Phenotypic and Genotypic Diversity and Symbiotic Effectiveness of Rhizobia Isolated from *Acacia* sp. Grown in Morocco

K. Fikri-Benbrahim[1*], M. Chraibi[1], S. Lebrazi[1], M. Moumni[2], and M. Ismaili[3]

ABSTRACT

Phenotypic and genotypic diversity as well as symbiotic effectiveness of twenty one symbionts isolated from nodules of four Acacia species (*Acacia melanoxylon*, *A. mearnsii, A. saligna* and *Acacia tortillis*) grown in Morocco were evaluated. Growth in various media (YMA, YMA-BBT, YMA-RC), colony morphology, tolerance to stress factors (temperature, pH, salt concentrations, various heavy metals) and different carbon sources were used as phenotypic markers. The genetic characterization was studied by Random Amplification of Polymorphic DNA (RAPD) and *16S rRNA* gene sequencing. The symbiotic effectiveness of rhizobial isolates on acacia was evaluated using plant nodulation assay in controlled conditions. Results showed that twelve isolates were fast growing, all of the twenty one isolates tolerated pH between 4.8 and 8.8 and temperatures of 14, 22, 28, and 37°C, while only five could grow at 44°C and three at 55°C. Highest resistance to heavy metals was recorded for arsenate, mercury, chromium; in contrast, low resistance was noted for copper (15 isolates resisted to10 µg mL^{-1}). Most of the isolates were able to grow in a wide range of carbohydrates. The genotypic analysis of the tested isolates showed that they could be regrouped into three principal clusters based on their RAPD profiles. *Acacia saligna* was principally nodulated by *Bradyrhizobium* sp. while the other species were nodulated by different rhizobial species. Both phenotypic and genotypic studies showed great rhizobial isolate's diversity. The symbiotic effectiveness tests showed that two isolates (AsF and AsAg$_1$) had high N$_2$ fixing potential on *Acacia saligna*.

Keywords: Nitrogen fixation, Nodules, Resistance to heavy metals, Symbionts, *16S rRNA*.

INTRODUCTION

High soil nutrient depletion by intensive agricultural practices leads to soil desertification, irreversible degradation of vegetation cover, and decline in soil fertility, enhanced by over-exploitation of forest areas for wood and fodder and by erosion (Hermès *et al.*, 2014). To solve these problems, it is recommended, among others, to integrate trees in the crop system (intercropping), especially in forestry managements including reforestation and pasture management programs, and also to use green manures (Fikri Benbrahim *et al.*, 2004).

Nitrogen-fixing trees such as acacias are used in arid and semi-arid areas to prevent desertification and erosion and to improve soil fertility, thanks to their ability to adapt to adverse conditions and tolerate environmental stresses such as cold, low rainfall, heavy metals, and salinity (Zahran *et al.*, 2003).

---

[1] Laboratory of Microbial Biotechnology, Faculty of Sciences and Technology, Sidi Mohamed Ben Abdellah University, P. O. Box: 2202, Imouzzer Road, Fez, Morocco.
[*] Corresponding author; e-mail: kawtar.fikribenbrahim@usmba.ac.ma
[2] Laboratory of Cellular Genomic and Molecular Investigations Techniques, Biology Department, Faculty of Sciences, Moulay Ismail University, BP 11201, Zitoune 50 000 Meknes, Morocco.
[3] Environment and Soil Microbiology Unit, Biology Department, Faculty of Sciences, Moulay Ismail University, BP 11201, Zitoune 50 000 Meknes, Morocco.

Acacias have also multipurpose uses such as wood production for *A. melanoxylon* and tanning properties for *A. mearnsii* and *A. tortillis*; soil rehabilitation and fodder source for subsistence and commercial livestock production for *A. saligna* (Fikri Benbrahim *et al*., 2014).

Moreover, the symbiotic nitrogen fixing bacteria have an important role particularly in sustainable low-input agriculture and land rehabilitation (Ben Romdhane *et al.,* 2006).

Currently, about 122 species belonging to eleven genera of Alpha- proteobacteria and three genera of Betaproteobacteria have been described as rhizobia and are distributed as follows: *Aminobacter, Azorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Microvirga, Ochrobactrum, Phylobacterium, Rhizobium, Sinorhizobium/Ensifer, Shinella* (Class of α-proteobacteria), *Burkholderia, Cupriavidus and Herbaspirillum* (Class of β-proteobacteria) (Laranjo *et al*., 2014); showing a great diversity.

This study aimed to enhance biological nitrogen fixation by *Acacia* sp., by selecting bacteria resistant to various stress factors, through phylogenetic studies using phenotypic and molecular techniques and to produce an efficient inoculum adapted to different environmental conditions.

MATERIALS AND METHODS

Root Nodule Survey

Root nodules were collected from four *Acacia* species: *Acacia melanoxylon*, *A. mearnsii, A. saligna* and *A. tortilis,* growing in different geographical sites in Morocco such as Asilah, Larache and Tangier in the North West, Fez and Meknes in the center, and Agadir in the South West (Table 1). These nodules were collected from acacia's roots according to methods developed by Vincent (1970) and Beck *et al.* (1993).

Isolation of Rhizobia from *Acacia* plant's Nodules

Healthy, unbroken, pink root fresh nodules were selected; surface sterilized by the

Table 1. Geographic origin and geodesic coordinates of the Acacia host plants from which studied *Rhizobium* were isolated.

Strain number	Strain name	*Acacia* species (A)	Geographic origin	Coordinates °N, °W		
				Latitude	Longitude	Altitude
1	As A	*A. saligna*	Asilah	35° 27' 54'' N	6° 02' 05'' W	32 m
2	As A1	*A. saligna*	Asilah	35° 27' 54'' N	6° 02' 05'' W	32 m
3	As A1r	*A. saligna*	Asilah	35° 27' 54'' N	6° 02' 05'' W	32 m
4	As Ag 1	*A. saligna*	Agadir	30° 25' 12'' N	9° 33' 53'' W	16 m
5	As Ag 2	*A. saligna*	Agadir	30° 25' 12'' N	9° 33' 53'' W	16 m
6	As A$_2$	*A. saligna*	Asilah	35° 27' 54'' N	6 02' 05'' W	32 m
7	As F	*A. saligna*	Fez	34° 02' 13'' N	4° 59' 59'' W	104 m
8	As K	*A. saligna*	Kenitra	34° 15' 39'' N	6° 34' 48'' W	25m
9	As L'2	*A. saligna*	Larache	35° 11' 35'' N	6° 09' 20'' W	44m
10	As L2r	*A. saligna*	Larache	35° 11' 35'' N	6° 09' 20'' W	44m
11	As L2	*A. saligna*	Larache	35° 11' 35'' N	6° 09' 20'' W	44m
12	Mel	*A. melanoxylon*	Meknes	33° 53' 36'' N	5° 32' 50'' W	548m
13	Mol	*A. mearnsii*	Meknes	33° 53' 36'' N	5° 32' 50'' W	548m
14	As Ag 3	*A. saligna*	Agadir	30° 25' 12'' N	9° 33' 53'' W	16 m
15	At Ag 4	*A. tortilis*	Agadir	30° 25' 12'' N	9° 33' 53'' W	16 m
16	At Ag 5	*A. tortilis*	Agadir	30° 25' 12'' N	9° 33' 53'' W	16 m
17	As S.Y	*A. saligna*	Sidi Yahia	34° 18' 23'' N	6° 18' 22'' W	19m
18	As S.Yr	*A. saligna*	Sidi Yahia	34° 18' 23'' N	6° 18' 22'' W	19m
19	As T1	*A. saligna*	Tangier	35°46' 50'' N	5°48'49'' W	80m
20	As T2	*A. saligna*	Tangier	35°46' 50'' N	5°48'49'' W	80m
21	As T3	*A. saligna*	Tangier	35°46' 50'' N	5°48'49'' W	80m

standard method (Van Berkum *et al.*, 1996), then aseptically crushed with a glass rod in some NaCl (9g/L) drops to make it slurry (Beck *et al.,* 1993)**.** Nodule's bacterial suspension obtained was streaked on Yeast-Mannitol-Agar (YMA) (Vincent, 1970) supplemented with Congo Red (CR) (0.0025% w/v). Plates were incubated at 28°C for 3 to 7 days. The culture's purity was verified by repeated streaking of a single isolate's colony into YMA medium (Jordan, 1984**)**. Finally, pure cultures were preserved in 20% glycerol at -80°C until further use according to Zahran *et al.* (2012).

Isolates Authentication

Bacterial isolates were re-inoculated to *Acacia saligna* plants in Gibson tubes, in a growth chamber at 28 /25°C (day/night), with a photoperiod of 16 hours light/8 hours dark, a light intensity of 400 lux. *Acacia saligna's* seeds were surface sterilized according to Vincent (1970), scarified with H_2SO_4 at 95% for 45 minutes, then germinated on 9% agar, at 28°C for 2 to 3 days. The seedlings were placed aseptically in Gibson tubes supplemented with a nitrogen free plants nutrient solution (Gibson, 1980**)**. Each tube was inoculated with an isolate at exponential growth conditions ($\sim 10^8$ UFC mL^{-1}). Un-inoculated plants were used as controls. Three replicates were prepared for each treatment. After six months, plants were harvested, nodules separated from roots, then rhizobia was re-isolated from nodules.

Phenotypic Characterization

Colony morphology was examined on YMA agar plates, incubated at 28°C for 3 to 7 days, based on their size, color, shape, mucosity, transparency, borders and elevation (Vincent, 1970); then, Gram stain reaction was conducted to perform a microscopic examination.

The isolates were grown on YMA medium supplemented with Bromothymol Blue (BBT) at (0.0025% w/v) (Vincent, 1970)

and incubated at 28°C for 7 to 10 days, to distinguish between acid and alkaline producing rhizobia and to have an idea about their growth rate. Indeed, change of the agar medium to yellow indicates acid production (Keneni *et al.*, 2010). Moreover, the ability to change the pH distinguishes fast-growing bacteria (yellow) from slow growing ones (dark blue color) for which medium's acidification is late (3-5 days).

Effect of Intrinsic Factors

Isolate's ability to use different carbon sources was tested on Yeast Mannitol Broth (YMB) containing one of the following sugars: fructose, galactose, glucose, sorbitol, lactose, maltose, raffinose, starch, dextran or mannose at a concentration of 1% (W/V) instead of mannitol (Bekele *et al.*, 2013; Razika *et al.*, 2012**)**.

Each sugar was tested in triplicate and used as a stock solution previously filter-sterilized (Somasegaran and Hoben, 1994). An YMB medium containing mannitol without bacterial suspension was used as control. The other tubes were inoculated with 200 μL of exponential cultures (10^8 UFC mL^{-1}) and incubated at 28°C under agitation in a rotary shaker (125 rpm) for 3 days. The use of sugars as carbon source was recorded by measuring the culture's optical density at 600 nm.

Another test was conducted to assess resistance of the studied isolates to the following heavy metals: $AsNa_2HPO_4$ (6, 60, 100, 300 μg mL^{-1}), $CuSO_4$ (10, 50, 100 μg mL^{-1}), Hg_2Cl_2 (5, 10, 20, 40, 50 μg mL^{-1}); $NiCl_2$, $6H_2O$ (20, 40, 60, 80 μg mL^{-1}); $ZnSO_4$, $7H_2O$ (10, 20, 30, 40, 50 μg mL^{-1}), and $K_2Cr_2O_7$ (25, 50, 100, 150 μg mL^{-1}).

Stock solutions of different metals were filter sterilized (Millipore 0.2 μm) and a suitable sample of each stock solution was added to a previously autoclaved YMA medium, to have the target concentrations in μg mL^{-1}. This medium was inoculated by 10 μL of rhizobia culture (10^8 UFC mL^{-1}) of each isolate and incubated at 28°C for one week (Küçük *et al.,* 2006).

Effects of Extrinsic Factors

The isolates were grown on YMA medium with different NaCl concentrations (0.5, 1.5, 2, and 5%) and incubated for one week at 28°C (Ben Romdhane *et al.,* 2006).

The isolates were grown on YMA at different pH values: 4.8, 5.8, 6.8, 8.8 and incubated for one week at 28°C (Küçük *et al.,* 2006).

The isolates were incubated at 6, 14, 22, 28, 37, 44 and 54°C for one week (Hung *et al.,* 2005).

Genotypic Characterization

The polymorphism of the tested isolates was studied by Random Amplification of Polymorphic DNA (RAPD). Total genomic DNA was extracted according to Ivanova *et al.* (2000). Then, PCR amplification of the isolated DNA was performed according to Berrada *et al.* (2012) by using a mixture of equal volumes of 1/20 dilution in distilled water of two primers pairs: 2a/2s (A) and 3a/2s (B), at a final concentration of 1 mM for each primer.

(A)　　2a:　　5'GCCCGTTTGA3'/2s: 5'GCGGGCTTGA3'

(B)　　3a:　　5'AGGCGAGTCA3'/2s: 5'GCGGGCTTGA3'

Finally, the amplification products were separated by electrophoresis in a 2% agarose gel, prepared in 1X TBE buffer (10.8 g Tris, 3.4 g boric acid, 0.92 g EDTA, qsp 1,000 mL H_2O) added with few drops of Ethidium bromide 0.5 µg mL^{-1}), by deposition of 8 µL DNA mixed with 2 µL loading buffer (50% glycerol, 50% TBE 10X, Bromophenol blue).

At the end of DNA migration, bands obtained in the agarose gel were compared.

Amplification and Sequencing of *16S rRNA* Gene

The genomic DNA was extracted using a thermal shock technique by suspending a colony of each strain in 50 µL of sterile distilled water and stirring well, cooling at -20°C for 20 minutes then transferring immediately at 95°C for 3 minutes. To ensure complete cell lysis, the operation was repeated three times. After centrifugation at 7,000 rpm for 10 minutes, the supernatant was recovered and 2 µL was used for the amplification reaction.

Amplification of *16S rRNA* Gene

Amplification of the *16S rRNA* gene, of each bacterial isolate, was performed with the universal primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and Rs16 (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991)**,** and carried out in a 20 µL total reaction volume containing template DNA (100 ng), Taq buffer (10X), MgCl$_2$ (25 mM), dNTP (1 µM), Fd1 (10 µM), Rs16 (10 µM) and Taq DNA polymerase (5 U µL^{-1}).

The PCR temperature profile used was 94°C for 5 minutes followed by 35 cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. PCR products were confirmed by size and specificity by horizontal agarose gel electrophoresis 1% (w/v) stained by ethidium bromide's aqueous solution (1 mg mL^{-1}) and photographed under UV illumination.

The *16S rRNA* gene amplification for five isolates resulted in a single band of interest revealed by electrophoresis for each isolate.

The PCR amplification products were purified to eliminate dNTP excess and primers, then, sequencing was performed. The obtained sequences were compared with the *16S RNA* gene's sequences database via the National Center for Biotechnology Information "NCBI" using the BLAST (Basic Local Alignment Search Tool). Based on sequence homology, the degree of relationship was determined.

Study of the Symbiotic Effectiveness of Different Rhizobial Isolates

Twelve bacterial isolates (AsAg$_1$, AsAg$_2$, AsA, AsF, AsK, AsL$_2$, Mel, Mol, AsAg$_3$, AsAg$_5$, AsSY and AsT) were compared by inoculating them separately to *A. saligna*

plantlets grown in Gibson tubes, maintained in a growth chamber 28°C day/25°C night, 16 hour light/8 hour dark photoperiod and 400 lux (light intensity) and filled as needed with Munns nitrogen free nutrient solution (Beck *et al.,*1993; Somasegaran and Hoben, 1994).

Each inoculation treatment was conducted in triplicate. The plantlets were cultivated for 6 months, then, shoot and root dry matter yields, nodulation parameters, aerial part's nitrogen percentage and total nitrogen content were measured. For this purpose, shoot and root of plants were separated, oven dried at 70°C for 48 hours, and weighted. Nodules were also separated from the root system, counted and weighted. The Kjeldahl method was used to determine the nitrogen percentage and aerial plant's N content.

RESULTS

Microscopic observation showed that the twenty one isolates were Gram-negative**.**

Most colonies were detected after 24 hours of incubation and presented character's differences: Seventeen isolates produced circular, convex, smooth, non-puncture, translucent, milky or creamy colonies. Four isolates produced punctate, not translucent/transparent colonies. High production of mucus was observed in most of the studied isolates.

The low consistency, translucent and gummy appearance was observed in the fast-growing strains; while the thick, viscous consistency milky hue was found in slow growing ones.

Furthermore, the majority of colonies obtained in YMA-CR were pink and/or white with a low frequency of red color.

Color change of the pH indicator to yellow was detected after 24 hours for ~45% of the tested isolates (9), after 48 hours for 14% (3), 72 hours for 23% (5), and after 96 hours for 18% (4 isolates).

All of the sugars added to the YMB medium were assimilated by the isolates (Figure 1). Optical density measurements showed that glucose, fructose, mannose and maltose provided good rhizobial development for most isolates; while a reduced growth was observed with lactose.

All the isolates tolerated low concentrations of the six heavy metals tested. But, at higher concentrations, the isolates were negatively affected (Figure 2, Table 2).

High tolerance was recorded against Arsenate (As), mercury (Hg) and Chromium (Cr). Low tolerance was recorded for Copper (Co) and for high Nickel (Ni) concentrations ($\geq$40 µg mL^{-1}).

All studied isolates had normal growth at pHs 4.8, 5.8 and 8.8 (Table 2).

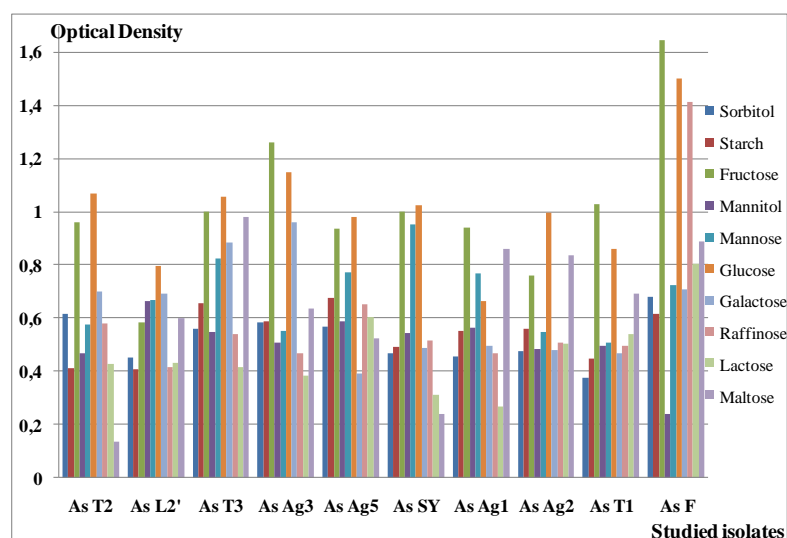**Figure 1.** Use of ten different carbohydrates by ten tested isolates.

Table 2. Phenotypic characters of the studied rhizobial isolates.

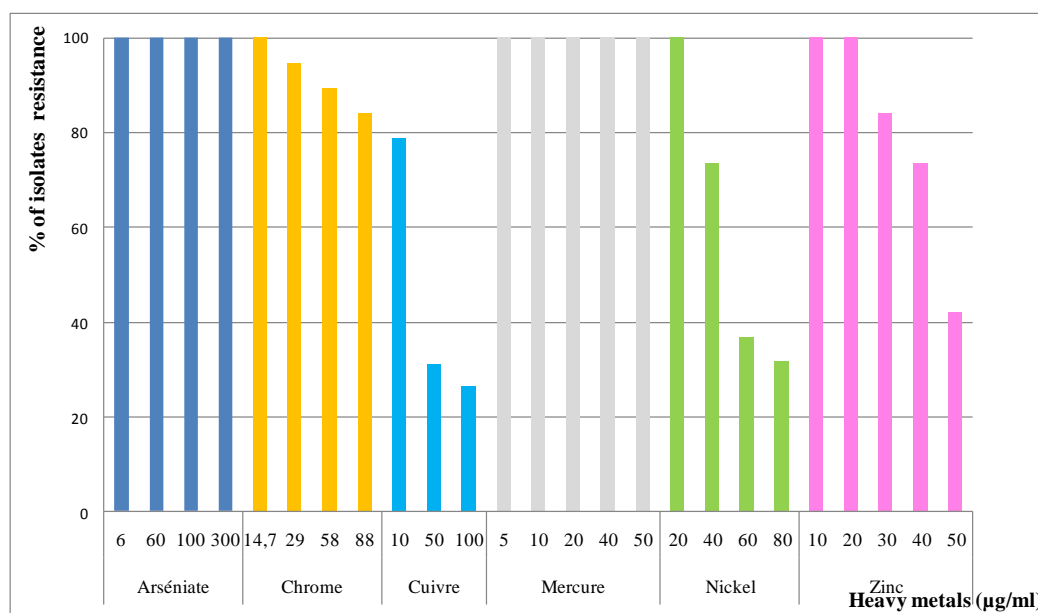| Isolates	Heavy metals																					pH				NaCl (%)					Temperature (°C)					
	Arsenate ($\mu g\ ml^{-1}$)				Chrome ($\mu g\ ml^{-1}$)				Copper ($\mu g\ ml^{-1}$)			Mercury ($\mu g\ ml^{-1}$)					Nickel ($\mu g\ ml^{-1}$)				Zinc ($\mu g\ ml^{-1}$)																	
	6	60	100	300	14.7	29	58	88	10	50	100	5	10	20	40	50	20	40	60	80	10	20	30	40	50	4.8	5.8	6.8	8.8	0.5	1	1.5	2	6	14	22	28	37	44	54
As A	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As A₁	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As A₁r	+	+	+	+	+	-	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
As Ag₁	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
As Ag₂	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
As A₂	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-
As F	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
As K	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As L'₂	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As L₂r	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As L₂	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Mel	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Mol	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
As Ag	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-
As Ag	+	+	+	+	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
As Ag	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As S.Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As S.Yr	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
As T₁	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
As T₂	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
As T₃	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

Figure 2. Tolerance of the twenty one isolates to different concentrations of heavy metals.

All isolates tolerated salt stress at 2% NaCl. Moreover, fast-growing isolates were generally more tolerant of high NaCl concentrations than slow growing ones (Table 2).

Most of the isolates were able to grow from 6 to 37°C with optimal growth temperature between 14 and 37°C (100%). However, 76.19% of the tested strains were able to grow at 6°C, 26.31% at 44°C and 14.28% at 54°C (Table 2).

A preliminary study of isolate's polymorphism by Random Amplification of Polymorphic DNA (RAPD) using two different primer pairs (2a/2s and 3a/2s)

showed that:

The isolates: AsF, $AsAg_2$, AsA_1 and AsA_2 had the same profile (Table 3).

Mol and Mel isolated from two different acacia species [*A. mearnsii* (Mol) and *A. melanoxylon* (Mel)] also had the same polymorphism profile.

Similarly, isolates $AsAg_3$ and AsSY gave the same RAPD profile (Table 3).

Based on these results, a representative isolate was selected from each group for sequencing.

Sequencing and comparison of the obtained sequences with those available in databases using the BLASTn program indicated that the

Table 3. Polymorphism obtained from two different primers couples (2a/2s and 3a/2s).

2a/2s		3a/2s	
Groups	Band's number	Groups	Band's Number
1/ As F, As Ag $_2$, As A$_1$, As T$_1$, As A$_2$	4	1/ As F, As Ag $_2$, As A$_1$, As T$_1$, As A$_2$	3
2/ Mol, As Ag $_5$, As Ag$_4$, Mel	2	2/ As SY, As Ag$_3$, Mol, Mel	7
3/ Cyc	3	3/ Cyc	2
4/ As L'$_2$	1	4/As Ag$_1$	5
5/ As Ag $_3$, As S.Y, Mel'	4	5/ As L'$_2$	3
		6/ As L$_2$, Mel'	2
		7/ As Ag $_5$	4
		8/ As Ag $_4$	2

207

isolate AsAg$_2$ can be assigned to the Genus *Bradyrhizobium* at 99% identity. Moreover, isolate AsT$_2$ can be assigned to the Genus *Brevundimonas* at 99% identity. Both isolates were sequenced in the innovation city (USMBA).

The other strains were deposited in the Coordinated Collection of Moroccan Microorganisms (CCMM) and identified as *Rhizobium radiobacter*, respectively, for Mol isolate (B79) and AsSY isolate (B82) and as *Rhizobium* sp. for AsT isolate (B83).

Results of shoot dry matter yield showed a highly significant difference between inoculated isolates (P= 0.0005) (Figure 3). Inoculation with AsF and AsAg$_1$ isolates induced a better plant growth with a shoot dry matter of 2 times and 2.5 times those obtained for plants inoculated respectively with AsK and AsL, which proved to be less efficient. Then, AsAg$_2$, AsSY and AsA$_2$ gave yields approaching twice those obtained with As L. Finally, AsT, Mol, As Ag$_3$ and Mel gave yields of about 1.5 times the yield obtained with the isolate AsL.

Roots dry matter yield also showed highly significant difference (P= 0.0146) between inoculated isolates (Table 4). AsAg$_1$ showed the highest Root Dry Weight (RDW), which was 1.35 times of that shown by AsA and AsF,

about 1.5 times of that found by AsT, AsAg$_2$, AsSY, Mol, AsK and AsAg$_1$, about 2 times that of Mel and 2.85 times that of AsL which showed the lowest *RDW*.

A highly significant difference was observed for nodulation parameters between inoculated isolates [P= 0.0004 for Nodule Number (Nod N) and P= 0.0090 for Nodule Dry Weight (NDW)]. As *F* gave the highest Nod N which was 1.4 times that obtained by As Ag$_1$; about 1.6 times that obtained by AsK, more than twice those formed by AsA$_2$ and Mol and more than 3 times that found with inoculation by AsL (Table 4).

The highest *NDW* was obtained with the isolates AsF and AsAg$_1$, for which *NDW* was 1.15 times that obtained for AsAg$_2$, about 1.35 times that of AsT, 1.5 times that of AsAg$_3$, more than 75% higher than Mel, about 100% higher than those obtained by AsAg3, AsA$_2$, Mol, and AsL, and about 170% higher than AsK.

Furthermore, nodules were observed in *A. saligna* inoculated with *Rhizobium* isolates from other species (*A. mearnsii* and *A. melanoxylon*). They were more efficient than isolates obtained from the same acacia species, including AsK and AsL.

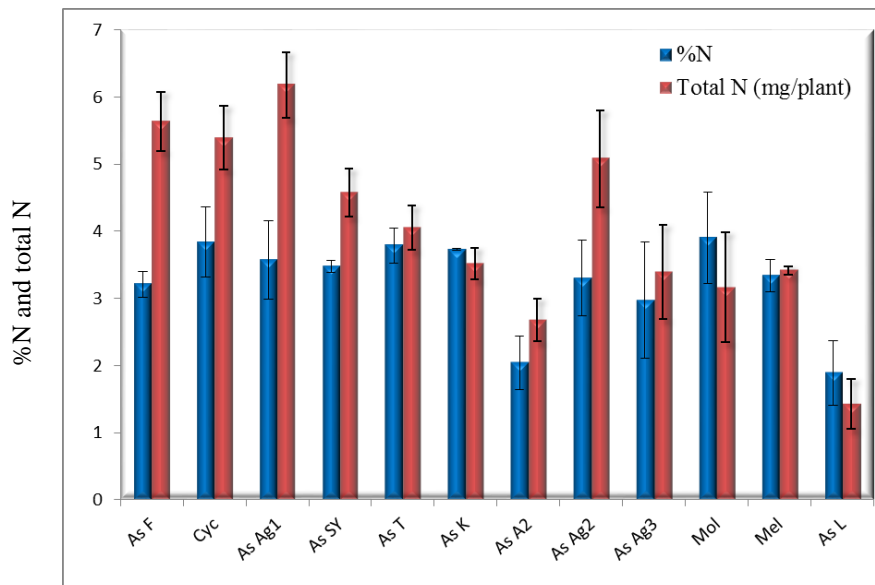The percentage of nitrogen in the aerial parts varied from 1.9% for AsL to 3.9% for AsT,

Figure 3. N percentage and shoot total N of *A. saligna* inoculated with different rhizobial tested isolates (the bars correspond to the standard deviation). *LSD* %N= 1.44%, *LSD* total N= 2.11 mg plant^{-1}.

Table 4. Growth's parameters (SDW: Shoot Dry Weight, RDW: Root Dry Weight), Nodule Number (Nod N) and Nodule Dry Weight (NDW) values of *Acacia saligna* inoculated by twelve studied isolates.

Inoculated strains	SDW (mg Plant^{-1})	RDW (mg Plant^{-1})	Nod N/Plant	NDW (mg Plant^{-1})
As F	175.33±7.75	56±13.11	36.67 ± 1.2	17 ± 2.89
Cyc	143 ± 10.69	48.33 ± 7.17	23 ± 2.65	9± 2
As Ag$_1$	178.33 ± 15.65	76 ± 9.61	25.67 + 3.28	17± 0.58
As S.Y	131 ± 8.69	50.33 ± 5.04	29.67 ± 1.86	12 ± 2.89
As T	109.67 ± 3.18	63 ± 6.03	31.33 ± 1.76	12.67± 3.28
As K	94.33 ±6.64	46.33±4.91	22.33 ±3.53	6.33±1.69
As A$_2$	138.33 ±21.26	56.33 ±7.31	26.67 ± 5.24	8.33 ±1.20
As Ag$_2$	153.33 ± 20.28	55.33± 1.76	32.33+2.02	14.66±1.45
As Ag$_3$	105.33 ±17.64	53±3.78	18± 2	11±1.52
Mol	107.66 ±3.75	46.66±5.45	24.33±4.05	7.83±0.44
Mel	103.66±9.56	41.66±9.49	20.66±5.45	9.5±1.75
As L	72±3.21	26.66±2.72	11±2.88	7.33± 2.4
Statistical test	*P=* 0.0005	*P=* 0.0146	*P=* 0.0004	*P=* 0.0090
LSD	*36.15*	*20.15*	*9.06*	*6.04*

without showing any significant difference between the different isolates at α= 5% (P= 0.2557).

While, the shoots total nitrogen content showed significant differences between the studied isolates (P= 0.0040) (Figure 3). Plants inoculated with AsAg$_1$, AsF and AsAg$_2$ accumulated more nitrogen in their aerial parts (> 5 mg plant^{-1}), followed by those inoculated by AsSY, AsT (> 4 mg plant^{-1}); Mel, AsAg$_3$, Mol and AsK (3 mg plant^{-1}< total N< 3.5 mg plant^{-1}), and finally AsL (< 1.5 mg plant^{-1}).

Moreover, good correlations were obtained for aerial dry matter yield with total nitrogen content (0.85) and nodule dry weight (0.51), respectively, and between *NDW* and total N (0.54).

DISCUSSION

In this research, we tried to identify and study the phenotypic and genotypic characteristics of rhizobial isolates nodulating *Acacia*. The tested isolates were confirmed to form nodules in *Acacia saligna.*

The morphological characteristics observed for these isolates and their colonies appearance were consistent with *Rhizobium* characteristics (Vincent, 1970). Furthermore, the very low Congo red absorption by studied isolates

enabled us to reveal their purity and to confirm their rhizobial characteristics according to Vincent (1970) and Jordan (1984).

Results of the BBT test showed that 59% of the isolates were acid producing rhizobia and could potentially be fast growing rhizobia.

The ability to use a wide range of carbohydrates as carbon source is consistent with previous works (Vincent, 1970; Jordan, 1984; Lindstrom and Lehtomaki, 1988; Somasegaran and Hoben, 1994). The reduced growth observed with lactose could be explained by the necessity of its transformation to glucose and galactose before joining the glucolysis pathway, while the other carbohydrates join it directly. Otherwise, the tested isolates do not require mannitol as the only C source, which confirms the results of Struffi *et al.* (1998) who have also shown a difference in carbohydrate assimilation degree.

The tolerance to low concentrations of heavy metals and the sensitivity induced by their high concentrations can be explained by their ability to affect microorganism's growth and physiology, especially for cadmium, lead, mercury, zinc, copper (Kopittke *et al.,* 2005). Similarly, these metals could affect the processes of nitrogen fixation and legume nodulation.

The high resistance to As, Hg, and Cr is consistent, in part, with the literature showing

that *Rhizobium* is resistant to high concentrations of As, Zn, and Hg (Carrasco *et al.*, 2005) and discordant with the same study concerning the sensitivity to Cu and resistance to Cr. This could be explained by an adaptation of the studied isolates to Cr following a probable contamination of soil from which their original nodules were collected and by a loss of the plasmid containing resistance gene to Cu after a long storage.

The isolates resistance to low Ni concentrations can be explained by its importance as an essential element for both rhizobia and host plants growth.

Furthermore, the differences observed concerning the tolerance of our isolates to heavy metals and their previously proved toxic irreversible effect on rhizobial growth and symbiotic performance (Gillet *et al.*, 1998) show the importance of determining the content of heavy metals in the soil before inoculation with any strain to ensure rhizobial resistance capacity, survival, and multiplication abilities in this soil.

Tolerance to a wide pH range is in agreement with several previous studies which state that rhizobia grow at intermediate pH values (Vincent, 1982; Jordan, 1984; Jourand *et al.*, 2004). Tolerance of rhizobia to acidic pH was shown by other authors (Graham *et al.,* 1991; Maatallah *et al.*, 2002), who indicated that calcium increases the capacity of several *Rhizobium* to survive in acidic soils. Moreover, Graham *et al.* (1994) reported that the cytoplasmic pH of acid-tolerant strains is less strongly affected by external acidity, due to the rhizobial ability to maintain intracellular pH between 7.2-7.5 even at external acidic pH.

Differences in the lipopolysaccharide's composition or cellular polyamine's accumulation have been associated with the cell growth in acid conditions (Brian *et al.*, 2007). The composition and structure of the outer membrane could also be a tolerance factor to acidic pH (Graham *et al.*, 1994). Even if soil acidity provide a serious problem during different symbiosis steps (Hungria *et al.*, 2000) and the microsymbiont is generally the most sensitive partner to pH, some rhizobial strains can tolerate acidity better than others (Graham, 2005).

The salt tolerance observed is consistent with previous findings and can be explained by an adaptation mechanism consisting of intracellular accumulation of osmoprotectors (low molecular weight organic solutes) (Miller and Wood, 1996) despite the sensitivity of rhizobia to salinity, particularly during the symbiotic process.

In addition, salt-tolerant strains can show their adaptation to osmotic stress by an ion's concentration increase and soil moisture variation during dry periods. The best tolerance observed for fast-growing isolates is consistent with previous studies (Küçük *et al.,* 2006; Berrada *et al.*, 2012). So, these isolates may be suitable candidates for using in saline soils, which are frequently observed in some irrigated areas in Morocco and contribute largely to land degradation and desertification.

Results of temperature tolerance are consistent with previous studies (Zahran *et al.*, 1994; Mohamed *et al.,* 2000; Berrada *et al.*, 2012) which showed that rhizobia are mesophilic bacteria growing between 10 and 37°C with an optimal growth temperature at 28°C. Strains able to grow at high temperatures seem to have some tolerance mechanism such as the ability to produce a thermo-tolerant 65 kb protein which is not produced during water or salt stresses (Zahran *et al.*, 1994). Moreover, the extreme temperatures do not affect bacteria in the same way in their natural habitat due to the soil components – micro-organisms interactions.

The RAPD results were shown to be very useful to differentiate very closely related isolates. In fact, the results showed that those isolates could be regrouped into three major clusters including: AsF, $AsAg_2$, AsA_1 and AsA_2; Mol and Mel; $AsAg_3$ and AsSY. For every cluster discriminated by RAPD, one isolate was randomly selected for *16S rRNA* gene sequencing.

The molecular analysis results might suggest a variability of nitrogen-fixing bacteria nodulating *Acacia saligna,* which seems to be related to the geographical origin of their host: Mol and AsSY isolated from acacias growing in the Gharb region (Northern Morocco) were found to belong to the same species *Rhizobium radiobacter*, while $AsAg_2$ from Agadir

(Southern Morocco) was affiliated with *Bradyrhizobium* sp.

Moreover, these study results showed that *Acacia* nodules from the same provenance can contain a mixture of *Rhizobium* with other soil bacteria such as the case of nodules of the plant collected in Tangier for which *Rhizobium* sp. and *Brevundimonas* sp. were identified, in different nodules. Since, *Brevundimonas* sp. have not been previously isolated from legume root nodules, it can be suggested that *Brevundimonas* represent nodule endophyte which can increase nitrogen fixing ability and efficiency of *Rhizobium*. Indeed, the genus *Brevundimonas* was proposed by reclassification of two *Pseudomonas* species **(**Segers *et al*., 1994**)** and comprises 12 species of Gram-negative, aerobic, motile bacilli. It is generally an environmental organism and infrequently causes human infections. Several species of *Brevindumonas* have been previously reported in soil such as *B. kwangchunensis*, *B. terrae* and *B. lenta* (Yoon *et al*., 2006a; 2006b; 2007). Furthermore, a previous study showed that the co-inoculation of *Bradyrhizobium japonicum* and Phosphate Solubilizing *Pseudomonas spp.* significantly increased plants growth, yield, and nodulation parameters, as well as plants N and P contents in soybean **(**Argaw, 2012**).** Another study showed a synergistic interaction between some rhizobia and *P. fluorescens,* with biocontrol activity potential, which improved plant growth and/or suppressed damping-off disease in bean plants (Samavat *et al*., 2011). However, further studies remain necessary to precisely identify the *Brevindumonas* species and to determine clearly if it represents new nitrogen fixing bacteria or a nodule endophyte.

Although AsSY, AsT$_2$ and AsAg$_2$ were isolated from the same species (*Acacia saligna)*, they belong to different *Rhizobium* species. This might be explained by changes in the nature of rhizobia nodulating the same legume living in different geographical and climatic zones previously reported in other studies (Gu *et al.,* 2007; Lafay and Burdon, 2007). Thus, the nitrogen-fixing symbiotic interaction study in temperate regions of the southeast of Australia indicated that the majority of root nodules forming bacteria belong to *Bradyrhizobium* genus; however,

some authors have suggested that, in dry areas, plant-microbe interactions are more likely dominated by faster-growing rhizobial genera such as *Rhizobium* (Hoque *et al*., 2011). Also, a previous study conducted by Lammel *et al*., (2013) showed that the roots of exotic *A. dealbata* were nodulated only by *Bradyrhizobium* sp. genotype.

Another study showed that *A. saligna* was also nodulated by *Rhizobium* variable populations in North and South of Algeria, but slow-growing strains of *Bradyrhizobium japonicum* and *B. betae* were principally found in the north, and fast-growing strains of *Sinorhizobium meliloti* and *Sinorhizobium gallicum* were found in the south (Amrani *et al*., 2010), which is discordant with our results. Anyway, among the different acacia species, *A. saligna* was found to be efficiently nodulated by soil rhizobial populations which are widespread in North Africa where the species was introduced more recently (Breton *et al*., 2008; Amrani *et al*., 2010).

In addition, species of *Rhizobium radiobacter* (previously named *Agrobacterium*) were also detected in this study. Some authors have reported that this species contains two types of agrobacteria, *Agrobacterium radiobacter* (avirulent) and *A. tumefaciens* (tumor forming) (Young *et al*., 2001; Willems, 2006). Bala and Giller (2001) found that some acacia species were able to form effective nodules with agrobacteria isolates. *Agrobacterium* strains have been isolated previously from nodules of *Acacia mellifera* and *Acacia nilotica* (Rincón-Rosales *et al*., 2009). Odee *et al*. (2002) indicated that agrobacteria were often found in association with root nodules as a co-occupant with rhizobia.

The results of isolate's symbiotic effectiveness showed not only a differential effect of the studied isolates on *Acacia* seedlings growth but also a significant diversity of rhizobia in Moroccan soils. Indeed, isolates AsAg$_3$, AsAg$_1$ and AsAg$_2$ were all from Agadir region, but showed significantly different dry matter yields; with a respective increase of 16 and 69% for AsAg$_1$ and AsAg$_2$ compared to plants inoculated by AsAg$_3$.

In addition, isolates from nodules of the same plant species (*A. saligna*) having geographical origin variability, showed yield difference: of 1.46 times than that showed by AsK (Kenitra) and 2.44 times that given by AsL (Larache).

Differences of nodule parameters suggest the existence of differences in efficiency between the studied isolates. Moreover, nodulation of *A. saligna* by isolates from other acacia species and their high efficiency confirm that it is promiscuous with broad host spectrum and a relatively moderate specificity for *Rhizobium*. This is consistent with previous findings (Dreyfus and Dommergues, 1981; Duhoux and Dommergues 1985; Sanginga *et al.,* 1987).

Furthermore, it can be concluded that there is a great diversity of *Rhizobium* in Moroccan soils which is concordant with the dry matter yields results and confirm the need to exploit this diversity, to select high performing and especially well suited fixing isolates to extreme natural conditions.

The highest dry weight yields, nodulation parameters, and accumulated total nitrogen were produced by AsF and AsAg$_1$ isolates. So, it can be concluded that their nitrogen fixation efficiency was the best.

Correlations existing between different parameters suggest that a fairly large part of the nitrogen accumulated by the studied plants comes from biological nitrogen fixation.

Finally, our results showed diversity between root-nodulating bacteria of acacias trees based on their phenotypical and genotypical characterizations. Also, most of the rhizobial isolates tested appeared to be well adapted to harsh environmental conditions. Therefore, selection of efficient isolates can be a suitable tool for acacia inoculation which will be of major interest in ecological and economic programs to increase and enhance production and nitrogen fixation in semi-arid and arid areas. In fact, as a nodulating tree legume, acacia participates in improving soil stability and fertility through nitrogen-fixing symbiosis with both fast and slow growing rhizobial species (Dreyfus and Dommergues, 1981).

REFERENCES

1. Amrani, S., Noureddine, N.E., Bhatnagar, T., Argandona, A., Joaquin, J. N. and Vargas, C. 2010. Phenotypic and Genotypic Characterization of Rhizobia Associated with *Acacia saligna* (Labill.) Wendl. In Nurseries from Algeria. *Syst. Appl. Microbiol.,* **3**: 44–51.
2. Argaw, S. 2012. Evaluation of Co-inoculation of *Bradyrhizobium japonicum* and Phosphate Solubilizing *Pseudomonas* spp. Effect on Soybean (*Glycine max* L. (Merr.)) in Assossa area. *J. Agr. Sci. Tech.,* **14**: 213-224.
3. Bala, A. and Giller, K. E. 2001. Symbiotic Specificity of Tropical Tree Rhizobia for Host Legumes. *New Phytol.,* **149**: 495–507.
4. Beck, D.P., Materon, L.A. and Fandi, F.A. 1993. *Practical Rhizobium-legume Technology Manual.* ICARDA Ed., Syria, 389 PP.
5. Bekele, H., Dechassa, N., and Argaw, A. 2013. Effects of Different Carbon and Nitrogen Sources in Broth Culture on the Growth of *Rhizobium leguminosarum* bv. phaseoli and Symbiotic Effectiveness of Haricot Bean (*Phaseoulus vulgaris* L.) in Eastern Hararghe Soils of Ethiopia. *Afr. J. Microbiol. Res.*, **7**: 3754-3761.
6. Ben Romdhane, S. B., Nasr, H., Samba-Mbaye, R. and Neyra, M. 2006. Genetic Diversity of *Acacia tortilis ssp. Raddiana* Rhizobia in Tunisia Assessed by *16S* and *16S-23S rDNA* Genes Analysis. *J. Appl. Microbiol.*, **100**: 436- 445.
7. Berrada, H., Nouioui, I., Iraqui, M. H., El Ghachtouli, N., Gtari, M. and Fikri Benbrahim, K. 2012. Phenotypic and Genotypic Characterizations of Rhizobia Isolated from Root Nodules of Multiple Legume Species Native of Fez, Morocco. *Afr. J. Microbiol. Res.,* **6(25)**: 5314-5324.
8. Breton, C., Guerin, J., Ducatillion, C., Medail, F., Kull, C. A. and Berville, A. 2008. Taming the Wild and 'Wilding' the Tame: Tree Breeding and Dispersal in Australia and the Mediterranean. *Plant Sci.*, **175(3):** 197-205.

9. Brian, W., Marcos, O. and Chandra, P. 2007. Polyamines in Bacteria: Pleiotropic Effect Yet Specific Mechanisms. *Adv. Exp. Med. Biol.,* **603(2):** 106-115.

10. Carrasco, J. A., Armario, P., Pajuelo, E., Burgos, A., Caviedes, M. A., Lopez, R., Chamber, M. A. and Palomares, A. J. 2005. Isolation and Characterization of Symbiotically Effective Rhizobium Resistant to Arsenic and Heavy Metals After the Toxic Spillat the Aznalcóllar Pyrite Mine. *Soil Biol. Biochem.,* **37(6):** 1131-1140.

11. Cummings, S. P., Gyaneshwar, P., Vinuesa, P., Farruggia, F. T., Andrews, M., Humphry, D. and James, E. K. 2009. Nodulation of *Sesbania* species by Rhizobium (Agrobacterium) Strain IRBG74 and Other Rhizobia. *Environ. Microbiol.*, **11(10):** 2510-2525.

12. Jourand, P., Giraud, E., Béna, G., Sy, A., Willems, A., Gillis, and M. De Lajudie, P. 2004. *Methylobacterium nodulans* sp. nov., for a Group of Aerobic, Facultatively Methylotrophic, Legume Root-nodule-forming and Nitrogen-fixing Bacteria. *Int. J. Syst. Evol. Microbiol.*, **54(6):** 2269-2273.

13. Dreyfus, B. L. and Dommergues, Y. R. 1981. Nodulation of *Acacia* Species by Fast and Slow Growing Tropical Strains of *Rhizobium. App. Environ. Microbiol.,* **41:** 97-99.

14. Duhoux, E. and Dommergues, Y. R. 1985. The Use of Nitrogen Fixing Trees in Forestry and Soil Reforestation in the Tropics. In: "*Biological Nitrogen Fixation in Africa*", (Eds.): Ssali, H. and Keya, S. O. Nairobi MIRCEN, Nairobi, Kenya, PP. 384-400.

15. Fikri-Benbrahim, K., Ismaili, M., Fikri Benbrahim, S. and Tribak, A. 2004. Problems of Environmental Degradation by Desertification and Deforestation: The Impact of the Phenomenon in Morocco. *Sécheresse,* **15(4):** 307-320.

16. Fikri-Benbrahim, K., Berrada, H., El Ghachtouli, N. and Ismaili, M. 2014. Acacia: Promising Nitrogen Fixing Trees for Sustainable Development in Arid and Semi-Arid Areas. *Int. J. Innov. Appl. Stud.*, **8(1):** 46-58.

17. Gibson, A. H. 1980. Methods for Legumes in Glasshouse and Controlled Environment Cabinets. In: "*Methods for Evaluating Biological Nitrogen Fixation*", (Ed.) Bergersen, F. J. Wiley, New York, PP. 139-184.

18. Gillet, E. K., Witter, E. and Grath, S. P. 1998. Toxicity of Heavy Metals to Microorganisms and Microbial Processes in Agricultural Soil: A Review. *Biol. Biochem.,* **30:** 1389-1414.

19. Graham, P. H., Draeger, K. J., Ferrey, M. L., Conroy, M. J., Hammer, B. E., Martinez, E., Aarons, S. R. and Quinto, C. 1994. Acid pH Tolerance in Strains of *Rhizobium* and *Bradyrhizobium*, and Initial Studies on the Basis for Acid Tolerance of *Rhizobium tropici* UMR 1899. *Can. J. Microbiol.*, **40:** 198-207.

20. Graham, P. H., Sadowsky, M. J., Keyser, H. H., Barnet, Y. M., Bradley, R. S., Cooper, J. E., De Ley, D. J., Jarvis, B. D. W., Roslycky, E. B., Strijdom, B. W. and Young, J. P. W. 1991. Proposed Minimal Standards for the Description of New Genera and Species of Root and Stem Nodulating Bacteria. *Int. J. Syst. Bacteriol.,* **41:** 582-587.

21. Graham, P. H. 2005. Biological Dinitrogen Fixation: Symbiotic. In: Sylvia, D.M., Fuhrman J, Hartel P. G., Zuberer D. A. eds. "*Principles and Applications of Soil Microbiology*", 2nd Edn. Prentice Hall, Upper Saddle River NJ, pp 405-432.

22. Gu, J., Wang, E. T. and Chen, X. W. 2007. Genetic Diversity of Rhizobia Associated with *Desmodium* Species Grown in China. *Lett. Appl. Microbiol.*, **44:** 286–292.

23. Hermès, M. N., Jacques, B. K., David, B. M., John, B. B., Cikuru, M. and Dieudonné, M. B. 2014. Contribution à la Caractérisation des Evénements Dommageables du Sol, ses Problèmes et ses Perspectives à Irhambi/Katana. *Int. J. Innov. Appl. Stud.,* **7(4):** 1617-1624.

24. Hoque, M. S., Broadhurst, L. M. and Thrall, P. H. 2011. Genetic Characterization of Root-nodule Bacteria Associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across South-eastern Australia. *Int. J. Syst. Evol. Microbiol.*, **61(2):** 299-309.

25. Hung, M. H., Bhagwath, A. A., Shen, F. T., Devasya, R. P. and Young, C. C. 2005. Indigenous Rhizobia Associated with Native Shrubby Legumes in Taiwan. *Pedobiol.,* **49(6):** 577-584.

26. Hungria, M. and Vargas, M. A. 2000. Environmental Factors Affecting N_2 Fixation in Grain Legumes in the Tropics, with an Emphasis on Brazil. *Field Crop. Res.,* **65:** 151-164.

27. Ivanova, E. P., Chun, J., Romanenko, L. A., Matte, M. E., Mikhailov, V. V., Frolova, G. M., Huq, A. and Colwell, R. R. 2000. Reclassification of *Alteromonas distincta* Romamenko *et al.* 1995 as

Pseudoalteromonas distincta comb. nov. *Int. J. Syst. Evol. Microbiol.,* **50**: 141–144.

28. Jordan, D. C. 1984. Family III Rhizobiaceae Conn. 1938-254. In: *"Bergey's Mannual of Systematic Bacteriology"*, (Eds.): Krieg, N. R and Holt, J. G. The Williams and Wilkins Co., Baltimore, **1:** 235-244.

29. Keneni, A., Prabu, P. C. and Assefa, F. 2010. Characterization of Acid and Salt Tolerant Rhizobial Strains Isolated from Faba Bean Fields of Wollo, Northern Ethiopia. *J. Agr. Sci. Tech.,* **12:** 365-376.

30. Kopittke, P. M., Dart, P. J. and Menzies, N. W. 2005. Toxic Effects of Low Concentrations of Cu on Nodulation of Cowpea *Vigna unguiculata. Plant Soil,* **279:** 287-296.

31. Küçük, Ç., Kıvanç, M. and Kınacı, E. 2006. Characterization of *Rhizobium* sp. Isolated from Bean. *Turk. J. Biol.,* **30:** 127-132.

32. Lafay, B. and Burdon, J. J. 2007. Molecular Diversity of Legume Root-nodule Bacteria in Kakadu National Park, Northern Territory, Australia. *PloSone*, **2:** 277.

33. Lammel, D. R., Cruz, L. M., Carrer, H. and Cardoso, E. J. 2013. Diversity and Symbiotic Effectiveness of Beta-rhizobia Isolated from Sub-tropical Legumes of a Brazilian Araucaria Forest. *World J. Microbiol. Biot.*, **29 (12):** 2335-42. doi: 10.1007/s11274-013-1400-7.

34. Laranjo, M., Alexandre, A. and S. Oliveira. 2014. Legume Growth–promoting Rhizobia: An Overview on the *Mesorhizobium* genus. *Microbiol. Res.*, **169(1):** 2-17.

35. Lindstrom, K. and Lehtomaki, S. 1988. Metabolic Properties, Maximum Growth Temperature and Phage Sensivity of *Rhizobium* sp. (Galega) Compared with Other Fast-growing Rhizobia. *FEMS Microbiol. Lett.,* **50**: 277-287.

36. Maâtallah, J., Berraho, E., Sanjuan, J. and Lluch, C. 2002. Phenotypic Characterization of Rhizobia Isolated from Chickpea (*Cicer arietinum* L.) Growing in Moroccan Soils. *J. Agron.,* **22:** 321-329.

37. Miller, K. J. and Wood, J. M. 1996. Osmoadaptation by Rhizosphere Bacteria. *Annu. Rev. Microbiol.,* **50:** 101-136.

38. Mohamed, S. H., Smouni, A., Neyra, M., Kharchat, D. and Maltouf, A. F. 2000. Phenotypic Characteristics of Root-nodulating Bacteria Isolated from Libyan *Acacia* spp. *Plant Soil*, **224**: 171-183.

39. Odee, D. W., Haukka, K., Mcinroy, S. G., Sprent, J. I., Sutherland, J. M. and Young, J. P. W. 2002. Genetic and Symbiotic Characterization of Rhizobia Isolated from Tree and Herbaceous Legumes Grown in Soils from Ecologically Diverse Sites in Kenya. *Soil Biol. Biochem.,* **34:** 801-811.

40. Razika, G., Amira, B., Yacine, B. and Ammar, B. 2012. Influence of Carbon Source on the Production of Exopolysaccharide by *Rhizobium sullae* on the Nodultion of *Headysarum coronarium* L. Legume. *Afr. J. Microbiol. Res.*, **6:** 5940-5946.

41. Rincón-Rosales, R., Lloret, L., Ponce, E. and Martínez-Romero, E. 2009. Rhizobia with Different Symbiotic Efficiencies Nodulate *Acaciella angustissima* in Mexico, Including *Sinorhizobium chiapanecum* sp. nov. which Has Common Symbiotic Genes with *Sinorhizobium mexicanum. FEMS Microbiol. Ecol.*, **67(1):** 103-117.

42. Samavat, S., Samavat, S., Besharati, H., and Behboudi, K. 2011. Interactions of Rhizobia Cultural Filtrates with *Pseudomonas fluorescens* on Bean Damping-off Control. *J. Agr. Sci. Tech.* **13:** 965-976.

43. Sanginga, N., Mulongoy, K. and Ayanaba, A. 1987. Evaluation of Indigenous Strains of *Rhizobium* for *Leucaena* de Wit in Nigeria. In: Les Arbres Fixateurs d'Azote et L'Amélioration Biologique de la Fertilité du Sol. *Colloques et Séminaires,* ORSTOM, Paris, PP. 416-436.

44. Segers, P., Vancanneyt, M., Pot, B., Torck, U., Hoste, B., Dewettink, D., Falsen, E., Kersters, K. and De Vos, P. 1994. Classification of *Pseudomonas diminuta* Leifson and Hugh 1954 and *Pseudomonas vesicularis* Büsing, Döll, and Freytag 1953 in *Brevundimonas* gen. nov. as *Brevundimonas diminuta* comb. nov. and *Brevundimonas vesicularis* comb. nov., Respectively. *Int. J. Syst. Bacteriol.* **44(3):** 499-510.

45. Somasegaran, P. and Hoben, H. J. 1994. *Handbook for Rhizobia: Methods in Legume-Rhizobia Technology.* Springer-Verlag, New York, 450 PP.

46. Struffi, P., Corich, V., Giacomini, A., Benguedouar, A., Squartini, A., Casella, S. and Nuti, M. P. 1998. Metabolic Properties, Stress Tolerance and Macromolecular Profiles of Rhizobia Nodulating *Hedysarum coronarium. J. Appl. Microbiol.,* **84:** 81-89.

47. Van Berkum, P., Beyene, D. and Eardly, B. D. 1996. Phylogenetic Relationships among *Rhizobium* Species Nodulating the Common Bean (*Phaseolus vulgaris* L.). *Int. J. Syst. Bacteriol.*, **46:** 240-244.

48. Vincent, J. M. 1970. *A Manual for Practical Study of Root Nodule Bacteria*. IBP Handbook 15, Blackwell Scientific Publications, Oxford.

49. Vincent, Y. H. 1982. *Nitrogen Fixation in Legumes*. Ed. Academic/Res., Australia, PP. 1-11

50. Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.,* **17:** 697–703.

51. Willems, A. 2006. The taxonomy of rhizobia: an overview. *Plant Soil,* **287:** 3–14.

52. Yoon, J.H., Kang, S.J., Lee, J.S. and T.K. 2006b. *Brevundimonas terrae* sp. nov., Isolated from an Alkaline Soil in Korea. *Int. J. Syst. Evol. Microbiol.,* **56:** 2915–2919.

53. Yoon, J. H., Kang, S. J., Oh, H. W., Lee, J. S. and Oh, T. K. 2006a. *Brevundimonas kwangchunensis* sp. nov., Isolated from an Alkaline Soil in Korea. *Int. J. Syst. Evol. Microbiol.,* **56**: 613–617.

54. Yoon, J. H., Kang, S.J., Lee, J. S., Oh, H. W. and Oh, T. K. 2007. *Brevundimonas lenta* sp. nov., Isolated from Soil. *Int. J. Syst. Evol. Microbiol.,* **57:** 2236–2240.

55. Young, J. M., Kuykendall, L. D., Martinez-Romero, E., Kerr, A. and Sawada, H. 2001. A Revision of *Rhizobium* Frank 1889, with an Emended Description of the Genus, and the Inclusion of all Species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et 1998 New Combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis*. *Int. J. Syst. Evol. Microbiol.,* **51(1):** 89-103.

56. Zahran, H. H., Rasanen, L. A., Karsisto, M. and Lindström, K. 1994. Alteration of Lipopolysaccharide and Protein Profiles in SDS-PAGE of Rhizobia by Osmotic and Heat Stress. *World J. Microb. Biot.,* **10:** 100-105.

57. Zahran, H. H., Abdel-Fattah, M., Ahmad, M. S. and Zaki, A. Y. 2003. Polyphasic Taxonomy of Symbiotic Rhizobia from Wild Leguminous Plants Growing in Egypt. *Folia Microbiol.,* **48:** 510-520.

58. Zahran, H. H., Abdel-Fattah, M., Yasser, M. M., Mahmoud, A. M. and Bedmar, E. J. 2012. Diversity and Environmental Stress Responses of Rhizobial Bacteria from Egyptian Grain Legumes. *Aust. J. Basic Appl. Sci.,* **6(10):** 571-583.

تنوع فنوتیپی و ژنوتیپی و کارآیی همزیستی ریزوبیوم های جدا شده از گونه اقاقیای کاشته شده در مراکش

ک. فکری بنبراهیم، م. شرایبی، س. لبرازی، م. مومنی، و م. اسماعیلی

چکیده

در این پژوهش، تنوع فنوتیپی و ژنوتیپی و کارآیی همزیستی ۲۱ ریزوبیوم جدا شده از چهار گونه اقاقیا (شامل *Acacia melanoxylon، A. mearnsii، A. saligna* و *Acacia tortillis*) که در مراکش کاشت می شود ارزیابی شد. به این منظور، رشد این جدایه های در محیط های مختلف رشد (YMA، YMA-BBT، YMA-RC)، شکل کلنی آنها، مقاومت به عوامل تنش زا(درجه حرارت، اسیدیته یا pH، غلظت نمک، عناصر سنگین گوناگون) و نیز منابع کربنی مختلف به عنوان نشانگر های فنوتیپی استفاده شدند. برای شناسایی ویژگی های ژنتیکی از تکثیر پلی مورفیکی DNA (RAPD) و توالی ژنی 16S rRNA استفاده شد. کار آیی همزیستی جدایه های ریزوبیوم ها روی اقاقیا با آزمون گره بندی روی گیاه (nodulation) در شرایط کنترل شده ارزیابی شد.نتایج نشان داد که ۱۲ جدایه

رشد سریع داشتند و همه ۲۱ جدایه قادر به تحمل اسیدیته بین ۴/۸ تا ۸/۸ و درجه حرارت های ۱۴، ۲۲

۲۸، و ۳۷ درجه سانتی گراد بودند در حالیکه فقط ۵ جدایه در ۴۴ درجه سانتی گراد و سه تای آنها در

۵۵ درجه سانتی گراد توانایی رشد نشان دادند. بیشترین مقاومت به عناصر سنگین برای آرسنات، جیوه و

کروم ثبت شد ولی در برابر مس کمترین مقاومت مشاهده شد(۱۵ جدایه تا غلظت ۱۰ میکروگرم در

میلی لیتر مقاومت کردند). همچنین، بیشتر جدایه ها قادر بودند که در طیف گسترده ای از کربوهیدرات

ها رشد کنند. نیز، تجزیه ژنو تیپی جدایه های آزمون شده حاکی از آن بود که می توان آن ها را بر

مبنای پروفیل راپید (RAPD) در سه خوشه اصلی دسته بندی کرد. گره بندی روی *Acacia*

saligna عمدتا به وسیله*Bradyrhizobium* sp انجام می شد در حالیکه گره بندی روی گونه های

دیگر توسط گونه های مختلف ریزوبیوم صورت می گرفت. هر دو مطالعات ژنوتیپی و فنوتیپی تنوع

زیادی در جدایه های ریزوبیوم ها نشان داد. نیز، در آزمون کارآیی همزیستی آشکار شد که دو جدایه

As F و As Ag₁ توانایی زیادی برای تثبیت نیتروژن روی *Acacia saligna* دارند.

216