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Phenotypic and Genotypic Diversity of Microsymbionts Nodulating *Medicago sativa* (L.) in the Algerian Sahara

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Abstract

This work was conducted to evaluate the phenotypic and phylogenetic diversity of 48 rhizobial strains. All rhizobial strains exhibited a broad tolerance to salinity and pH. In general, they grew well at 28°C and 37°C but poorly at 4°C and 45°C. The rhizobial strains showed an array of antibiotic sensitivity patterns. The numerical (UPGMA) analysis of phenotypic traits and the phylogenetic tree of concatenated housekeeping genes produced highly similar results. Phylogenetic analysis of *recA* and *glnII* showed that all the isolates were affiliated to the genus *Sinorhizobium*, but belong to two distinct groups: Group I, originating in Ghardaïa, was close to the species *S. meliloti* and *S. kummerowiae*. Group II, originating in Ouargla and El Oued, clustered separately from sequences of known *Sinorhizobium* species, which suggests they could be a new lineage. The classifications resulting from the *nodC* gene reflect host specificity, while phylogeny based on chromosomal genes is independent of the host plant. Based on the studies documented in the literature, the genetically characterized rhizobial strains can be used as an effective inoculant for the improvement of forage yields in Saharan regions.

Keywords: Abiotic stress, phylogeny, alfalfa, rhizobia, arid environment.

1. Introduction

Alfalfa (*Medicago sativa*) is one of the oldest forage crops and contributes immensely to world food production (Massimi et *al.*, 2017). It is vital due to its high protein content, high biomass yield, excellent nutritive value and high digestibility. It is widely planted throughout the world, especially in the arid and semi-arid areas (Zhang and Wang, 2015). This forage crop provides fixed nitrogen to agricultural ecosystems and reduces dependence on synthetic N fertilizers (Mouradi, 2016; Ahmad et *al.*, 2016). However, in adverse conditions such as high salinity and drouth, the survival of rhizobia is greatly affected (Domínguez-Ferreras et *al.*, 2006) and therefore, nodulation and effectiveness in alfalfa can be significantly reduced (Brígido et *al.*, 2013; del Pozo et *al.*, 2017; Azib et *al.*, 2020).

Very little is known about the diversity of rhizobial strains nodulating Saharan varieties of alfalfa, despite the alfalfa-sinorhizobia symbiosis being one of the best studied plant-microorganism interactions. So far, only two closely related species are known to be able to nodulate alfalfa: *Sinorhizobium meliloti* and *S. medicae* (Tabares-da Rosa *et al.,* 2019).

The development of polyphasic taxonomy (phenotypic, genotypic and phylogenetic characteristics) and the use of *16S rRNA* as a taxonomic marker has led to many changes in the taxonomy of rhizobia (Zakhia and de Lajudie, 2006). A highly conserved gene like *16S rRNA* is not suitable for the discrimination of closely related *Sinorhizobium* (or *Ensifer*) species (Martens et *al.*, 2007). To overcome these limitations, the multilocus sequence analysis (MLSA) of several protein encoding housekeeping genes (*atpD*, *recA* and *glnII*, etc.) has been suggested as alternative phylogenetic markers (Stackebrandt et *al.*, 2002).

The aim of this study was to investigate phenotypic and genotypic diversity of 48 strains nodulating alfalfa in 14 Algerian Saharan sites affected by salt and drought. Firstly, phenotypic characterization for tolerance to salinity, temperature, pH and antibiotics was assessed; secondly, housekeeping genes *recA* and *glnII*, and symbiotic gene *nodC* were used to *establish* phylogeny of these strains.

2. Materials and methods

2.1. Nodule collection and isolation of rhizobia

During the period from February to March of 2014, root nodules of alfalfa plants were collected from 14 sites (Table 1) and rapidly dried and kept in tubes containing desiccant according to the method described by Somasegaran and Hoben (1985).

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Desiccated nodules were rehydrated before sterilization. Nodules were placed in small beakers with clean cool water and left in the refrigerator to soak overnight. Then, they were surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo-chlorate for 3-4 minutes. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals (Somasegaran and Hoben, 1985).

The rhizobia were isolated following the standard method on yeast extract mannitol medium (YEM) (Vincent, 1970). Each nodule was crushed in a drop of sterile distilled water and suspension was streaked onto YEM Agar. Bacterial colonies appeared after incubation at 28°C for 3-5 days. A single representative colony, for each sample, was restreaked on freshly prepared YEM plates in order to obtain pure cultures.

Table 1. Site description, and strain names, location, type of climate, soil type, salinity of irrigation water and year of sampling.

Site	Station	Strains	Geographical position	Soil texture	Salinity of irrigation water (g/l)*	Period of sampling
Ouargla	Hassi Ben Abdallah	O114, O144, O152, O172	Lat. 32°00’77’’N		2 to 42 to 4	February 2014
			Long. 5°46’27’’E	Sandy	2 to 4	
	Oum Erraneb	O211, O213,	Lat. 32°05’03’’N			February 2014
		O223	Long. 5°34’46’’E		2 to 4	
	ITAS	O313, O321, O344	Lat. 31°94’11’’N			March 2014
			Long. 5°29’54’’E			
	Chott Ain Beida	O413, O422, O434, O442, O452, O461	Lat. 31°97’76’’N Long.5°38’96’’E			March 2014
Ghardaïa	Daya Ben Dahoua	G131, G132, G122, G124	Lat. 32°53’53’’N		1 to 41 to 1.5	March 2014
			Long. 4°40’35’’E		1 to 1.5	
	Mansoura	G211, G241, G242	Lat. 31°98’25’’N	Sandy	1 to 1.5	March 2014
			Long. 3°57’52’’E		1 to 1.5	
	Oued Laroui	G312, G315, G321	Lat. 32°57’01’’N			March 2014
			Long. 3°62’86’’E			
	Sebseb	G42, G422, G424,	Lat.32°17’01’’N			March 2014
		G431, G432	Long. 3°57’52’’E			
	Guerrara	G514, G522	Lat.32°67’89’’N			March 2014
			Long. 4°73’77’’E			
El Oued	Tenedla	E114, E131, E141	Lat.33°67’58’’N		2 to 62.5 to 6	April 2014
			Long.6°03’72’’E	Sandy	2 to 6	
	El-Meghaier	E213, E222, E251	Lat.33°56’25’’N		2 to 4	April 2014
			Long. 5°92’71’’E			
	Djamaa	E353	Lat.33°52’30’’N		2 to 4	April 2014
			Long.6°02’32’’E			
	Guemmar	E414, E421, E432, E441, E452	Lat.33°51’07’’N			April 2014
			Long.6°78’26’’E			
	Reguiba	E52, E532, E543	Lat.33°56’25’’N Long. 6°71’74’’E			April 2014

*: values taken from OSS (2003).

2.2. Nodulation tests

All the cultures obtained were tested for nodulation in the host plant *Medicago sativa* (Alfalfa). Isolates were used to inoculate surface sterilized alfalfa seeds growing in tubes containing Jensen’s N-free agar medium (Jensen and Hauggaard-Nielsen, 2003) and evaluated after six weeks according to presence or absence of nodules (Gibson, 1980). The experiment was conducted in a plant growth chamber with 16/8 h day/night and 22°C.

Two day old seedlings were transferred into test tubes (15 cm × 2 cm) with one seedling per tube, containing 10 ml of agar slant medium and inoculated with 1 ml of standardized bacterial suspension (OD600nm of 0.9).

2.3. Phenotypic characterization

Isolates that induced nodulation in alfalfa were used in this study. Two closely related *Sinorhizobium meliloti* strains, Sm1021 and Sm2011 received from Dr. Helene Berges, Plant Genomic Center (CNRGV, INRA-France), were used as reference strains.

The tolerance of isolates to NaCl was tested by using YEM supplemented with 1.7, 40, 80, 160, 320, 640 and 1280 mM NaCl. The tolerance to pH was assessed by adjusting the pH to 4.0, 5.0, 6.0, 6.8, 8.0 and 9.0 through the addition of acid or base to the YEM (Vincent, 1970). Isolates were examined for tolerance to temperature by

incubating at 4, 28, 37 and 45°C as described by Niste et *al*. (2015).

The resistance to ten antibiotics (µg/disc): fusidic acid: 10 µg (FA), amikacin: 30 µg (AK), amoxicillin: 25 µg (AMX), chloramphenicol: 30 µg (C), colistin: 10 µg (CS), erythromycin: 15 µg (E), kanamycin: 30 µg (K), penicillin: 6 µg (P), spiramycin: 100 µg (SP) and vancomycin: 30 µg (VA), was tested on YEM plates by adding antibiotic discs on the surface of the agar.

The growth was recorded after 72 h of incubation at 28°C in liquid YEM by measuring the OD at 600 nm (Wei *et al.*, 2004) and on solid YEM by counting the colonies appearing on the plates. Tests tubes containing 10 ml of liquid YEM were inoculated with 0.1 ml of a fresh culture of each isolate and incubated under shaking (200 rpm). On solid YEM, supplemented with 1.5% agar, inoculation is carried out by streaking on Petri plates (Vincent, 1970; Somasegaran and Hoben, 1985).

2.4. DNA extraction and PCR amplification and purification

Total genomic DNA of the isolates was extracted using DNeasy® Blood and Tissue Kit columns in accordance with the manufacturer's protocol (QIAGEN Ltd.) from cells grown for 3 days in yeast extract mannitol broth (YMB) at 28°C under shaking (200 rpm). After extraction, the DNA was quantified to determine its approximate quantity and relevance for further analysis using agarose gel electrophoresis.

Housekeeping genes *glnII* and *recA*, and symbiotic gene *nodC* were amplified by PCR using the following primers: GSII-1F (5'-AACGCAGATCAAGGAATTCG-3') and GSII-4R (5'-GCGACGATCTGGTAGGGGT-3') (Turner and Young, 2000); recA_41F (5'-TTCGGCAAGGGMTCGRTSATG-3') and recA_640R (5'-ACATSACRCCGATCTTCATGC-3') (Vinuesa *et al.*, 2005); nodC_for540 (5'-TGATYGAYATGGARTAYTGGCT-3') and nodC_rev1160 (5'-CGYGACARCCARTCGCTRTTG-3') (Sarita *et al.*, 2005). The quantity of DNA was determined by using a NanoDrop spectrophotometer (NanoDrop ND1000). The PCR reaction was carried out in a 25 µl volume containing 2.5 µl 10×Standard Reaction Buffer with $MgCl_2$ (Biotools), 2 µl DNA, 1 µl *Taq* DNA polymerase (Biotools), 0.5 µl dNTP, 1 µl of each primer and 17 µl of distilled water.

The thermal program for PCR reactions of *glnII* and *recA* was carried out at 95°C for 90s; 35 cycling times at 95°C for 45s, 55°C for 45s and 72°C for 2min and a final cycle was 72°C for 7min. For *nodC*, it was at 95°C for 3min; 35 cycling times at 94°C for 1min, 55°C for 1min and 72°C for 2min and a final cycle was 72°C for 7min. Unincorporated primers and dNTPs were removed from PCR mixes with PCR Clean-up (Macherey-Nagel). PCR products were verified by electrophoresis in 1% agarose gel submerged in TBE buffer (Del Papa *et al.*, 1999) and visualized with a Gel Doc EZ system (Bio-Rad). Sequencing reactions were outsourced to Stabvida (Lisbon, Portugal).

2.5. Phylogenetic analysis

The quality of the sequences was checked and edited manually using BioEdit 7.2.5 (Hall, 1999) and automatically using DNA Baser Assembler v4.36.0 (2013) (Heracle BioSoft, http://www.DnaBaser.com).

Initially, a blast search (Altschul *et al.*, 1990) conducted using the National Center of Biotechnological Information (NCBI) website was carried out for preliminary identification and *recA*, *glnII* and *nodC* gene sequences of the reference species related to our strains were downloaded. The phylogenetic analyses were performed using MEGA 6.06 software (Tamura *et al.*, 2013). A neighbor-joining tree was constructed using the Kimura two-parameter model of evolution (Kimura, 1980) and support of internal branches was assessed using 1000 bootstrap replications.

2.6. Statistical analysis

The phenotypic characters results were analyzed by utilizing XLSTAT software (version 2016.02.28451). Bacterial growth in liquid medium was subjected to analyses of variance (ANOVA) and treatment means compared using Tukey's HSD (honest significant different) test. Numerical analysis of phenotypic traits was evaluated by UPGMA algorithm to infer a dendrogram on the basis of growth (+) or no growth (-) for each of the isolate in solid medium.

3. Results

3.1. Morphologic characterization and authentication

After 3 days of incubation at 28°C, all isolates formed visible colonies on YEM Agar medium. Colonies were whitish and translucent, varying in diameter from 1 to 3 mm, circular, convex, with a regular outline and a smooth surface. Furthermore, the isolates formed nodules on the roots of alfalfa plants six weeks after inoculation.

3.2. Phenotypic characterization

The results show that all strains exhibited a broad spectrum of tolerance to salinity. All strains were able to grow in the presence of 1.7 mM to 640 mM NaCl (Table 2). In contrast, no strain grew at 1280 mM NaCl. It is worth mentioning that tolerance to a given concentration of NaCl does not necessarily mean good growth of the strains. Analysis of variance shows significant differences between the salt concentrations of 80, 160, 320 and 640 mM with the average growth of the strains decreased with increasing salt concentrations (Figure 1a).

At low salinities (1.7 and 40 mM), strains G132 and G424, from Ghardaïa, had the best growth. At 80 and 160 mM, strains O152, O211 and E141 from Ouargla and El Oued were the most resistant. At the highest concentrations, the E543 and E452 strains from the El Oued region performed the best (Table 3).

Table 2. Results of strains tolerance to some environmental stress factors and antibiotics.

Strains	Temperature (C°)			pH			NaCl (mM)		Antibiotic									
	4	28 and 37	45	4	5	6 to 9	1.7 to 640	1280	FA	AK	AMX	C	CS	E	K	P	SP	VA
O114	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	-	+
O144	-	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-
O152	-	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	-
O172	-	+	-	-	+	+	+	-	-	+	-	+	+	-	+	-	+	+
O211	-	+	-	-	+	+	+	-	-	+	-	+	+	-	+	-	+	+
O213	-	+	-	-	+	+	+	-	-	+	-	+	+	-	+	-	+	+
O223	-	+	-	-	+	+	+	-	+	+	-	+	+	-	-	-	+	-
O313	-	+	-	-	+	+	+	-	-	-	-	-	+	-	-	-	+	+
O321	-	+	-	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+
O344	-	+	-	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+
O413	-	+	-	+	+	+	+	-	-	+	-	-	+	-	+	-	-	+
O422	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
O442	-	+	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+
O452	-	+	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+
O461	-	+	-	-	+	+	+	-	-	-	-	-	+	-	+	-	+	-
E114	-	+	-	-	+	+	+	-	-	+	-	-	+	-	+	-	+	+
E131	-	+	-	-	-	+	+	-	+	+	-	+	+	-	+	-	-	+
E141	-	+	-	-	-	+	+	-	+	+	-	-	+	-	+	-	+	+
E213	-	+	-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	+
E222	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-	+	+
E251	-	+	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-
E353	-	+	-	-	+	+	+	-	-	+	-	+	+	-	-	-	-	+
E421	-	+	-	-	-	+	+	-	-	+	-	-	+	-	+	-	-	+
E432	-	+	-	-	-	+	+	-	+	+	-	-	+	-	+	-	+	+
E441	-	+	-	-	+	+	+	-	+	+	-	-	-	-	+	-	-	+
E452	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
E521	-	+	+	-	+	+	+	-	-	+	-	-	+	-	+	-	+	+
E532	-	+	+	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+
E543	-	+	-	-	+	+	+	-	+	+	-	-	+	+	+	-	+	+
G131	-	+	-	-	+	+	+	-	+	-	+	+	-	+	-	+	-	+
G132	-	+	-	-	+	+	+	-	-	-	+	+	+	+	-	+	+	-
G241	-	+	-	+	+	+	+	-	+	-	+	-	-	-	+	+	+	-
G242	-	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	+	+
G122	-	+	-	-	+	+	+	-	+	-	+	-	-	-	-	+	+	+
G124	-	+	+	+	+	+	+	-	+	-	+	-	-	-	-	+	+	+
G211	-	+	-	-	+	+	+	-	-	-	+	+	-	+	-	+	-	+
G312	-	+	+	-	+	+	+	-	-	-	+	+	-	+	-	+	+	-
G315	-	+	-	-	+	+	+	-	+	-	+	-	-	+	-	+	+	+
G321	-	+	-	+	+	+	+	-	+	-	+	+	-	+	-	+	+	+
G421	-	+	+	-	-	+	+	-	+	-	+	+	-	+	-	+	+	+
G422	-	+	+	-	+	+	+	-	+	-	+	+	-	+	-	+	+	+
G424	-	+	-	+	+	+	+	-	-	-	+	-	-	-	-	+	+	+
G431	-	+	-	+	+	+	+	-	+	-	+	-	-	+	-	+	+	+
G432	-	+	+	+	+	+	+	-	+	-	+	-	-	+	-	+	+	+
G514	-	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
G522	-	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	+	+
Sm1021	+	+	+	-	-	+	+	-	+	-	+	-	+	+	+	+	-	+
Sm2011	+	+	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	+

The resistant strains for the different factors were coded as "+" and the sensitive strains as "-".

Growth of rhizobial strains differed with differences in pH values. They tolerated alkaline and neutral pH better than acidic pH (Figure 1c). The strains were affected by excessively acidic pHs and registered growth rates of 32.43% and 81.63% at pH 4 and 5, respectively. At slightly acidic, neutral and alkaline pH, all rhizobial strains grew well. The ANOVA test showed significant differences between the growths of strains at different pH (Figure 1d). Eleven strains (O152, O413, O422, O452, G241, G124, G321, G424, G431, G4311 and G514), from Ouargla and Ghardaïa, were resistant to pH 4.

Table 3. Selection of strains tolerant to different NaCl concentrations.

	NaCl Concentrations						
Strains	**1.7 mM**	**40 Mm**	**80 mM**	**160 mM**	**320 mM**	**640 Mm**	**1280 mM**
	G132 [a]	G132 [a]	O152 [a]	E141 [a]	G241 [a]	E543 [a]	-
	O211 [ab]	G424 [ab]	O211 [a]	O223 [ab]	E521 [a]	E452 [a]	-
	O114 [abc]	E222 [abc]	O172 [ab]	O172 [abc]	E141 [a]	O461 [ab]	-
	O452 [abc]	G122 [abc]	O223 [ab]	O321 [abcd]	E114 [ab]	O442 [abc]	-
	G321 [abcd]	O152 [abcd]	E222 [ab]	E114 [abcd]	G124 [ab]	E251 [abcd]	-
	O144 [abcde]	O172 [abcde]	E521 [ab]	O313 [abcd]	O313 [ab]	E213 [abcde]	-
	O313 [abcde]	E131 [abcde]	E131 [ab]	G422 [abcde]	O211 [abc]	O144 [abcdef]	-

For each parameter, the means in the same column followed by the same letter are not significantly different, as determined by Tukey's HSD test at P = 0.05.

At 28 and 37°C, all strains showed good growth, produced visible colonies on solid medium (Figure 1e) and high optical densities (OD) (Figures 1f). At 4°C, only the two reference strains, Sm2011 and Sm1021, were able to grow. Increasing the temperature to 45°C significantly reduced growth and only 15 isolates (O144, O152, E251, E521, E532, G242, G321, G421, G422, G431, G432, G514, G522, Sm2011 and Sm1021) were thermotolerant (Table 2).

The strains showed different resistance profiles to antibiotics. They exhibited strong resistance to spiramycin 100 µg (SP), vancomycin 30 µg (VA) and colistin 10 µg (CS), while their resistance was low, but comparable, for the other antibiotics (Figure 1b). Strains from Ghardaïa and reference strains are, generally, more tolerant to antibiotics (61.17% and 65% respectively) than those from Ouargla and El Oued that show low levels of resistance (around 35% and 43.5%), apart from a few that are resistant to a single antibiotic.

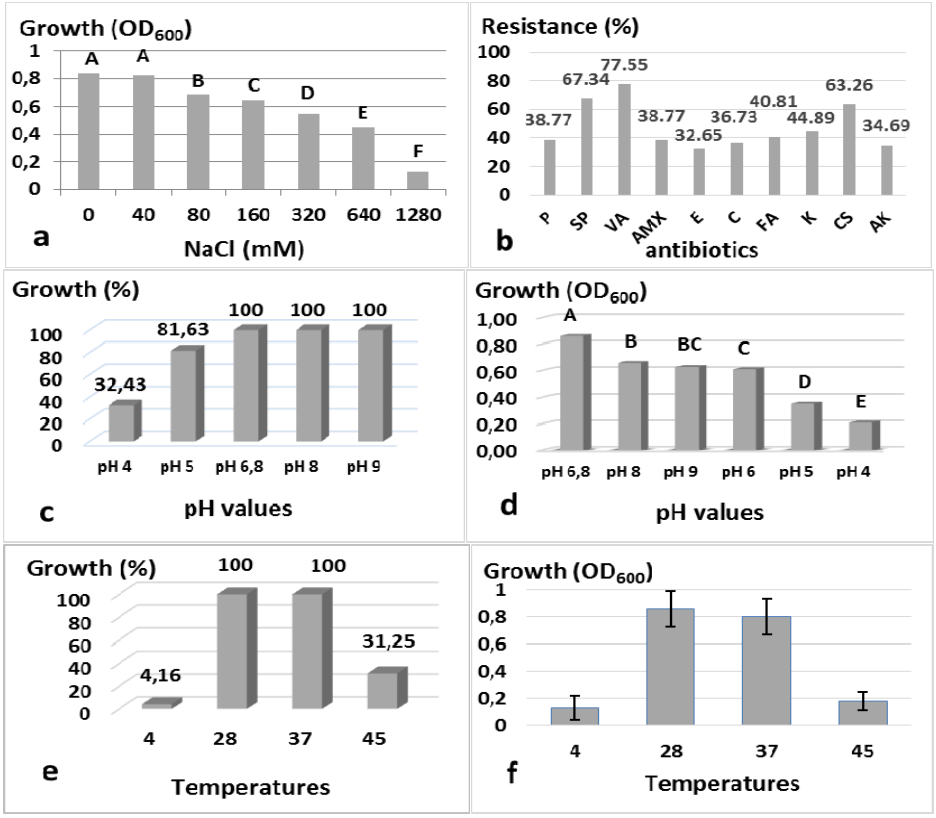

Figure 1. Growth of strains under: salinity (a), antibiotics (b), different pH (c, d) and temperatures (e, f) (done at the Saharan Bioresources Laboratory of the University of Ouargla, in 2017).

3.3. Numerical analysis of phenotypic traits

The 27 phenotypic characters of the strains were used to construct a dendrogram using the UPMGA method (Figure 2). At about 60% dissimilarity, rhizobial strains have been classified into three phenotypic groups. Group 3 has 29 strains, all from the Ouargla and El Oued regions. Group 1 consists of 17 stains exclusively from the Ghardaïa region. The two reference strains Sm1021 and Sm2011 were in a separate group (group 2), closer to group 1 than to group 3.

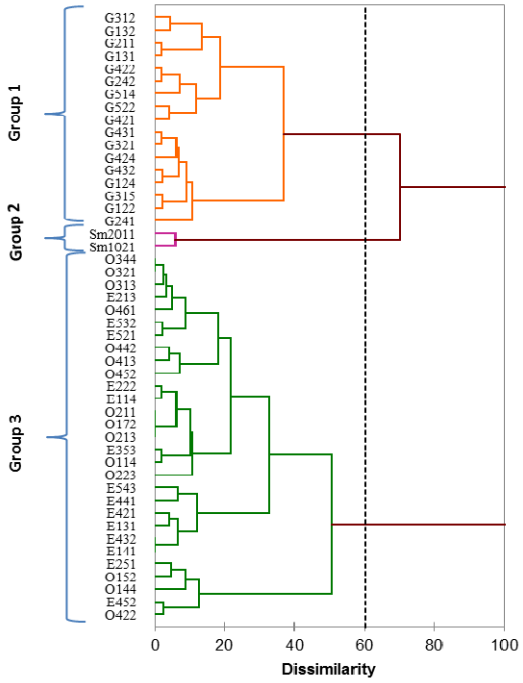

Figure 2. Dendrogram showing the phenotypic diversity of 48 strains constructed using the UPGMA method, based on a binary matrix of 27 physiological characters.

3.4. Phylogenetic analysis

Forty-eight strains were chosen for the phylogenetic examination of housekeeping genes (*recA* and *glnII*) and the symbiotic gene *nodC*. Phylogenetic trees were constructed for each gene utilizing the Neighbor-joining method and Kimura's two-parameter model. Bootstrap analysis was based on 1000 replications.

3.4.1. Housekeeping gene phylogenies

Strains were more closely related to *S. meliloti* and *S. kummerowiae* (De Lajudie *et al.*, 1994; Wei *et al.*, 2002) than to other species according to the housekeeping gene phylogenies (Table 4). Sequence analysis of *recA* and *glnII* respectively revealed 98–100% and 97–99% similarities with type strains *S. meliloti* USDA 1002^T, 98-99% and 97-100% with type strains *S. kummerowiae* CCBAU 71714^T and 91% and 91-93% with type strains *S. medicae* A321^T (Rome *et al.*, 1996).

Table 4. Sequence similarities for *recA*, *glnII* and *nodC* genes relatedness among the 48 strains and related type strains.

	Gene marker and sequence similarity with type strains %							
	glnII		***recA***		***glnII+recA***		***nodC***	
Type strain	**Similarity %**	Stains **numbers**	**Similarity %**	**Isolate numbers**	**Similarity %**	**Isolate numbers**	**Similarity %**	**Isolate Numbers**
S. meliloti USDA 1002^T	99% 98% 97%	14 10 24	100% 99% 98%	05 06 37	99% 98% 97%	14 27 07	99% 97% 96% 89%	18 08 18 04
S. kummerowiae CCBAU 71714^T	100% 99% 98% 97%	04 10 33 01	99% 98%	04 44	99% 98%	13 35	96% 95% 94% 90%	02 13 29 04
S. medicae A321^T *S. medicae* USDA 1037	91% 92% 93%	01 46 01	91%	48	92% 91%	47 01	100% 99% 96% 90%	02 13 29 04

glnII gene phylogeny

The phlogenetic tree corresponding to *glnII* (Figure 3) showed that the strains clustered into two groups with high bootstrap support (99 for group I and 72 for group II). Group I consisted of 14 strains, originating exclusively from Ghardaïa, closely related to reference strains *S. kummerowiae* CCBAU 71714^T and *S. meliloti* USDA 1002^T. There were 13 strains (G421, G422, G242, G424, G 431, G432, G514, G211, G242, G315, G321, G122

and G124) clustered with *S. kummerowiae* CCBAU 71714^T at sequence similarities of 99.82 to 100% and one strain (G312) clustered only with *S. meliloti* USDA 1002^T at sequence similarity of 99.52%. Group II contained 34 strains, coming from the El Oued and Ouargla (except G131, G132 and G522), which were separated from the reference strains. Similarities between the strains in this group and type strains *S. kummerowiae* CCBAU 71714^T and *S. meliloti* USDA 1002^T were 97.69 to 98.58% and 97.46 to 98.19%, respectively.

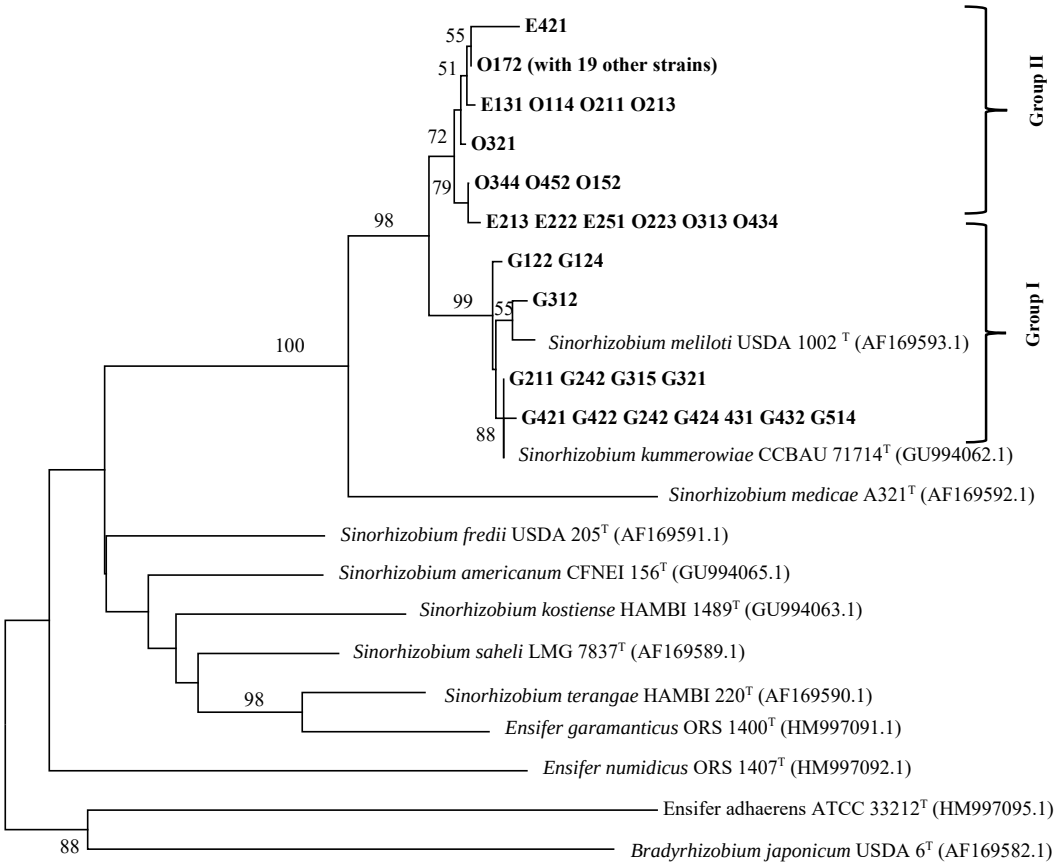

Figure 3. Neighbor-joining phylogenetic tree constructed from *glnII* gene (555 bp) showing the relationship among strains nodulating alfalfa and related species of the *Sinorhizobium–Ensifer* group. Bootstrap values (1000 replicates; only values over 50 % are given) are indicated above the branches. *Bradyrhizobium japonicum* USDA6^T was used as an outgroup. Type strains are indicated with a superscript^T.

recA gene phylogeny

The strains clustered into 2 groups with very high bootstrap values (97 for group I and 93 for group II) (Figure 4). There were 14 strains from Ghardaïa in group I, and they were closely related to type strains *S. meliloti* USDA 1002^T and *S. kummerowiae* CCBAU 71714^T. There were 10 strains (G122, G124, G211, G241, G242, G312, G315, G32, G421 and G422) clustered with *S. meliloti* USDA 1002^T at sequence similarities of 100% and 4 strains clustered with the reference strain *S. kummerowiae* CCBAU 71714^T at sequence similarity of 99.24%. Thirty-four strains in group II from the El Oued and Ouargla regions (except G131, G132 and G522) are not grouped with any of the known reference strains and form an individualized clade on the tree. The similarities between these strains and *S. meliloti* USDA 1002^T and *S. kummerowiae* CCBAU 71714^T were 98.22 to 98.67%.

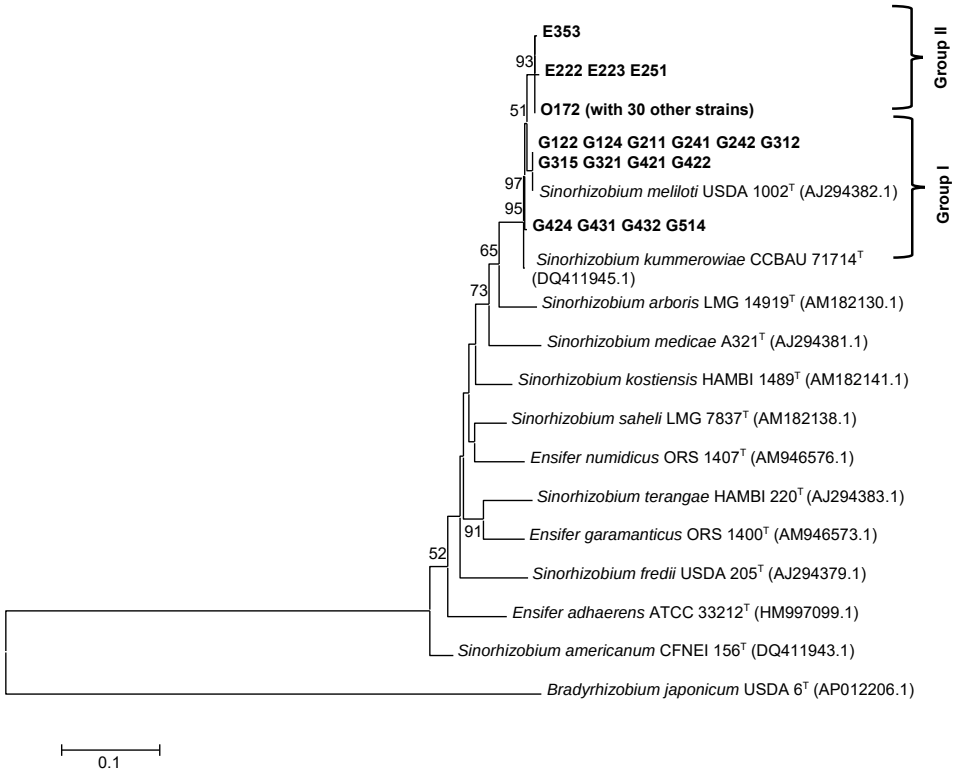

Figure 4. Neighbor-joining tree constructed from *recA* gene (430 bp) showing phylogenetic relationships of strains nodulating alfalfa and related species of the *Sinorhizobium–Ensifer* group. Only values over 50 % are indicated above the branches.

Concatenated housekeeping gene phylogeny

In order to refine the phylogeny of the studied strains, a phylogenetic tree was constructed from concatenated *glnII* and *recA* gene sequences (Figure 5). The grouping results were similar to those of the individual gene trees. The 48 strains clustered into 2 different groups. Fourteen strains belong to group I with a bootstrap value of 98. Seven strains (G122, G124, G211, G242, G315, G321 and G312) were grouped with the *S. meliloti* USDA 1002^T at sequence similarity of 99.48 to 99.69% and seven others (G431, G432, G514, G424, G241, G421 and G422) were linked with *S. kummerowiae* CCBAU 71714^T at similarity rates of 99.38 to 99.69%. It should be noted that all the strains composing group I came from the region of Ghardaïa. Group II, composed of 34 strains coming exclusively from the El Oued and Ouargla regions (except G131, G132 and G522), formed a clearly separated group from the reference strains with 97 bootstrap support and suggested that these novel strains may represent a distinct lineage from defined species. Similarities between the group 2 strains and the closest reference strains are 97.73–98.25% with *S. meliloti* USDA 1002^T and 98.04–98.56% with *S. kummerowiae* CCBAU 71714^T, respectively.

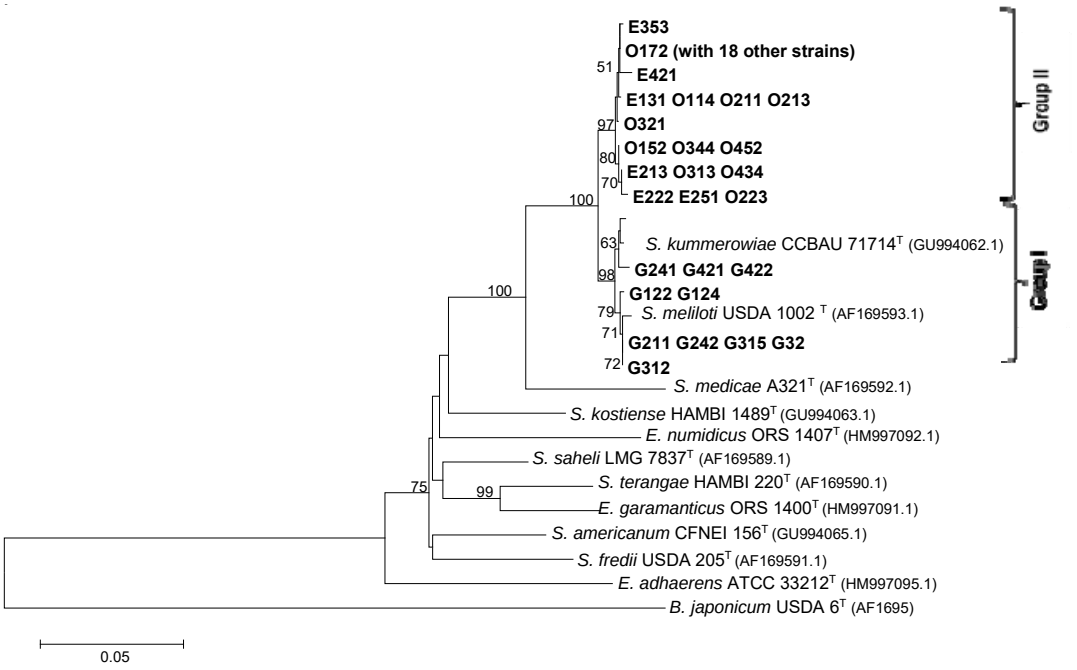

Figure 5. Neighbor-joining tree constructed from concatenated housekeeping genes *glnII* and *recA* (985 bp) showing phylogenetic relationships of strains nodulating alfalfa and related species of the *Sinorhizobium–Ensifer* group. Bootstrap values (1000 replicates; only values over 50 % are given) are indicated above the branches.

3.4.2. nodC gene phylogeny

The *nodC* phylogenetic tree showed three well-supported distinct groups, at bootstrap value of 100 for groups I and II, and 99 for group III, as presented in Figure 6. The group I and II strains from different areas were clustered with type strains *S. meliloti* USDA 1002^T at similarity of 97.05 to 99.66% and *S. medicae* A321^T at similarity of 99.65 to 100%, respectively. The strains G424, G431, G432 and G514 composing group III displayed high sequence identities with *S. meliloti* LAIII42 (99.66% similarity) and came from the region of Ghardaïa. Thus, the strains used in this study belong to two types of symbiovars: *meliloti* (groups I and II) and *medicaginis* (group III) described by Villegas *et al.* (2006).

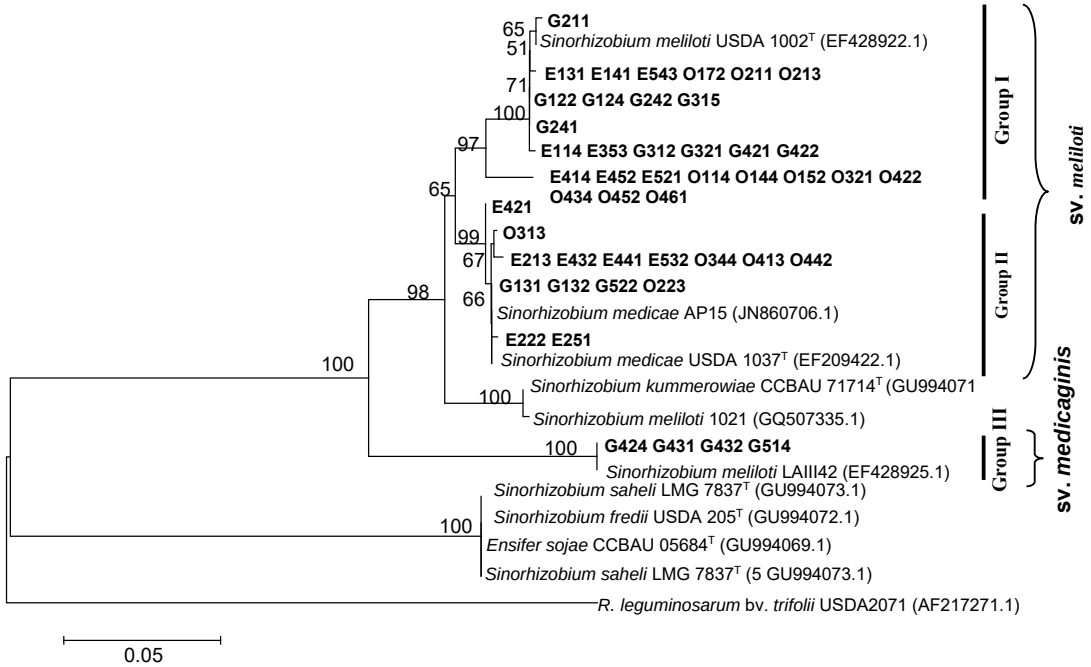

Figure 6. Neighbor-joining tree constructed from *nodC* genes showing phylogenetic relationships of strains nodulating alfalfa and related species of the *Sinorhizobium–Ensifer* group. Bootstrap values (1000 replicates; only values over 50 % are given) are indicated above the branches.

4. Discussion

In this study, we analyzed for the first time a collection of 48 strains obtained from *Medicago sativa* root nodules collected from three different regions in the North eastern Algerian Sahara. This study included both phenotypic and phylogenetic analyses and was the first time such an investigation was conducted on rhizobial isolates from this region.

The phenotypic characterization showed that morphological and growth characteristics of the strains were in agreement with those already described in the literature (Vincent, 1970; Latrache et *al*., 2017). The results of the plant nodulation tests showed that all strains could produce nodules.

Salinity is an important stress factor for rhizobia, as it inhibits their growth and development (Graham, 1998; Farissi *et al.*, 2014). In the present study, all strains were able to tolerate salt concentrations from 1.7 mM to 640 mM (Table 2). In contrast, no strains were able to grow at 1280 mM. Our results are in agreement with those of Mohammad *et al.* (1991), Embalomatis *et al.* (1994) and Jebara *et al.* (2000) who indicated that strains of *S. meliloti* were tolerant of NaCl concentrations between 300–700 mM in American, Greek and Tunisian soils respectively. Likewise, a tolerance of up to 800 mM of NaCl was observed in rhizobia collected in the Sebkha of Misserghine (north-western Algeria) (Merabet *et al*., 2006). Elboutahiri *et al*. (2010) were able to isolate strains of *S. meliloti* capable of growing at 1711 mM NaCl. These were sampled in areas heavily affected by salinity in southern Morocco.

At 80 and 160 mM, the most resistant strains were O152, O211 and E141 and at the highest concentrations, strains E543 and E452 performed best. Adaptation to salinity in a few *Rhizobium* species is the result of intracellular accumulation of low molecular weight organic solutes called osmolytes, as described by Boscari et al. (2002)

At pH of 4.0 and 5.0, strains were sensitive thus confirming the results of Elboutahiri *et al.* (2010) and Thami-Alami *et al.* (2010) that strains tolerated acidic pH of 5.5 to 6.0 where most isolates grow (Latrache *et al*., 2017). Our results are also in agreement with those of Abolhasani *et al.* (2010), Thami-Alami *et al.* (2010) and Hameed *et al*. (2014) who indicate that the strains of *S. meliloti* nodulating alfalfa were all resistant to the alkaline pH 8.0 and 9.0. At pH 6.8, all strains showed maximum growth, which is in agreement with Rodrigues *et al.* (2006) and Shetta *et al.* (2011) who stated that the optimum pH for rhizobia multiplication is between 6.5 and 7.0.

At temperatures 28°C and 37°C, we recorded 100% growth. Our results agree with those of Zahran (1999) and Dekak (2018) who found that rhizobia are mesophilic bacteria and optimum growth of most strains is between 28°C and 31°C. At 4°C and 45°C, the strains that were able to grow only produced a few small colonies on solid medium and low OD's in liquid medium. High and low temperatures have been reported to be among the main factors limiting growth and nitrogen fixation by rhizobia (Niste *et al.*, 2015).

We found that the strains show different antibiotic resistance profiles. The sensitivity to antibiotics, which is higher in some strains, can be attributed to the less abundant microbial biomass in the rhizosphere (Grego *et al.* 1995). The more pronounced resistance in Ghardaïa strains can be explained by the use of large amounts of intensive livestock manure in this region as noted by Guessoum *et al.* (2014), where antibiotics are commonly added to animal feed to treat diseases and promote growth (McManus, 1997).

Many studies have described *S. meliloti* and *S. medicae* as the only rhizobia capable of nodulating alfalfa. Phylogenetic analysis of *recA* and *glnII* showed that instead it is *S. meliloti* and *S. kummerowiae*, which are the microsymbionts of *M. sativa* in the Saharan regions of Algeria. The strains were more closely related to *S. meliloti* and *S. kummerowiae* than to other species. Sequence analysis of *recA* and *glnII* revealed high similarities with type strains *S. meliloti* USDA1002^T and *S. kummerowiae* CCBAU71714^T. Our results converge with those of Wei *et al.* (2002) and Toularoud *et al.* (2016) who mentioned that alfalfa could be efficiently nodulated by *S. kummerowiae* in Chinese and Turkish soils. The presence of *S. kummerowiae* in the study areas was reported recently by Arbi *et al.* (2015) and Chaïch *et al.* (2017) as dominant microsymbiont, with *S. meliloti*, of the spontaneous legumes *Medicago littoralis*, *Melilotus indicus* and *Genista saharae*.

The concatenated housekeeping gene phylogeny showed that the strains were grouped into 2 different groups. Group I comprises strains from Ghardaïa, strongly related to type strains *S. kummerowiae* CCBAU 71714^T and *S. meliloti* USDA 1002^T. The thirty four strains composing group II were separated from the reference strains, and this suggested that these novel strains may represent a distinct lineage from defined species. As suggested by Toularoud *et al.* (2016), housekeeping gene phylogenetic analyses may help to further resolve the taxonomic relationship between *S. kummerowiae* and *S. meliloti*, which may belong to a single species. The grouping results were, generally, similar to those of the individual gene (Figures 3 and 4).

The results obtained were very similar to those of the concatenated housekeeping gene phylogenetic tree (Figure 5). The strains originating from the region of Ghardaïa are phylogenetically and phenotypically linked to the species *S. kummerowiae* and *S. meliloti* and separated from those of the regions of Ouargla and El Oued. These results revealed geographic variation in the rhizobial population composition as shown in many works (Fierer and Jackson, 2006; Talebi *et al*., 2008).

Neighbor-joining phylogenetic tree of *nodC gene sequences* revealed three distinct well-supported clusters (Figure 6). Twenty-four strains were in group I that consisted of strains related to *S. meliloti* USDA 1002^T. Group II consisted of thirteen strains related to the type strain *S. medicae* A321^T. Four separate strains forming group III showed 100% similarity to *S. meliloti* LAIII42. To label resulting groups, we used the system of symbiovars proposed by Rogel *et al.* (2011) and De Meyer *et al.* (2011). So, the *Sinorhizobium* isolates used in this study belonged to two symbiovar types, *meliloti* (group I and II) and *medicaginis* (group III) as described by Villegas *et al.* (2006). These results are explained by the fact that the tested strains and *S. meliloti* and *S. medicae* have the same host plant.

Several studies have shown that the evolutionary history of chromosomal genes may be different from that of symbiotic genes. Our results are in agreement with those of Laguerre *et al.* (2001) who indicated that the classification resulting from the analysis of the symbiotic *nodC* gene reflects host specificity, while the phylogeny based on chromosomal genes is independent of the host plant. Symbiovars can be shared by different species due to lateral transfer of symbiotic genes (Rogel *et al.*, 2011).

5. Conclusion

This study showed that the bacteria isolated from the root nodules of *Medicago sativa,* cultivated in the Algerian Sahara, are genetically and phenotypically diverse. Phenotypic analysis showed that many strains have interesting characteristics. This may allow them to be used as an effective inoculum for Saharan soils, which are subjected too many edaphoclimatic stress conditions. Phylogenetic analysis showed that our strains are strongly related to *S. meliloti* and *S. kummerowiae*, which are the effective symbiotic partners of alfalfa in the study area. There was a similarity between the genotypic and phenotypic profiles suggesting the existence of a relationship between the groups of strains and their geographic distribution.

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References

Abolhasani M, Lakzian A, Tajabadipour A and Haghnia G. 2010. The study salt and drought tolerance of *Sinorhizobium* bacteria to the adaptation to alkaline condition. *Aust J Basic & Appl Sc*, **4**: 882-886.

Ahmad E, Zaidi A, Khan MS (2016) Effects of plant growth promoting rhizobacteria on the performance of greengram under field conditions. Jordan J Biol Sci 9: 79-88.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol*, **215**: 403-410.

Arbi SB, Chekireb D, Quatrini P, Catania V, Cheriet D and Ouartsi A. 2015. Phenotypic and genotypic characterization of root nodules rhizobia of *Medicago littoralis* Rhode and *Melilotus indicus* (L.) All. growing in the Oasis of Touggourt, Oued Righ Valley, in the Algerian Sahara. *Symbiosis*, **66**: 75-87.

Azib S, Chelouf H, Attab S and Bouras N. 2019. Improvement of alfalfa growth under water stress by inoculation with *Sinorhizobium meliloti* strains from the Algerian Sahara. *International Journal*, **75**(7): 35-43.

Boscari A, Mandon K, Dupont L, Poggi MC and Le Rudulier D. 2002. BetS is a major glycine betaine/proline betaine transporter required for early osmotic adjustment in *Sinorhizobium meliloti*. *J Bacteriol*, **184**: 2654-2663.

Brígido C, Nasciment FX, Duan J, Glick BR and Oliveira S. 2013. Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Mesorhizobium* spp. reduces the negative effects of salt stress in chickpea. *FEMS Microbiol Lett*, **349(1)**: 46-53.

Chaïch K, Bekki A, Bouras N, Holtz MD, Soussou S, Mauré L, Brunel B, De Lajudie P and Cleyet-Marel JC. 2017. Rhizobial diversity associated with the spontaneous legume *Genista saharae* in the northeastern Algerian Sahara. *Symbiosis*, **71**: 111-120.

Dekak A, Chabi R, Menasria T and Benhizia Y. 2018. Phenotypic characterization of rhizobia nodulating legumes *Genista microcephala* and *Argyrolobium uniflorum* growing under arid conditions. *J. Adv. Res*, **14**:35–42.

De Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins DM, Dreyfus B, Kersters K and Gillis M. 1994. Polyphasic Taxonomy of Rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Evol Microbiol*, **44**: 715-733.

De Meyer SE, Van Hoorde K, Vekeman B, Braeckman T and Willems A. 2011. Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). *Soil Sci Am J*, **43**: 2384-2396.

Del Papa MF, Balagué LJ, Sowinski SC, Wegener C, Segundo E, Abarca FM, Toro N, Niehaus K, Pühler A, Aguilar OM, Martínez-Drets G and Lagares A. Isolation and characterization of alfalfa-nodulating rhizobia present in acidic soils of central Argentina and Uruguay. *Appl Environ Microbiol*, **65**: 1420-1427.

del Pozo A, Ovalle C, Espinoza S, Barahona V, Gerding M and Humphries A. 2017. Water relations and use-efficiency, plant survival and productivity of nine alfalfa (*Medicago sativa* L.) cultivars in dryland Mediterranean conditions. *Eur J Agron*, **84**: 16-22.

Domínguez-Ferreras A, Pérez-Arnedo R, Becker A, Olivares J, Soto MJ and Sanjuán J. 2006. Transcriptome profiling reveals the importance of plasmid *pSymB* for osmoadaptation of *Sinorhizobium meliloti*. *J Bacteriol*, **188(21)**: 7617-7625.

Elboutahiri N, Thami-Alami I and Udupa SM. 2010. Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco. *BMC Microbiol*, **10**: 1-13.

Embalomatis A, Papaxosta D and Katinakis P. 1994. Evaluation of *Rhizobium mdiloti* strains isolated from indigenous populations in Northern Greece. *J Agron Crop Sci*, **172**: 73-80.

Farissi M, Ghoulam C and Bouizgaren A. 2014. The effect of salinity on yield and forage quality of alfalfa populations in the Marrakech region (Morocco). *Fourrages*, **219**: 271-275.

Fierer N and Jackson RB. 2006. The diversity and biogeography of soil bacterial communities. *Proc Natl Acad Sci*, **103**: 626-631.

Gibson A. 1980. Methods for legumes in glasshouses and controlled environment cabinets. In: Bergersen F.J. (Eds.), **Methods for Evaluating Biological Nitrogen Fixation**. John Wiley and Sons Ltd., Chichester, UK, pp. 139–184.

Graham PH. 1998. Biological dinitrogen fixation: symbiotic. Principles and applications of soil microbiology, **2**: 222-241.

Grego S, Quatrini P, Badalucco L, De Cesare F, Zanotti C and Cacciari I. 1995. Souches résistantes de *Rhizobium* dans une rhizosphère caractérisée d'Acacia au nord et au sud du Sahara. In : Ed. Pontanier R., M'Hiri A., Akrimi N., Aronson J., Le Floc'h E. (Eds.), **L'homme peut-il refaire ce qu'il a défait ?**. John Libbey Eurotext, Paris, 201-210.

Guessoum H, Benbrahim F, Halilat M, Laouar F, Bensalama M and Darem S. 2014. Pollution biologique des eaux phréatiques de la région de Ghardaïa (Cas de Sebseb). *Int J Adv Sci Technol*, **3**: 35-43.

Hall TA.1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser*, **81**: 95-98.

Hameed RA, Hussain NN and Aljibouri AM. 2014. Phenotypic Characterization of Indigenous Iraqi *Sinorhizobium meliloti* Isolates for Abiotic Stress Performance. *J Life Sci*, **8(1)**: 1-9.

Jebara M, Aouani ME, Mhamdi R, Ghrir R and Mars M. 2000. Effet du sel sur des isolats de *Sinorhizobium* sp. de Tunisie *in vitro* ou en association avec *Medicago* sp. *Cah Agric*, **9**: 99-102.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*, **16**: 111-120.

Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P and Amarger N. 2001. Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology*, **147**: 981-993.

Latrach L, Mouradi M, Farissi M, Bouizgaren A and Ghoulam C. 2017. Physiological characterization of rhizobial strains nodulating alfalfa (*Medicago sativa*) isolated from soils of Southeastern Morocco. *Appl J Envir Eng Sci*, **3(4)**:353-364.

Martens M, Delaere M, Coopman R, De Vos P, Gillis M and Willems A. 2007. Multilocus sequence analysis of *Ensifer* and related taxa. *Int J Syst Evol Microbiol*, **57**: 489-503.

Massimi M, Haseeb M, Kanga L and Legaspi J. 2017. Enhancement of silage sorghum and corn production using best management practices. In: Proceeding, Association of 1890 Research Directors Symposium, Georgia, Atlanta, United States of America.

McManus MC. 1997. Mechanisms of bacterial resistance to antimicrobial agents. *Am J Health-Syst Pharm*, **54**: 1420-1433.

Merabet C, Bekki A, Benrabah N, Baba-Hamed Bey M, Bouchentouf L, Ameziane H, Rezki MA, Domergue O, Cleyet-Marel JC, Avarre JC, Béna G, Bailly X and De Lajudie P. 2006. Distribution of *Medicago* species and their microsymbionts in a saline region of Algeria. *Arid Land Res Manag*, **20:** 219-231.

Mohammad R, Akhavan-Kharazian M, Campbell W and Rumbaugh M. 1991. Identification of salt-and drought-tolerant *Rhizobium meliloti* L. strains. *Plant Soil*, **134**: 271-276.

Mouradi M, Bouizgaren A, Farissi M, Latrach L, Qaddoury A and Ghoulam C. 2016. Seed osmopriming improves plant growth, nodulation, chlorophyll fluorescence and nutrient uptake in alfalfa (*Medicago sativa* L.) rhizobia symbiosis under drought stress. *Sci Hortic*, **213**: 232–242.

Niste M, Vidican R, Rotar I and Pop R. 2015. The Effect of temperature stress on *Rhizobium trifolii* and *Sinorhizobium meliloti* strains *in vitro*. ***Bull Univ Agric Sci Vet Med Cluj Napoca*, 72(1)**: 297-298.

OSS. 2003. **Système aquifère du Sahara septentrional: gestion commune d'un bassin transfrontière**. Rapport de synthèse, Observatoire du Sahara et du Sahel, Tunisie.

Rodrigues CS, Laranjo M and Oliveira S. 2006. Effect of heat and pH stress in the growth of chickpea mesorhizobia. *Curr Microbiol*, **53**: 1-7.

Rogel MA, Ormeno-Orrillo E and Romero EM. 2011. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst Appl Microbiol*, **34**: 96-104.

Rome S, Fernandez MP, Brunel B, Normand P and Cleyet-Marel JC. 1996. *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int J Syst Evol Microbiol*, **46**: 972-980.

Sarita S, Sharma PK, Priefer UB and Prell J. 2005. Direct amplification of rhizobial *nodC* sequences from soil total DNA and comparison to *nodC* diversity of root nodule isolates. *FEMS Microbiol Ecol*, **54**: 1-11.

Shetta N, Al-Shaharani T and Abdel-Aal M. 2011. Identification and characterization of *Rhizobium* associated with woody legume trees grown under Saudi Arabia condition. *Am Eurasian J Agric Environ Sci*, **10**: 410-418.

Somasegaran P and Hoben HJ. 1985. **Methods in legume-*Rhizobium* technology**. University of Hawaii NifTAL Project and MIRCEN, Department of Agronomy And Soil Science Hawaii Institute Tropical Agriculture Human research.

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ ... and Whitman WB. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol*, **52:** 1043-1047.

Tabares-da Rosa S, Signorelli S, Del Papa M, Sabatini O, Reyno R, Lattanzi F, Rebuffo M, Sanjuán J and Monza Galetti J. 2019. *Rhizobium* inoculants for alfalfa in acid soils. *Agrocienc Urug*, **23(2)**: 1-13.

Talebi MB, Bahar M, Saeidi G, Mengoni A and Bazzicalupo M. 2008. Diversity of *Sinorhizobium* strains nodulating *Medicago sativa* from different Iranian regions. *FEMS Microbiol Lett*, **288**: 40-46.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, **30**: 2725-2729.

Thami-Alami I, Elboutahiri N and Udupa S. 2010. Variability in natural populations of *Sinorhizobium meliloti* in Morocco. *Options Mediterr*, *Série A. Séminaires Méditerranéens*, **92**: 265-269.

Toularoud AS, Asersec AA, Alikhania HA, Jouzanid GRS, Rahmanid HA, Khavazid K, Räsänene LA and Lindström K. 2016. Taxonomic diversity and symbiotic efficiency of rhizobial strains obtained from nodules of *Medicago sativa* growing in Iran. *Ann Biol Res*, **7**: 29-38.

Turner SL and Young JPW. 2000. The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol*, **17**: 309-319.

Villegas MDC, Rome S, Mauré L, Domergue O, Gardan L, Bailly X, Cleyet-Marel JC and Brunel B. 2006. Nitrogen-fixing sinorhizobia with *Medicago laciniata* constitute a novel biovar bv. *medicaginis* of *S. meliloti*. *Syst Appl Microbiol*, **29**: 526-538.

Vincent JM. 1970. **A manual for the practical study of the root-nodule bacteria**. Blackwell Science Ltd., UK.

Vinuesa P, Silva C, Werner D and Martínez-Romero E. 2005. Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol Phylogenetics Evol* **34**: 29-54.

Wei GH, Wang ET, Tan ZY, Zhu ME and Chen WX. 2002. *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. *Int J Syst Evol Microbiol,* **52**: 2231-2239.

Wei W, Jiang J, Li X, Wang L and Yang S. 2004. Isolation of salt-sensitive mutants from *Sinorhizobium meliloti* and characterization of genes involved in salt tolerance. *Lett Appl Microbiol*, **39**: 278-283.

Zahran HH. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol Rev*, **63**: 968-989.

Zakhia F and De Lajudie P. 2006. La taxonomie bactérienne moderne: revue des techniques-application à la caractérisation des bactéries nodulant les légumineuses BNL. *Can J Microbiol*, **52**: 169-181

Zhang F and Smith DL. 1996. Inoculation of soybean *Glycine max*.L. Merr. with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. *Plant Soil*, **179**: 233-241.

Zhang WJ and Wang T. 2015. Enhanced salt tolerance of alfalfa (*Medicago sativa*) by *rstB* gene transformation. *Plant Sci*, **234**: 110-118.