | 8         | 5         | 1    |
| Chl 100                                 | 8         | 5         | 1    |
| Tet 10                                  | 5         | 12        | 0    |
| Tet 25                                  | 1         | 5         | 0    |
| Tet 50                                  | 0         | 0         | 0    |
| Tet 100                                 | 0         | 0         | 0    |
| Kan 10                                  | 10        | 14        | 1    |
| Kan 25                                  | 10        | 9         | 0    |
| Kan 50                                  | 8         | 3         | 0    |
| Kan 100                                 | 2         | 0         | 0    |
| Str 10                                  | 9         | 9         | 1    |
| Str 25                                  | 9         | 4         | 0    |
| Str 50                                  | 9         | 2         | 0    |
| Str 100                                 | 5         | 1         | 0    |
| pH tolerance                            | 4.8       | 10        | 27   |
| Tolerance to temperature (°C)            | 5.8       | 10        | 27   |
|                                          | 6.8       | 10        | 27   |
|                                          | 8.8       | 10        | 27   |
|                                          | 11        | 10        | 27   |
|                                          | 4         | 10        | 27   |
|                                          | 14        | 10        | 27   |
|                                          | 28        | 10        | 27   |
|                                          | 37        | 10        | 25   |
|                                          | 44        | 9         | 20   |
|                                          | 55        | 0         | 0    |
| Tolerance to NaCl (%)                    | 0.5       | 10        | 27   |
|                                          | 1         | 10        | 27   |
|                                          | 2         | 10        | 27   |
|                                          | 5         | 10        | 26   |
|                                          | 7         | 6         | 26   |
|                                          | 10        | 4         | 13   |

Intrinsic resistance to heavy metals

Results of rhizobial isolate’s tolerance to different heavy metals concentrations are reported in Table 3. The majority of isolates were able to tolerate low concentrations of the tested heavy metals, but at higher concentrations bacterial growth was negatively affected. Indeed, the highest resistance was recorded for As with a growth rate of 100% at 3mmol l\(^{-1}\), followed by Cr, Zn and Pb (68%, 31% and 29% at 3mmol l\(^{-1}\)). The resistance of isolates to different tested concentrations of cadmium and copper seems to be similar. These two metals are considered as the most growth inhibitors which induced a lethal effect on all isolates at
concentrations of 2mmol l\textsuperscript{-1}. Moreover, the isolates’ growth is inhibited for nickel at a concentration of 3mmol l\textsuperscript{-1}.

**Intrinsic resistance to antibiotics**

Strong resistance was recorded for ampicillin, chloramphenicol, kanamycin and streptomycin, while the lowest resistance was observed for tetracycline. At a concentration of 10 µg / ml, the majority of the isolates showed good resistance with a growth rate of 71% for ampicillin and kanamycin and 73% for chloramphenicol. While at the same concentration of tetracycline and streptomycin, only 44 to 50% of isolates were able to grow in the presence of these antibiotics. At high concentrations (50 and 100 µg / ml), the inhibitory effect was highly marked especially in the case of three antibiotics, tetracycline, kanamycin and streptomycin. Resistance percentages of 50% and 39%, respectively, were noted for the other two antibiotics (ampicillin and chloramphenicol) (Figure 1).

**Numerical analysis**

For the numerical Analysis, the most discriminating characters of this analysis were grouped and presented in table 4. All phenotypic characteristics were treated by XLSTAT software to build the dendrogram represented in Figure 2. At 45% of similarity degree, the isolates were divided into two delimited clusters with an independent lineage represented by the strain I26, which presented a great capacity to tolerate the extrinsic stress factors (temperatures from 4-44 °C, 10% NaCl and pH 4.8 - 6.8) but was less resistant to intrinsic factors: sensitivity towards high concentrations of some heavy metals and antibiotics (Table 4).

**Table 5.** Plant growth parameters: (SDW: Shoot dry weight, RDW: root dry weight), nodule number (Nod N) and nodule dry weight (NDW) values of *Acacia* trees evaluated six months after plants inoculation.

| isolate | SDW g/plant | RDW g/plant | Nod N /plant | NDW g/plant |
|---------|-------------|-------------|--------------|-------------|
| I1      | 15.32±0.55  | 9.97±0.74   | 81.3±3.53    | 0.365±0.04  |
| I2      | 7.32±0.89   | 7.32±0.57   | 61±8.48      | 0.212±0.02  |
| I3      | 7.25±1.15   | 4.84±1.71   | 37±3.53      | 0.132±0.01  |
| I5      | 5.44±0.77   | 3.12±0.14   | 57±3.53      | 0.148±0.03  |
| I6      | 8.72±0.33   | 6.01±1.22   | 56±5.65      | 0.143±0.01  |
| I14     | 8.45±0.08   | 4.37±0.19   | 52±4.24      | 0.143±0.01  |
| I16     | 5.54±1.15   | 2.89±0.20   | 56±5.65      | 0.129±0.004 |
| I20     | 6.68±1.35   | 3.24±0.55   | 53±4.94      | 0.103±0.01  |
| I23     | 3.44±0.26   | 1.05±0.09   | 31±4.94      | 0.124±0.02  |
| I25     | 8.30±1.44   | 6.91±1.03   | 42±9.89      | 0.147±0.004 |
| I28     | 13.25±0.55  | 8.75±0.28   | 67±4.24      | 0.141±0.02  |
| NC      | 12.02±1.13  | 5.82±0.24   | 0.00±0.00    | 0.00±0.00   |
| C0      | 1.26±0.06   | 0.37±0.14   | 0.00±0.00    | 0.00±0.00   |
| P-value | 0.0000      | 0.0000      | 0.0000       | 0.0000      |
| F       | 38.23       | 30.4        | 41.82        | 36.51       |

**Table 6.** Sequence analysis of 16S rDNA for six rhizobial strains nodulating *Acacia* sp.

| Isolate | Sequence (bp) | Accession (no) | Homology to the reference strains | Identity (%) |
|---------|---------------|----------------|----------------------------------|--------------|
| I1      | 471           | KX519318.1     | Rhizobium pusenseMB17a            | 98           |
| I3      | 537           | KY426385.1     | Rhizobium sp.Moz90               | 99           |
| I5      | 603           | EF638791       | Rhizobium sp. LAR-14             | 99           |
| I6      | 650           | HQ836158.1     | Rhizobium sp. SAB12b             | 99           |
| I16     | 549           | KJ748400.1     | Rhizobium sp. DG22               | 99           |
| I28     | 491           | LC133652.1     | Phyllobacterium sp. JCM 28305    | 98           |

J PURE APPL MICROBIO, 12(1), MARCH 2018.
The first cluster contains ten isolates and the second one twenty seven isolates

The first cluster is formed by N₂ fixing bacteria isolated from *A. saligna* and *A. horrida* and is divided at 47% of similarity degree in two subclusters. The subcluster’s I isolates (65% of similarity) tolerate different stress factors (salinity, pH and temperature), resist to high antibiotic concentrations except Tetracycline, but are less resistant to heavy metals especially cadmium and copper. The subcluster’s II isolates present a similarity level of 58% and show tolerance / resistance to both extrinsic and intrinsic stress factors excluding I4 and I6 which are sensitive for low concentrations of chloramphenicol.

The 2nd cluster which is more heterogeneous is divided in four subclusters at 48% of similarity. The subcluster I contains two isolates with 55% of similarity, presenting sensitivity to high concentrations of antibiotics and heavy metals. These two isolates are tolerant to different abiotic stresses with the exception of the high concentration of NaCl (10%).

The subcluster II’ contains eight isolates with 63% of similarity, presenting sensitivity to high concentrations of antibiotics and moderate resistance to heavy metals except I18, and having a good tolerance to abiotic stress. The subcluster III’ consists of seven isolates (67% of similarity) tolerant to high salinity percentage (7-10%) and...
temperatures (37-44°C), to acid/alkaline medium. The isolates of this subcluster present high resistance to heavy metals but are more sensitive to the majority of tested antibiotics.

The subcluster IV’ contains ten isolates, at 63% of similarity, generally tolerant to extrinsic stress factors, sensitive to low concentrations of cadmium, nickel and copper, and highly sensitive to the tested antibiotics.

**Evaluation of the rhizobial isolate’s symbiotic effectiveness**

The symbiotic effectiveness of the strains was evaluated based on shoot dry mass, nodule’s number and dry mass, and total nitrogen of the plant. The obtained results showed that acacia could be nodulated by all isolates tested. A large variability of the infective capacity of the isolates was demonstrated (Table 5). Indeed, even if the plants inoculums concentration of different isolates was the same (10^8 UFC/ml), the average nodules number formed by plant ranged from 31 to 81. The most infective isolate was I1 with 81±3.53 nodules formed by plant, while the less infective isolate (I23) was able to induce the formation of 31 nodules.

Plant response to inoculation reveals different relative efficiencies (RE) (Figure 3). The results showed that the shoot dry weight (SDW) of plants inoculated with the isolates I1 and I28 exceeded the SDW of plants fertilized with mineral nitrogen (Table 5). In fact, shoot biomass, used as an indicator of relative effectiveness, indicated that isolate I1 was the most effective with an 127% dry biomass of the NC control followed by I28 with 110% of RE. The least effective isolate was I23 with only 29% of the dry biomass of the C0 control. The dry biomass of C0 control was 10% of the NC control.

Regarding the performance in root dry weight (RDW), the results showed a significant difference between inoculated isolates, the highest RDW was detected in I1 while the lowest RDW was showed by I23 with values of 9.97 and 1.05 g/plant respectively (Table 5).

The percentage of total nitrogen derived from atmospheric N2 and fixed by acacia was evaluated in the rhizobium inoculation field trial. In fact, it varied from 2.1% for isolate I20 to 4.9% for the isolate I23, with significant difference between the different isolates (P= 0.000) (Figure 4).

The shoot’s total nitrogen content showed also significant differences between the studied isolates (P = 0.000) (Figure 5). Plants inoculated with the isolate I1 accumulated more nitrogen in their aerial parts (0.75 g/plant) followed by the ones inoculated with the isolates I6and I28which accumulated 0.34 and 0.38 g/plant respectively.

These results showed not only a differential effect of the studied isolates on acacia’s growth but also showed a significant biodiversity of *Rhizobium* bacteria isolated from Moroccan soils.

**Fig. 3.** Relative efficiency of isolates nodulating acacia evaluated six months after plants inoculation
Molecular classification of the plant-growth promoting isolates

The sequencing of 16S rRNA genes of the tested isolates was repeatedly reported as the preferred method in order to characterize microbial communities, found in the plant rhizosphere\textsuperscript{20-21}. According to the sequences similarity, local rhizobial isolates are identified as members Rhizobium and Phyllobacterium genera (Table 6).

DISCUSSION

This study provides phenotypic characterization of rhizobial isolates from different moroccan Acacia tree species. The majority of isolates were fast growing. The response of the various isolates against different environmental stress was evaluated in order to show their preliminary variability based on phenotypical characteristics.

![Fig. 4. Total nitrogen percentage in the aerial parts of acacia inoculated with different isolates of Rhizobium, evaluated six months after inoculation. (C\textsubscript{0} = nitrogen-free control, NC nitrogen control fertilized with KNO\textsubscript{3})](image)

![Fig. 5. Total nitrogen content in the aerial parts of acacia inoculated with different isolates of Rhizobium, evaluated six months after inoculation](image)
In the present study, all isolates grew in medium salt concentrations (5.0%) and the majority of them (71%) could persist to high salinity reaching 10%. This NaCl range tolerance agree with previous reports showing that Rhizobium isolates from arid and saline area’s various collections are highly salt-tolerant and withstand at high NaCl levels up to 5-10 %. Furthermore, the fast growing isolates showed higher salt tolerance than slow-growers, which is consistent with previous studies\textsuperscript{15, 22}. However, other studies revealed that salt tolerance is not correlated with growth rate\textsuperscript{23} but is correlated with other physiological and biochemical mechanisms\textsuperscript{24}. Furthermore, salinity is one of the major factors restricting the symbiotic nitrogen fixation\textsuperscript{25} and nodulation in legumes. Therefore, these isolates may be performant candidates for inoculation in saline soils frequently observed in some irrigated areas in Morocco. Especially because salinity is among the main factors inducing desertification and lands degradation\textsuperscript{26}.

The results showed that most of the isolates in this study could grow within a wide temperature range (4°C to 44°C) which is concordant with Maâtallah et al\textsuperscript{27} finding that Rhizobium could grow within a wide temperature range (4°C to 44°C). The growth inhibition observed for all the tested isolates at high temperature (55°C) agree with Zahran et al\textsuperscript{28} and Fentahun et al\textsuperscript{29} findings. Furthermore, Rhizobia are known to be mesophilic and have optimum culture growth temperatures in the range of 28-31 °C\textsuperscript{30}. Maximum temperature degrees (Tmax) for free-living rhizobia ranged between 35-45 °C\textsuperscript{19, 28}. Even if the rhizobia grow at high temperatures it does not mean that it is an efficient N\textsubscript{2}-fixer\textsuperscript{29}. In fact, the exposure to high temperatures can lead to loss of symbiotic plasmid and consequently the loss of the bacterial infective capacity\textsuperscript{31}. So, high soil temperature can generate a major problem for biological nitrogen fixation in leguminous because the high temperature may control the bacterial infection in host plant.

According to our results, the isolates studied are generally slightly tolerant to acidity at pH 4.8-5.8, and mostly tolerant to alkalinity. This finding is concomitant with results of previous studies which indicated that Rhizobia were able to grow at a wide pH range in soil conditions\textsuperscript{12-24} and even at pH 12 in agar culture\textsuperscript{35}. The isolates which can survive on a wide pH range are candidates for further strain improvement to highly acidic or alkaline conditions. Several authors have reported the sensitivity of slow-growing rhizobial strains to higher acid pH than fast growing rhizobin\textsuperscript{36-37}. Other studies showed that fast-growing rhizobia are generally more sensitive to acidity than bradyrhizobia\textsuperscript{11}. Similarly, Marsudi et al\textsuperscript{38} reported that the slow growing Bradyrhizobium strains isolated from Acacia saligna were found to be sensitive to alkaline pH and tolerant to acid pH.

However, in our study, no obvious relationship between the isolates growth rate and their tolerance to different pH range was found. Mohamed et al\textsuperscript{39} also deduced in their results that there is no correlation between pH tolerance of strains and their growth’s rate. Regarding the intrinsic resistance to heavy metals, several studies showed that they might have been toxic to rhizobia when present in soil at moderate to high concentrations.

In our study, strong resistance has been recorded, on one hand, for arsenate, chrome, plumb and zinc. On the other hand, low resistance has been recorded for nickel, copper and cadmium. This result is consistent with the literature that showed that Rhizobium could withstand to high concentrations of arsenate, zinc and plumb\textsuperscript{40} and was less resistant to high concentrations of cadmium and cooper\textsuperscript{41}. The selection of isolates resistant to heavy metals presents actually great practical interest for bio-remediation investigations. Abd-Alla et al\textsuperscript{42} have shown that an excess of Cd, Zn, and Cu in soil can have an adverse effect for symbiotic microorganisms as well as for the symbiosis establishment. Similarly, Sepehri et al\textsuperscript{43} revealed that Cd can affect the symbiotic properties of Sinorhizobium meliloti strains and therefore S. meliloti-alfalfa symbiosis.

As with Cd, the tested isolates were found to be more sensitive to Cu than other metals, which is consistent with previous studies showing that high Cu concentrations may be toxic to soil microorganisms by affecting their structural diversity and their tolerance to metals\textsuperscript{44-45}.

Antibiotic resistance of bacterial strains isolated from acacia was tested against different antibiotics. In general, the isolates showed strong resistance to Ampicillin, Chloramphenicol and Kanamycin. However, the majority of the tested
isolates were sensitive to high concentrations of Streptomycin, Kanamycin and Tetracycline. This is in agreement with the results of Gauri et al.\textsuperscript{46} and Bakhoun et al.\textsuperscript{16}. Similar results were also found by Hilali et al. (2006)\textsuperscript{31} in a rhizobacterial population nodulating lupine in Morocco in which tetracycline, streptomycin and kanamycin were considered as inhibitors of the plasma membrane and protein synthesis, and which had the most radical effect on the isolates growth.

Graham et al.\textsuperscript{30} reported that the inhibitory effect of an antibiotic varies with its nature, its concentration in the medium, and the degree of inhibition varies from one species to another and from one strain to another. In similar studies, Kanamycin has been reported to be growth’s inhibitor of some strains of \textit{Bradyrhizobium} nodulating lupine\textsuperscript{47} and some strains nodulating different \textit{Acacia}’s species\textsuperscript{23}. However, in our study, tetracycline was recorded as the most detrimental for the isolates growth. The selection of isolates with multiple resistance to different antibiotics is very important because this property can be used as a great marker for strain’s identification and study of their diversity\textsuperscript{48}.

Previous studies mentioned that the strains associated with Acacia spp. in Africa can belong to \textit{Rhizobium}, \textit{Agrobacterium}, \textit{Ensifer}, \textit{Mesorhizobium} and \textit{Bradyrhizobium}\textsuperscript{49-52}. In this study, the identified strains isolated from Acacia tree was found to belong to \textit{Rhizobium} and \textit{Phyllobacterium} genus. In fact, it has been reported that \textit{Phyllobacterium} genus was able to nodulate some Acacia species\textsuperscript{53-54}.

The six strains were isolated from the same species (\textit{Acacia saligna}), and were found to belong to different rhizobial species. This is consistent with previous studies which reported that changes in the nature of rhizobia associated with the same legume can be affected by the different geographic and meteorological characteristics of each sampling site\textsuperscript{55-56}.

Also, it was found that I1 and I28 isolated from the same geographical region are classified in two different genera. However, a previous study showed that legume plants in the same geographic region had one rhizobial species in common\textsuperscript{57}.

All tested isolates were able to infect their host plant and to fix atmospheric nitrogen leading to plant shoot production above the noninoculated and nitrogen-free controls. In fact, the results of the experiments showed that acacia’s inoculation by different isolates have significant effects on the level of infection of \textit{A. saligna} (measured by the nodules number formed).

The results of isolate’s symbiotic effectiveness showed not only a differential effect of the studied isolates on \textit{Acacia} seedlings growth but also a significant diversity of Rhizobial isolates in Moroccan soils. Indeed, I1, I2, I3 and I28 were all isolated from the same geographical region (Oujda), but induced significantly different plant dry matter yields.

The results of this study also showed differences in N\textsubscript{2} fixation among the tested Acacia plants both for the %Ndfa and the total N fixed.

Several previous works also showed that selection and inoculation of effective rhizobium increased nodulation, shoot dry weight, height and total shoot nitrogen content\textsuperscript{58-59}.

These results are extremely encouraging for the use of acacias in land’s rehabilitation and/or restoration projects. Rhizobial isolates identified as effective in the greenhouse trial can be useful in field inoculation trials to improve soils fertility and transfer nitrogen to associated crops by symbiotic interaction Acacia/Rhizobia.

**CONCLUSION**

This study provides phenotypic and symbiotic characterization of rhizobial isolates from different Moroccan \textit{Acacia} tree species and showed their large physiological and biochemical diversity. Most of them were fast growing and their response to different environmental stress showed a variable resistance against stress factors, namely, temperature, pH, salinity and also to antibiotics and heavy metals, which allowed us to make a preliminary classification and characterization of these isolates based on their phenotypic similarities. Moreover, this analysis enabled to bring out a phenotypic groups containing intra and inter- cluster diversity. This phenotypic diversity and the high symbiotic efficiency of the tested Rhizobial strains can help us to precisely select the right candidates for any inoculation test according to requirements of different environmental
conditions considered in order to improve and to enhance plant’s crops and nitrogen fixation in semi-arid and arid areas.

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