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Preliminary characterization of slow growing rhizobial strains isolated from *Retama monosperma* (L.) Boiss. root nodules from Northwest coast of Algeria

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In this paper, we did some preliminary characterization of six slow growing rhizobial strains, isolated from *Retama monosperma* (L.) Boiss. root nodules sampled from 3 sites along the coast of Oran (CapeFalcon, Bousfer and MersElHadjadj) in Northwestern Algeria. Results of this study showed that all strains had a very slow growth rate in yeast malt (YM) agar medium, forming colonies less than 1 mm in diameter after seven days incubation. Catalase, oxidase and urease were positive, 3-ketolactose was negative. All strains resisted alkaline pH up to 9 and salinity equal to 2% (w/v) NaCl. They grew from 14 to 30°C with optimum growth at 28°C which is related to their place of presence (marine sand dunes Mediterranean climate). The strains used are, on the one hand D-glucose and D-galactose as carbon source and on the other hand L-leucine, L-tyrosine as nitrogen source. The nodulation tests performed pointed out that the total dry weight of the plant could go up to 1.7 g per plant and the maximum number of nodules was equal to 5.6 nodules per plant for the strain RMB1 from Bousfer site. The intrinsic antibiotic resistance level in all strains was tested against nine antibiotics; they revealed a variability of resistance against spectinomycin (10 $\mu g ml^{-1}$), erythromycin (15 $\mu g ml^{-1}$, rifampicin (30 $\mu g ml^{-1}$), streptomycin (500 $\mu g ml^{-1}$), kanamycin (30 $\mu g ml^{-1}$), naldixic acid (30 $\mu g ml^{-1}$), penicillin (6 $\mu g ml^{-1}$) and tetracycline (30 $\mu g ml^{-1}$) except for gentamycin (500 $\mu g ml^{-1}$). The results of electrophoresis on 0.7% agarose gel showed the presence of chromosomal DNA and absence of plasmids.

Key words: *Retama monosperma* (L.) Boiss., *Bradyrhizobium*, symbiosis, root nodules, nitrogen fixation, endemic legumes, Algeria.

INTRODUCTION

The plant family Leguminosae (Fabaceae) is the third largest family in the Angiosperms; leguminous shrubs are dominant in a range of arid and extreme ecosystems where they constitute a stable microhabitat facilitating herbaceous vegetation establishment and growth (Nilsen, 1992; Pugnaire et al., 1996a; Rodríguez-Echeverría et

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al., 2012). These plant species are of ecological interest in dune stabilization, soil fixation, and revegetation of semiarid and arid ecosystems (Herrera et al., 1993; Caravaca et al., 2002; Rodríguez-Echeverría and Perez-Fernandez, 2005). *Retama* species (subfamily Faboideae, tribe Genisteae) (Käss and Wink, 1997; Pardo et al., 2004) are distributed in Northern Africa, Canary Island, Southern Europe and Western Asia (Zohary, 1959). They were first classified in *Spartium* L. then in *Retama* Boiss (Lopez et al., 1998). They correspond to perennial herbaceous plants, 3 to 4 m high shrubs, which have an important ecological role in the formation of "fertility islands" where the growth of numerous annual and woody species are favoured (Pugnaire et al., 1996b). Thanks to their capacity to fix N_2 in symbiotic association with legume nodulating bacteria (LNB) (Valladares et al., 2002) and to its deep root system, which is functional at more than 20 m depth providing access to deep nutrient and water sources (Haase et al., 1996). *Retama* could be useful as a tool for restoration, providing long term plantations without artificial aid and allowing primary succession (Singh et al., 2002).

The ecological and economic importance of nitrogen fixation has justly earned research attention for the *Rhizobium*-legume symbiosis; based on previous studies on the association between rhizobia and *Retama* from different Mediterranean areas (Spain, Algeria and Morocco). It has been noticed that *Bradyrhizobium* is the dominant genus of symbiotic nitrogen-fixing bacteria associated with *Retama* species: *Retama monosperma* (L.) Boiss., *Retamaraetam* Webb and Berthel., *Retama sphaerocarpa* Boiss. (Rodríguez-Echeverría et al., 2003, 2014; Ruiz-Díez et al., 2009; Boulila et al., 2009; Guerrouj et al., 2013). Recently, the novel *B. retamae* species, which groups with *B. elkanii* and *B. pachyrhizi* and related *B. lablabi* and *B. jicamae* type strains included in *Bradyrhizobium* group II (Menna et al., 2009), has been isolated from *R. sphaerocarpa* and *R. monosperma* in Morocco and Spain (Guerrouj et al., 2013).

Although the wild geographical spread of the endemic species of *R. monosperma* (known as *Genista monosperma* or bridal broom) in some regions of the Mediterranean Basin (Northern Algerian, Moroccan coasts and the South West coast of Spain), its important ecological role in the fixation and stabilization of sand dunes, its resistance and adaptation to the harsh environmental conditions that characterize the dune habitat, such as scarcity of water and nutrients, substrate mobility, incidence of salt spray, high air and soil temperatures and intense light (Ranwell, 1972; Carter, 1988; Heslenfeld et al., 2004; Martínez et al., 2004; Maun, 2009).

Today and over the whole Oran coast, this species is under wild aggressive attacks caused by the advance of buildings, roads and other infrastructure constructions due to economic development without any protective measures for the vegetal or sprouts *R. monosperma* (L.) Boiss.

In order to protect and preserve this endemic plant for its numerous beneficial interests as fixing the atmospheric nitrogen (N_2) due to its symbiotic life with the bacteria of the *Rhizobiaceae* family, we took up an extensive study under the form of a synthesis (Hannane et al., 2014) under the theme "*Retama*-rhizobia symbiosis studies in some countries of the Mediterranean Basin", which led us to undertake preliminary microbiological studies that consisted in identifying and characterizing of isolates of root nodules of *R. monosperma* subsp. bovei var. oranensis (Maire) collected from different sites of Oran coast located in the North West of Algeria.

MATERIALS AND METHODS

Collection of root nodules

Root nodules from *R. monosperma* growing in Oran Northwestern Algeria were collected from three sites. Fragments of roots with attached nodules were excised with scalpel, and transported in distilled water in plastic vials to the laboratory. Thirteen bold, pink, active nodules were collected randomly and placed in screw cap vials (10 ml) containing calcium carbonate as desiccant below cotton at the bottom and stored at 4°C until isolation.

Isolation, purification and cultivation of bacteria

The root nodules (Figure 1) were surface sterilized by washing for 30 s with 95% ethanol, immersed in 5% sodium hypochlorite for 3 min and finally were washed six times by sterile double distilled water. Following sterilization, nodules were crushed aseptically in 1 ml sterile double distilled water, the nodules extract were streaked on surface of yeast extract mannitol agar YMA plates supplemented with 0.025 g/l of Congo red and finally were incubated at 28°C for 12 days (Hahn, 1966; Somasegaran and Hoben, 1994).

Single colonies were picked up and checked for purity by repeated streaking and by microscopic examination of the cellular morphology. Gram staining reaction was carried out by using a loopful of pure culture grown on YM medium and stained as per the standard Gram's procedure (Somasegaran and Hoben, 1994). Cell shape and dimensions were determined by microscopy. Isolates were designated according to the plant species from which they were isolated (RM isolated from *R. monosperma*) and stored as frozen stock cultures in YM medium containing 15% glycerol at -80°C (Manniatis et al., 1982).

Authentification and Symbiotic effectiveness of the isolates

Seed sterilization and growth conditions

The soil was analyzed according to Page et al. (1982). Location, soil characteristics and climatic data of *R. monosperma* fields used for the isolation of rhizobia are presented in Table 1. The nodulation ability of the isolates was checked by inoculating their host plant of origin as described by Vincent (1970). Seeds of *R. monosperma* were scarified by immersion in concentrated sulfuric acid (H_2SO_4 98%) for 4 h (Bouredja et al., 2011). After scarification, seeds were disinfected by soaking in 1% sodium hypochlorite for 3 min, and rinsed seven times with sterile distilled water. They were sown in

Figure 1. *Retama monosperma* plant (a) and root nodules (b, c and d). Pictures taken in Cape falcon site Oran Algeria, December 2011.

sterile Petri dishes containing 1.5% water agar [Agar type A6686 purchased from Sigma-Aldrich Co. (St. Louis, MO)], and incubated at 25°C. After seed germination non contaminated seedlings were transplanted to grow hydroponically in modified Gibson system (Vincent, 1970). The N-free nutrient solution medium was used as described by FAO (1983).

Seedlings inoculation

The nodule isolates were grown for 7 days in YM medium at 28°C for 150 rpm on a rotary shaker procured from Sigma-Aldrich Co. (St. Louis, MO). Cells were harvested by centrifugation at 10000 rpm at 4°C for 10 min and washed twice with sterile NaCl solution 0.85% (w/v). Growth was monitored by optical density measurement of the cell suspension at 600 nm using UV/Visible Spectrophotometer Type JENWAY 6305. Inoculation was performed after transfer with 1 ml of the appropriate isolate of rhizobial broth culture containing approximately 10^9 cells/ml. Controls, not inoculated, were included.

Plants were grown under 18 h light period (150- 250 iEm^2/sec^2) and a 6 h darkness at 23°C. Five replications were used for each isolate. After growth for 4 and 6 months of planting, symbiotic efficiency of the isolates was determined by measuring the shoots and total dry weight of test plants (Buttery et al., 1997; Somasegaran and Hoben, 1994; Wynne et al., 1980).

Physiological and biochemical characterization

Determination of NaCl tolerance

The salt tolerance of rhizobia was tested on YMA plates containing 2 and 3% (w/v) NaCl. The plates were inoculated with 10^8 cells/ml, and the growth was scored after 12 days at 28°C (Moschetti et al., 2005). Tests were done in triplicate.

pH tolerance

Tolerance to pH was determined by inoculating 10^8 cells/ml from exponentially growing YM liquid cultures into tubes containing 10 ml portions of YM liquid media which were adjusted to pH 4, 4.5, 5, 5.5, 6, 6.5, 6.8, 7, 7.5, 8, 8.5, 9, 9.5 and 10 by using 1 N HCl or

Table 1. Location, soil characteristics and climatic data of *R. monosperma* fields used for the isolation of rhizobia.

Host plant	Geographical origin	Altitude (m)	Strain name	Soil characteristics			Ecosystem and climatic data*
				Soil type	Soil pH	EC	
Retama monosperma	Cap falcon 35° 44 940 N 00° 47 250 W	38	RMC	Sandy	8.28	0.25	Ecosystem : Coastal dune Mediterranean type climate: Sub-humid
Retama monosperma	Bousfer(Hai Mardjadjou) 35° 43 898 N 00° 50 509 W	21	RMB	Sandy loam	8.5	0.21	Average temp. of warmest month 23.8 C°
Retama monosperma	Mers El Hadjadj 35° 47 112 N 00° 07 721 W	8	RMM	Sandy	8.34	0.23	Average temp. of coldest month 13.5 C° Annual rainfall 30.9 cm

E.C, Electrical conductivity (mmhos cm^{-1}at 25ºC); pH: the hydrogen ion concentration (soil water susp. 1:2.5). * Data from 2010 through 2011, obtained from OMN (office de la météorologie d'Oran).

NaOH. The tubes were incubated at 28°C for 12 days/ 150 rpm on a rotatory shaker and scored for growth. Tests were performed in triplicate and the Do measured at 680 nm.

Maximum growth temperature

Temperature tolerance of rhizobia was examined by inoculating rhizobia on YMA plates and incubating at 4, 14, 28 and 37°C (Lindstrom and Lehtomaiki, 1988). Before incubation, isolates were grown on YMB to 10^8 cells/ml. When test plates were used, inoculation was performed with 30 µl of these cultures. After 12 days, plates were examined for bacterial growth.

Tests for enzymatic activity

Production of 3-ketolactose was determined by the method of Bernaerts and De Ley (1963). The principle of this test is based on the ability of *Agrobacterium*, a common contaminant of *Rhizobium* to produce ketolactase enzyme which converts lactose to ketolactose. Ketolactose would be detected by Benedict's reagent. *Rhizobium* cultures were streaked on lactose medium in the centre. After incubation for 7 days at 28 ± 2°C, 5 ml of Benedict's reagent was poured in each Petri plate and kept at room temperature for 1-1½ h. *Agrobacterium* growth was surrounded by yellowish zone of Cu_2O, whereas no such yellow zone was observed around the growth of rhizobia. Acid and alkali production was determined in YMA medium with BTB bromothymol blue indicator (0.0025% w/v). Urease activity was determined on urea agar slants (Christensen, 1946) that were incubated for 7 days at 28°C, and 2% (w/v) urea aqueous solution was aseptically added to the basal medium after filtration through Whatman filter paper containing 0.012% phenol red as pH indicator. Change in color of medium from yellow to pink was taken as positive test for urease production (Lindstrom and Lehtomaki, 1988). Oxidase was determined by using the method of Kovaks (Kovaks, 1956). Catalase was determined by the method of Graham and Parker (1964). After incubation for 7 days, hydrogen peroxide solution 3% (H_2O_2) was added over the culture.

Appearance of effervescence within 20 s indicates positive catalase activity.

Carbon and nitrogen sources utilization

The use of different carbon and nitrogen sources was tested according to Amarger et al. (1997) and Wang et al. (1998) using basal medium. For carbohydrate utilization tests, the basal medium used was that of Bishop et al. (1976). The same basal medium was used to determine the use of amino acids as nitrogen sources at final concentrations of 10 mM/l. The medium was solidified with purified agar (Agar, Purified type A7049 purchased from Sigma–Aldrich Co. (St. Louis, MO). All carbohydrates (d-glucose, dextrin, d-galactose, l-fructose and sucrose) were sterilized by filtration with pore size of 0.22 µm as indicated in Amarger et al. (1997). Before they were added to cool melted agar medium, filter sterilized carbon sources were added to a final concentration of 0.1% (w/v). Inocula were prepared by removing cells from YMA slants with a cotton swab and suspending the cells to a density of approximately 10^8 cells/ml in sterile distilled water. A 10 fold dilution of each cell suspension was added to the wells of a multiple inoculator plate and inoculated onto the surfaces of carbohydrate containing agar plates. Bishop agar plates without carbohydrate served as controls. Duplicate plates of each carbohydrate were incubated at 28°C for 7 days and scored for growth.

All amino acids (L-leucine, L-arginine, L-tyrosine and L-proline) were prepared as filter sterilized stock solutions. When substrates were tested as nitrogen sources, they were added to the medium from which ammonium sulfate had been omitted and to which mannitol had been added as a carbon source at a concentration of 0.1% (w/v). Basal medium containing ammonium sulfate (0.01%. w/v), as a sole nitrogen source, was used as a positive control.

Intrinsic antibiotic resistance (IAR)

The intrinsic resistance of different bradyrhizobial isolates to nine antibiotics: spectinomycin (10 μgml^{-1}), erythromycine (15 μgml^{-1}), rifampicin (30 μgml^{-1}), gentamycin (500 μgml^{-1}), streptomycin (500 μgml^{-1}), kanamycin (30 μgml^{-1}), naldixic acid (30 μgml^{-1}), penicillin (6 μgml^{-1}) and tetracycline (30 μgml^{-1}) was determined using antibiotic discs according to the method described by Josey et al. (1979). Isolates were cultivated in YM liquid medium for 7 days at 28°C on a rotary shaker with 150 rpm. For each isolate, 1 ml of

liquid culture that contain about 10^9 cells/ml was added to 25 ml of melted and cooled (50°C) YM agar medium, and then poured in sterilized Petri dishes. Sterile antibiotic discs procured from Bio-Rad were placed on the surface of agar, and then plates were incubated at 28°C for 12 days and the susceptibility to antibiotics was recorded as a positive or negative result. There were three replicates of each test scored as: +, vigorous growth; ±, weak growth; or -, no growth. Resistance class was determined by the extent of growth on each antibiotic.

Plasmidic and chromosomic DNA extraction

Each nodule isolate was grown in 10 ml of YM broth to mid-exponential phase at 28°C with shaking on the orbital shaker purchased from Sigma-Aldrich Co. (St. Louis, MO) (Ausubel et al., 1989). The cells were subsequently used as a 1% inoculant for 25 ml of YM broth (YM minus mannitol) without shaking at 28°C to avoid an excess of polysaccharides (CASSE et al., 1979). After growth, cells were centrifuged at 8000 rpm for 10 min at 4°C using a compact centrifuges purchased from Sigma-Aldrich Co. (St. Louis, MO). The resulting pellet was resuspended in 1 ml of TE buffer pH 8 (50 mM Tris, 20 mM EDTA) (CASSE et al., 1979) and again centrifuged for 10 min at 8000 rpm at 4°C. After the tube was thoroughly drained, the resulting pellet was vigorously resuspended in 100 µl of 25% sucrose in 10 mM tris hydroxyl-methyl aminomethane (Tris), 1 mM ethylene diamine tetra-acetate (EDTA), pH 8.0 and placed on ice. A 50 µl sample of cell suspension was transferred to a 1.5 ml micro centrifuge tube on ice and mixed with 20 µl lysozyme mixture containing (1 mg/ml lysozyme from hen egg white procured from Sigma-Aldrich Co. (St. Louis, MO), in Tris-borate buffer (pH 8.2; 89 mM Tris base, 12.5 mM disodium EDTA, and 8.9 mM boric acid) for 2 to 5 min at room temperature.

Thirty micro liters of the SDS mixture: 0.2% SDS sodium dodecyl sulfate in Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid) was carefully layered on top of the bacteria lysozyme gently mixed with a toothpick, moving it from side to side (which was mixed by brief agitation). Complete mixing was avoided and the two layers were still distinguishable (Eckhardt, 1978).

After cooling to room temperature for 7 min, the lysate was neutralized with 10 µl of 2 M Tris pH 7.0, and extracted twice with two volumes of phenol: $CHCl_3$ the frozen phenol was liquefied at 50°C and mixed with chloroform (v/v) in TE buffer (50 mM Tris, 10 mM Na_2EDTA, pH 8), and twice with $CHCl_3$ (Barry et al., 1984).

The two phases were mixed by stirring at 300 rpm for 10 s and further stirred for 2 min at 100 rpm (CASSE et al., 1979). Following centrifugation (12000 rpm, 15 min), the clear aqueous phase was brought to 0.3 M sodium acetate (final concentration), and twice the volume of cold (-20°C) 95% ethanol was added to precipitate the DNA (Meyers et al., 1976). The tube was kept at -20°C for 2 to 3 h (or overnight if more convenient). The precipitated DNA was recovered by centrifugation at 12000 rpm at -10°C for 20 min. The ethanol was thoroughly drained from the tube, and the DNA was suspended in 100 µl of TES buffer (30 mM Tris, 5mM EDTA, 50 mM NaCl, pH 8.0). The DNA sample was analyzed immediately by agarose gel electrophoresis or stored at -20°C until ready for use (CASSE et al., 1979).

Agarose gel electrophoresis of DNA

The integrity of DNA was analyzed by electrophoresis with 0.7% agarose gels [Agarose Type I-A: Low EEO purchased from Sigma–Aldrich Co. (St. Louis, MO)] and comparison with known amounts of phage lambda DNA [λ DNA-HindIII Digest purchased from Sigma-Aldrich Co. (St. Louis, MO)](Sambrook et al., 1989).

Ethanol-precipitated DNA (2 to 25 µl) from cleared lysates was subjected to electrophoresis in 0.7% agarose dissolved in Tris borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid). A dye solution consisting of bromophenol blue (0.07%), SDS (7%), and glycerol (33%) in water was added at 5 µl per sample to DNA samples prior to electrophoresis. Electrophoresis was run during 6 h at 10°C at 80 V using Scientific - Gamme MultiSub™(Pistorio et al., 2003). According to Eckhardt (1978) and Wheatcroft et al. (1990) DNA bands were observed under UV illumination after staining of the gel with 0.5 to 1 µg/ml ethidium bromide for 15 min using UV transilluminator for electrophoresis on gel purchased from Herolab, equipped with Camera Type Bio-Pyramid MBP-01.

RESULTS AND DISCUSSION

Morphological and cultural characteristics

Fifty three bacterial isolates investigated in this study were isolated from shrubs legumes nodules *R. monosperma* subsp. bovei var. oranensis (Maire) collected from three different coastal dunes of Oran city (North western area of Algeria) (Table 3).

The gram staining technique showed that all isolates were Gram negative and rod shaped under the light microscope. The bacterial colonies of 6 nodulating isolates produced in YMA were circular and convex with entire smooth margins cream in color, and were not mucoid. They were classified as slow growers, according to the description of Jordan (1984) modified by Odee et al. (1997), because their colonies were ≤ 1 mm in diameter after 7 days at 28°C (Table 2) whereas, the isolates that were unable to form nodules with *R. monosperma* were mucoid, classified as fast growers (Odee et al., 1997), since their colonies were > 2 mm in diameter after 5 days in YMA medium at 28°C. Very probably they were combined to nodulating slow growers in the crushed nodule suspensions, and dominated the small colonies formed by the slow growers on the YMA Petri dishes at the first step of isolation. The colony morphology of these fast growers was very similar to that described for non nodulating agrobacteria.

Our results are in accordance with other previous investigations on the association between rhizobia and *Retama* from different Mediterranean areas (Spain, Algeria and Morocco); it has been noticed that *Bradyrhizobium* is the dominant genus of symbiotic nitrogen fixing bacteria associated with *Retama* species (Rodríguez-Echeverría et al., 2003, 2014; Ruiz-Díez et al., 2009; Boulila et al*.,* 2009; Guerrouj et al., 2013). The previous report on nodule isolates from *R. raetam* growing in arid areas of Tunisia yielded very contrasting results (Mahdhi et al., 2008). This study shows that the majority of this isolates are gathered in *Sinorhizobium* and *Rhizobium* branches. However, Zakhia et al. (2006) found that strains isolated from *R. raetam* grown in the infra-arid regions of Tunisia belonged to the branches containing the genera (*Bosea,Ochrobactrum, Starkeya, Microbacterium* and *Paracraurococcus*), but all these

Table 2. Morphological and cultural characters of bacterial isolates from *R. monosperma* root nodules.

Characters	Result
Shape	Circular
Size of colony	Slow growers 1 mm
Color on YMA	Cream, translucent slime, not mucoid, gum production
Elevation	Convex, raised
Surface margin	Regular entire margin
Oxygen demande	Aerobic
Gram's nature	Gram negative (-)
Color produced on YMA+ BTB	Blue alkali production
Color produced on YMA + Red Congo	pink transparent

Table 3. Number of isolates (N), Number of strains (S), strain richness (S/N) and Nodules number per plant.

Sampling site collection sites	Isolates (N)	Strain (S)	Strain richness (S/N)	Average number of nodules / plant
Cap falcon	35	2	0.05	RMC7 : 3.2 RMC34 : 4.3
Bousfer (Hai Mardjadjou)	10	2	0.20	RMB1 : 5.6 RMB4 : 4.2
Mers El Hadjadj	8	2	0.25	RMM2 : 2.0 RMM6 : 1.2

strains failed to nodulate their host of origin.

Authentification and symbiotic effectiveness of *R. monosperma* nodule isolates

The ability of the new rhizobial isolates to form symbioses with their original host plant was tested. The effective nodulation observed with rhizobial isolates, clearly indicated that only six isolates RMC7, RMC34, RMB1, RMB4, RMM2 and RMM6 were able to re-nodulate *R. monosperma*. It was noted that the nodules were pink, indicating leghemoglobin content, and the leaves of the nodulated plants were dark green which suggested that the six isolates were effective at N_2 fixation, while un-inoculated control plants were yellow and dead after 30 days.

The six rhizobial isolates indicated significant performance on nodulation status and plant growth parameters of *R. monosperma* inoculated plants compared to the un-inoculated control plants. Isolate RMB1 formed the uppermost number of nodules with an average of 5.6 nodules per plant (Table 3). While, the highest number of nodules was observed for *R. sphaerocarpa* inoculated with rsp from *R. sphaerocarpa* 15 nodules (Rodríguez-Echeverría et al., 2003). Mahdhi et al. (2006) reported that, most of the isolates from *R. raetam* root nodules formed 3 to 6 nodules per plant.

The shoot dry weight of *R. monosperma* inoculated plants that ranged from 0.4 to 1.30 g per plant were significantly higher than the un-inoculated control 0.3 g per plant, indicating that all plants benefited from forming symbiosis with the rhizobial isolates (Figure 2).

On the other hand, total dry weight indicated that isolate RMB1 was the most effective with 1.7 g per plant. The least effective isolate was RMM6 with only 0.6 g per plant of the total dry weight. The total dry weight of T control was 0.5 g per plant. Whereas, the maximum plant biomass was observed for *R. sphaerocarpa* plants nodulated by the strain rsp from *R. sphaerocarpa* 190 mg (Rodríguez-Echeverría et al., 2003). Mahdhi et al. (2008) proved that all isolates from *R. raetam* except one were able to induce nodules when inoculated to *R. raetam* and overall assessments clearly indicated that inoculation of the isolates enhanced the vegetative growth and dry matter.

Similar results were announced by Hatimi (1999) with isolates nodulating *R. monosperma* in Morocco and by Rodríguez-Echeverría et al. (2003) with bacteria nodulating *R. sphaerocarpa* from central western Spain.

Physio-biochemical characterization

Nodulation and N_2 fixation in symbiosis require that host and microorganism are compatible, but also that the soil

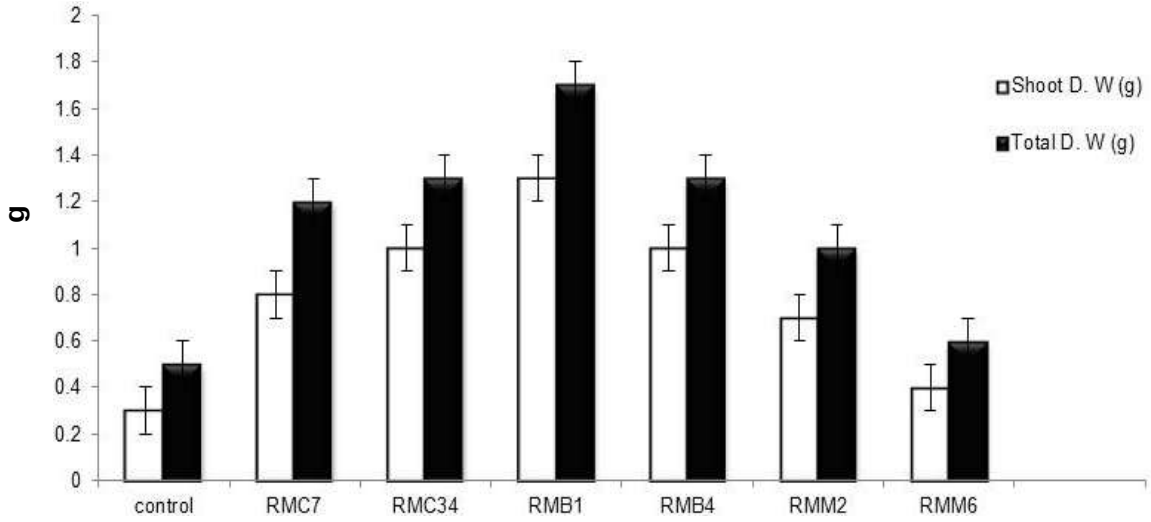

Figure 2. Shoot / total dry weight as a measure of the symbiotic effectiveness of the different Rhizobia isolates on *R. monosperma* (Values are means of 10 plants).

environment be appropriate for the exchange of signals that precede infection (Hirsch et al., 2003; Leibovitch et al., 2001; Zhang et al., 2002). Furthermore, microbial communities are able to develop a range of activities that are very important in maintaining biological balance and sustainability in soil particularly under stress conditions (Barea et al., 2002; Kennedy and Smith, 1995). In this study, physiological and biochemical characterization of rhizobial isolates from *R*. *monosperma* identified a highly tolerant to different abiotic stress conditions including salinity, pH and high temperature.

Determination of NaCl tolerance

It has been demonstrated that several factors associated with rhizobia are important in nodulation (Abdelmoumen et al., 1999; Bhattacharya et al., 2004; Hotter and Scott, 1997; Kulkarni and Nautiyal, 2000). Salinity stress is one of the most serious factors limiting the productivity of agricultural crops.

In this research, all slow growing rhizobia from *R. monosperma* are resistant to 2% NaCl and sensitive to 3% NaCl (Table 4). In contrast, the strains *B. retamae* isolated from *R. sphaerocarpa* and *R. monosperma* obtained from Morocco and Spain did not grow in presence of 1% NaCl (Guerrouj et al*.,* 2013). The relatively high tolerance to NaCl reported for two isolates RH 18 and RH 29 identified by Mahdhi et al. (2008) as *Sinorhizobium* were able to tolerate a high NaCl concentration from 1 to 3%. Moreover, Ruiz-Díez et al. (2009) recorded that isolates from *R. sphaerocarpa* RST-1 and RS-3 were able to grow at 0.1% NaCl, whilst RST-2 showed resistance at a high NaCl concentration equal to 2.5 to 3%. It is often believed that saline soils naturally select strains more tolerant to salinity. Besides, the ability of bacteria to grow well in high salt concentration has been shown to be dependent on the accumulation of internal solutes that maintain the osmotic balance of the cells and thus counteract the outflow of water molecules (Imhoff and Rodriguez, 1984; Miller and Wood, 1996), likewise many rhizobia respond to salt stress by increasing the intracellular concentration of K^+ and glutamate (Miller and Wood, 1996).

Generally, salinity stress decreased nodule number, fresh weight of both shoot and nodules as observed by Bordeleau and Prevost (1994). However, it was reported that as the number of nodules decreased, average size of the nodules increased with increasing salinity levels. Elahi et al. (2004) and Kassem et al*.* (1985) suggested that alfalfa and the symbiotic N_2 fixation process were more sensitive to NaCl than the rhizobia themselves.

pH tolerance

R. monosperma slow growing rhizobia tested showing a wide diversity in their pH tolerance (Figure 3). All strains grew in YM medium with pH values from 5.0 to 9.0, with an optimum growth at pH 7 and 7.5, as observed in the other strains isolated from *R. sphaerocarpa* and *R. raetam* respectively (Ruiz-Díez et al., 2009; Mahdhi et al., 2008). Our findings revealed that only one isolate RMM6 showed the capacity to grow at pH 4.5 (it was the most tolerant of acid pH). This result is in agreement with strains related to *B. canariense* were found only in soils with pH below 7.0, which agrees with the description of this species as well adapted to acid soils (Rodríguez-Echeverría et al., 2014). At low pH, some isolates exhibited an acido tolerant character since they grew

Table 4. Physiological and biochemical characterization of isolates.

Isolates	NaCl		Temperature				Utilization of carbohydrates					Utilization of amino acids				Enzymesproduction			
	2%	3%	4°C	14°C	28°C	37°C	d-Glucose	Dextrin	d-Galactose	l-Fructose	Sucrose	L-Leucine	L-Arginine	L-Tyrosine	L-Proline	Catalase	Oxidase	Urease	3-Ketolactose
RMC 7	+	-	-	+	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
RMC34	+	-	-	±	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
RMB1	±	-	-	±	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
RMB4	+	-	-	+	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
RMM2	±	-	-	+	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
RMM6	±	-	-	±	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-

+: Growth; -: No growth; ±:Weak growth.

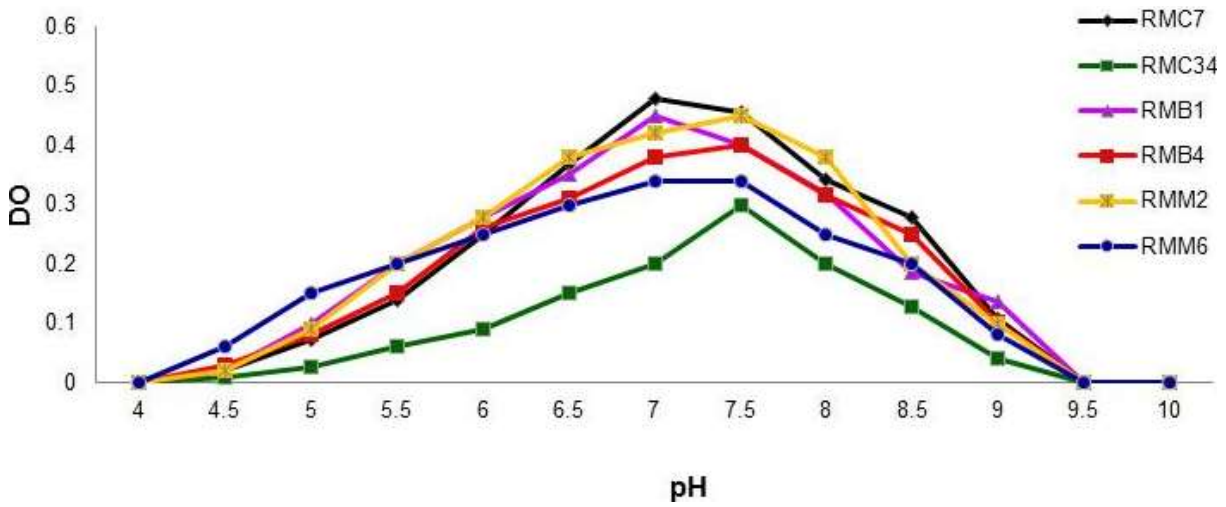

Figure 3. Effect of pH on growth of *R. monosperma* slow growing rhizobia.

without restriction in pH acid. Unlike other rhizobia genera, *Bradyrhizobium* species are not adversely affected by low soil pH (Graham et al., 1994). Van Rossum et al. (1994) compared 12 strains of *Bradyrhizobium* for their symbiotic performance with groundnut in acidic soils and found that some strains were totally ineffective under acidic stress pH of 5.0 to 6.5, whilst others performed well under these conditions. This fact could be explained by the different activity of H^+ ions in culture medium comparing to the soil where the charges of the colloids can partially neutralize the activity of the ions. Similar lack of correlation between the pH of the soils and bacteria in pure buffered media was also found before Hungria et al. (2001) and Priefer et al. (2001).

Isolate RST-1 *B. canariense* from *R. sphaerocarpa* could only grow at pH 7, the optimal

pH for rhizobial growth, while strain RST-2 *Phylobacterium myrsinacearum* was basic resistant, capable of growth up to pH 9 (Ruiz-Díez et al., 2009). Guerrouj et al. (2013) reported that *B. retamae* occurred only in soils with pH higher than 7.5, which suggests that this species could be better adapted to alkaline soils. However, Bordeleau and Prevost (1994) have noticed that, highly alkaline soils of pH above 8.5 tended to be high in contents of sodium chloride, bicarbonate and borate which reduce nitrogen fixation.

Maximum growth temperature

All isolates grew from 14 to 30°C with an optimum growth at 28°C. Growth is negative at 4 and 37°C (Table 4). Our results are in agreement with the results of other strains isolated from *R. sphaerocarpa* and *R. monosperma* from Spain and Morocco (Ruiz-Díez et al., 2009; Guerrouj et al., 2013), while the majority of isolates from *R. raetam* of arid areas in Tunisia were resistant to 35°C and some of them continued to grow at 40°C (Mahdhi et al., 2008). This may be a specific adaptation to high soil temperatures in the arid regions as described by Karanja and Wood (1988).

Some previous studies have shown that temperature affects several stages of symbiosis such as root hair infection, bacteroid differentiation, nodule structure and functioning (Roughley and Dart, 1970). Moreover, high soil temperature is associated with delaying or restricting nodulation in the subsurface region (Graham, 1992). Pankhurst and Gibson (1973) reported a rapid decline in nitrogenase activity and bacteroid degeneration at high root temperatures from 30 to 33°C, but Rai and Prasad (1983) indicated that temperature adapted *Rhizobium* strains and host cultivars could overcome this high temperature disruption of N_2 fixation. In this context, Michiels et al. (1994) found that the acetylene reduction activity of common bean plants was strongly diminished at 35°C when plants were inoculated by heat sensitive or heat tolerant strains.

Tests for enzymatic activity

Further conformity of rhizobia performed on ketolactose agar showed that all the six isolates were negative for the production of 3-ketolactose from lactose. Bernaerts and De Ley (1963) found that the production of 3-ketolactose from lactose is limited to species of *Agrobacterium,* a genus that is closely related to *Rhizobium.* Additionally, the rhizobial isolates in the current study were further tested on YMA plates containing BTB indicated that, isolates produced blue color colonies which proves the presence of alkali producers. This latter considered as slow growing rhizobia and formed circular, convex colonies. All of the slow-growing rhizobia from *R. monosperma* examined here were positive for catalase and oxidase, as confirmed by the liberation of effervescence of oxygen around the bacterial colonies and change in color of the oxidase strip, respectively.

These tests were not completely presented in previous studies related to bacteria nodulating *Retama* species, in exception urease test which has givena positive result with *B.retamae* (Guerrouj et al., 2013); this in accordance withour results.

Carbon and nitrogen sources utilization

All strains were able to use the majority of carbon and nitrogen sources, this is shown clearly in Table 4. Assimilation of d-glucose and d-galactose was positive. Assimilation of dextrine was negative. As pointed out by Glenn and Dilworth (1981), slow growing rhizobia tend to lack both uptake systems and catabolic enzymes for disaccharides. Our results were in agreement with the results of these authors in that the disaccharides l-fructose and sucrose were not metabolized by slow growing strains. Same results were observed for *B. retamae* by Guerrouj et al. (2013). The amino acids used in this study L-leucine and L-tyrosine as nitrogen source were utilized by the six isolates except L-proline and L-arginine. These results are in accordance with the findings of Guerrouj et al. (2013) and Mahdhi et al. (2008).

Intrinsic antibiotic resistance (IAR)

The suitability of *Rhizobium* strains resistant to high levels of antibiotics for studying *Rhizobium* ecology was first suggested by Obaton (1971) and used by Danso et al. (1973); Brockwell et al. (1995) and Kuykendall and Weber (1978). Hence, intrinsic antibiotic resistance (IAR) may indicate the ability of the *Rhizobium* to withstand antimicrobial substances present in the soil. It is simple characteristic which can be used for screening *Rhizobial* and *Bradyrhizobial* cultures (Chanway and Holl, 1986; Galiana, 1991).

The evaluation of intrinsic resistance to nine antibiotics of *R. monosperma* rhizobia showed that all strains were resistant to spectinomycin (10 $\mu g/ml^{-1}$), erythromycin (15 $\mu g/ml^{-1}$) rifampicine (30 $\mu g/ml^{-1}$) and sensitive to gentamycin (500 $\mu g/ml^{-1}$). Same results were obtained for the strains of *B. retama* isolated from *R. monosperma* and *R. sphaerocarpa* by Guerrouj et al. (2013). They show variable resistance level against streptomycin, kanamycin, naldixic acid, penicillin and tetracycline (Table 5). Four isolates RMC7, RMC34, RMM2 and RMM6 were resistant to kanamycin (30 $\mu g/ml^{-1}$) and streptomycin (500 $\mu g/ml^{-1}$), whereas RMB1 and RMB4 were sensitive. Most isolates were resistant to 30 $\mu g/ml^{-1}$ of nalidixic acid except for RMB4 and RMM2.

Table 5. Resistance of isolates against antibiotics.

Isolates	Antibiotics (µg / ml)								
	Spectinomycin (10)	Erythromycin (15)	Rifampicine (30)	Gentamycin (500)	Streptomycin (500)	Kanamycin (30)	Naldixic acid (30)	Penicillin (6)	Tetracycline (30)
RMC7	+	+	+	-	±	±	±	+	±
RMC34	+	+	+	-	±	±	±	±	-
RMB1	+	+	+	-	-	-	±	±	-
RMB4	+	+	+	-	-	-	-	-	-
RMM2	±	±	±	-	±	+	-	±	±
RMM6	±	±	±	-	±	±	±	+	±

+ Resistant: growth; - sensitive: no growth; ±, weak growth.

The majority of isolates were resistant to 6 µg/ml^{-1} of penicillin except one RMB4. Isolates RMC7, RMM2 and RMM6 were resistant to tetracycline (30 µg/ml^{-1}), but RMC34, RMB1 and RMB4 are sensitive. Abdel-Moneim et al. (1984) found that the sensitivity of *Rhizobium* towards antibiotics seemed to be a specific property of the isolates regardless of the species. Sinclair and Eaglesham (1984) reported that resistance to 4 inhibitors of protein synthesis was associated with little or no effectiveness in either fast or slow growing strains. They found a positive correlation between resistance to streptomycin and efficiency. In this way, resistance to streptomycin has been one of the most frequently used markers for rhizobia (Brockwell et al., 1995; Borges et al., 1990).

Plasmidic and chromosomic DNA extraction

Rhizobial genomes have basically two main components: the core and the accessory genome (Young et al., 2006). Chromosomes with conserved gene content and order (synteny) are considered as a core. Accordingly, plasmids constitute the accessory genome, and are more flexible than chromosomes, as defined by more frequent gene gains and losses, regardless of species (Mazur et al., 2011). We have been going through deep explorations searching for an inexpensive screening method for the detection of chromosomic and plasmidic DNA. This searching approach allowed us to better determine their size, which is in correlation with that could be applied to a wide variety of bacterial species.

Figure 4 shows the relative electrophoretic mobilities of chromosomic and plasmidic DNA extracted from *R. monosperma* isolates RMC7, RMC34, RMB1, RMB4, RMM2 and RMM6, together with the leader molecular (λ DNA-HindIII Digest) used to estimate DNA sizes. A plot of the logarithm of relative motilities through the gel against DNA logarithm size determined by electrophoresis migration provided consistent linear curve for DNA ranging in size from isolates. The logarithm of relative mobility (RM) widely associated to the logarithm of size and linear relationship was obtained. Calculation of linear correlation coefficient ended up with the formula $R^2 = 0.993$ and the equation of the regression line shown in Figure 5 is: Log RM = - 0.432 Log S + 3.009. This equation was used to calculate molecular weights of DNA extracted from different investigated strains. The standard deviation of calculated molecular weights takes into account deviation of the measured relative mobility and the error calculated from the regression line. All strains showed a common band of molecular weight MW ≥ 69168 pb; it's highly supposed to be chromosomic DNA. Strains sampling of "Cap falcon" area RMC7 and RMC34 demonstrated two common bands in molecular weight equals to 1327 and 913 pb, which is likely to be ARN spots since we have not used ribonuclease. Whereas, RMB1 strain of "Bousfer" site had a second band of strong mobility in addition to chromosomic DNA band, in molecular weight equals to 2027 pb. This band was relevant to the strain resistance regarding to penicillin and naldixic acid, or could be due to pH alcalin pH 8.5 resistance characterizing this site soil. While, there are no reports of a *Bradyrhizobium* strain carrying a symbiotic plasmid. The plasmids found in some strains of photosynthetic bradyrhizobia, which nodulate in a Nod factor independent manner (Giraud et al., 2007), are not symbiosis plasmids (Cytryn et al., 2008). Previous studies on *Bradyrhizobium* have been found that genes

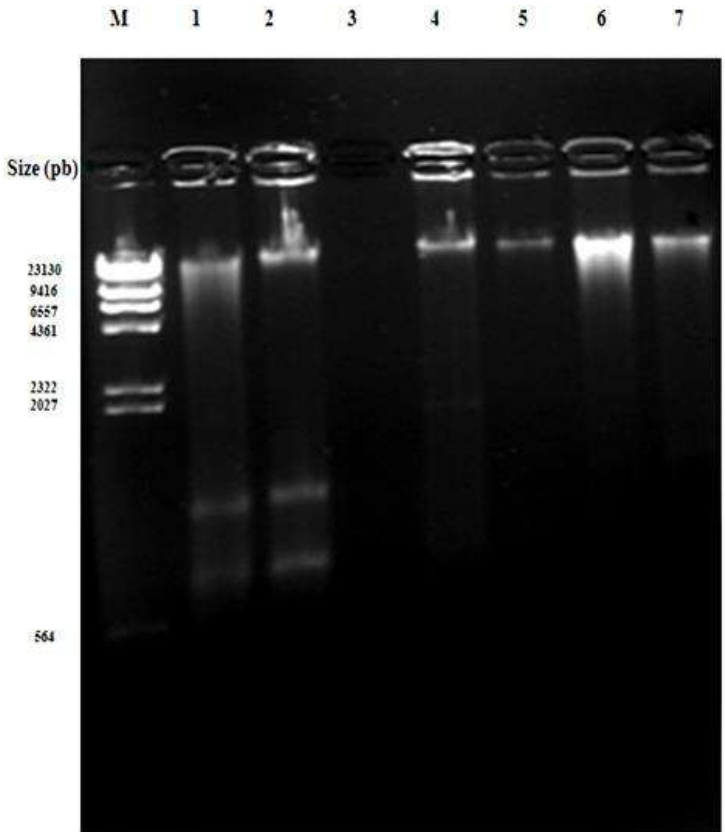

Figure 4. Agarose gel electrophoresis of plasmid DNA from *Retama monosperma* isolated M: *λ DNA-HindIII* Digest. Lane 1, RMC7; 2, RMC34; 3, empty; 4, RMB1; 5, RMB4; 6, RMM2; 7, RMM6.The numbers refer to the sizes of the DNA fragment in pb.

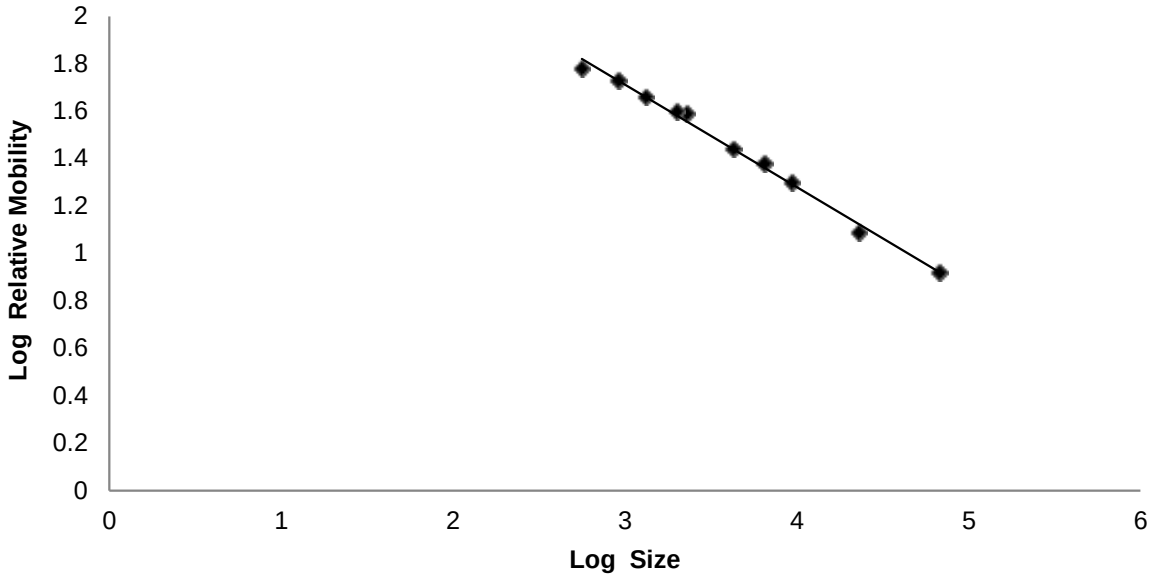

Figure 5. Relative mobility of DNA from *Retama monosperma* isolated in a 0.7% agarose gel vs. size of fragment *λ DNA-HindIII* Digest. The equation of the regression line is: Log RM = -0.432 Log S + 3.009 $R^2 = 0.993$.

involved in nitrogen fixation and symbiosis are located on the chromosome in 'symbiotic islands' (Kaneko et al., 2002). *B. japonicum* (the host plant is soybean) and *B. "cowpea*" (the host plants are tropical legumes) are called slow growing *Rhizobia* and their *nif* and *nod* genes are located on the chromosome (Long, 1989, Martinez et al*.,* 1990, Appelbaum et al., 1990). These results are in accordance with the finding of Yao et al. (2002) who reported that the symbiotic genes are chromosomal-borne and no plasmids were observed.

Conclusion

Throughout many microbiological researches undertaken by several authors, it has been shown that *Retama* species have positive effects on their environment and vegetation in Iberian Peninsula and the Mediterranean coast due to their capacity to establish N_2 fixing symbiosis with rhizobia. In this paper, the rhizobia naturally associated with *R. monosperma* collected from three sites of the Oranian coastal have shown a wide morphological and physio-biochemical diversity. Most of them showed resistance to unfavorable conditions (salinity, extreme pH and intrinsic antibiotic resistance); this may help *Retama* plants to become more adapted to harsh environmental conditions. More studies based on phylogenetic analysis performed by sequencing of housekeeping genes and the internal transcribed spacer region (ITS), were not pursued in this work need to be undertaken in order to elucidate the taxonomic relationships among bacteria species within the genus *Bradyrhizobium*.

Finally, the number of papers on natural populations of rhizobia that nodulate *Retama* species is relatively small, but all of them show the great diversity of these bacteria irrespective of the technique employed. In fact, they could be a source of valuables genotypes to improve strains of environmental importance. In this way, it would be possible to fight against ecological problems through the production of biofertilizers necessary to the restoration and revegetation of land degraded soils (polluted soil, arid and semiarid areas) through microclimate creation; this would also be a modest contribution to problems of the time such as global warming.

Further investigation of the rhizobia associated with *R. monosperma* using both core and symbiotic genes markers (*nif* and *nod*) and host range studies, are essential for a better understanding its biological evolution, the specificity of the symbiosis and the adaptation strategy of the two partners to various environmental conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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