

PHENOTYPIC DIVERSITY AND BIOCHEMICAL CHARACTERISTICS OF SELECTED RHIZOBIA NODULATING THE COMMON BEAN (*Phaseolus vulgaris* L.)

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ABSTRACT

Increasing interest in using rhizobia as biofertilizers in smallholder agricultural farming systems has prompted scientists to investigate rhizobia diversity, resulting in the identification of many strains. Fifty-five *Rhizobium* strains nodulating in the common bean (*Phaseolus vulgaris* L.) were isolated from soil samples from different areas of Tunisia and phenotypically characterized to determine their symbiotic nitrogen fixation capabilities. Their tolerance to pH, salinity, temperature and alkalinity, as well as their cultural and biochemical characteristics indicated wide physiological diversity. These phenotypic characteristics significantly affected rhizobia growth, and strains of interest were identified and used in inoculation trials. They were efficient and able to tolerate pH from 4 to 9, NaCl concentrations of 25 to 100 mM, temperature variation from 10 to 40 °C, and lime (CaCO₃) from 0.05 to 0.20 mM. Selected *Rhizobium* strains were identified as candidates for biofertilizer production for a variety of Tunisian soil types.

Additional keywords: Biodiversity, *Rhizobium*, salinity, temperature

RESUMEN

Diversidad fenotípica y características bioquímicas de cepas de *Rhizobium* nodulando el frijol (*Phaseolus vulgaris* L.)

El creciente interés en el uso de rizobios como biofertilizantes en sistemas agrícolas de pequeña escala ha llevado a investigar su diversidad, lo que ha resultado en la identificación de muchas cepas. En este estudio se aislaron 55 cepas de *Rhizobium* que nodulaban en frijol común (*Phaseolus vulgaris* L.) en muestras de suelo de diferentes áreas de Túnez y se caracterizaron fenotípicamente para determinar su capacidad de fijación simbiótica de nitrógeno. Su tolerancia a la salinidad, pH, temperatura y alcalinidad, así como las características culturales y bioquímicas indicaron una amplia diversidad fisiológica. Estas características fenotípicas afectaron significativamente el crecimiento de los rizobios, los cuales se identificaron y las cepas de interés se utilizaron en ensayos de inoculación. Estas cepas fueron eficientes y capaces de tolerar un pH de 4 a 9, concentraciones de NaCl de 25 mM a 100 mM, variaciones de temperatura entre 10 y 40 °C, y encalado (CaCO₃) de 0,05 a 0,20 mM. Se identificaron cepas de *Rhizobium* como candidatas para la producción de biofertilizantes para una variedad de tipos de suelos tunecinos.

Palabras clave adicionales: Biodiversidad, *Rhizobium*, salinidad, temperatura

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is a widely cultivated food crop, representing 50 % of the leguminous grain directly consumed by humans. It provides a good source of plant proteins, potentially reducing animal consumption. It is also an important energy

source. In addition to the dietary benefits, the bean can also benefit agricultural production; in this sense, Meng (2015) noted that the benefits of yield increase of bean are associated with biofertilizers.

One of the significant features of the common bean plant is its capability of establishing a symbiotic relationship with specific bacteria and

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developing biological N₂ fixation in root nodules that provides required N to the plant (Hungria et al., 2003; Mwenda et al., 2018; El Attar et al., 2019). The N₂ fixation process is associated with the host plant's physiological state in the *Rhizobium*-legume symbiosis. Therefore, a rhizobial strain may not express its full capacity for N₂ fixation, specifically if certain soil or environmental factors limit the vigour of the host legume. These factors may include extreme temperature or unfavourable soil pH or salinity, among others (Kaymakanova et al., 2008; Cao et al., 2014; Hungria et al., 2015). Stressors might exert multiple effects; for example, salinity might lead to water stress (affecting the photosynthetic rate) or directly impact nodule initiation and metabolism. The most challenging environments for rhizobia are marginal land that receives minimal precipitation, experiences high temperatures and has acidic soil with low nutrient concentrations and poor water holding capacity (Bargaz et al., 2012; Kumar et al., 2016; Chekani et al., 2018).

In some of these regions, inoculation of crops with non-native strains of common bean rhizobia has been utilized to improve crop production, but those areas experienced extremely low yields. The limited yield was not attributed to the nitrogen-fixing potential of the common bean–*Rhizobium* symbiosis but the symbiont's non-adaptability to the osmotic stress prevailing in those regions. Arid soils may contain strains adapted to the severe environmental stresses of pH variation, high soil temperature and drought (Musandu and Ogenko, 2001; Yanni et al., 2016). These strains should be considered for use as bioinoculants in regions facing severe water shortages. Their successful inoculation depends on their N₂ fixation capabilities and adaptability to adverse soil conditions, such as increased drought and salinity. The current research aims to examine the phenotypic diversity and nodulation ability of the adapted and efficient rhizobia nodulating *Phaseolus vulgaris* L. in Tunisia to select the best strain for the production of biofertilizer inoculants in the region.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Fifty-five rhizobia isolates were extracted from root

nodules of common beans (*Phaseolus vulgaris* L.) during the flowering stage. Following the criteria defined by Vincent (1970), one isolate was extracted from each nodule. Yeast extract–mannitol (YEM) medium was used to cultivate, purify and isolate these rhizobia. The strains were stored in tubes at 4 °C on YEM agar media. Table 1 provides a list of all isolates.

Symbiotic traits. A common bean variety, 'Coco Blanc' registered in the official Tunisian catalogue, was used as plant material. 'Coco Blanc' seeds were sterilized for 1 minutes with calcium hypochlorite (3 %), then washed gently four times with distilled water. They were allowed to germinate in Petri dishes with sterile moistened blotting paper for three days. The Laboratory of Agricultural Sciences and Techniques, National Institute of Agronomic Research in Tunisia (INRAT, Tunisia) provided local and introduced rhizobia and common bean seeds (Table 1). Rhizobial inoculants prepared as liquid cultures in YEM medium (10⁹ cells·mL⁻¹) were introduced by soaking seedlings for 30 minutes in the inoculants before transplanting into plastic growth pots (0.5 kg of sterile perlite). The nodulation parameters and their effects on nodule number and biomass were measured for 55 infective strains. Symbiotic testing of the rhizobia strains was replicated four times. Irrigation was performed twice weekly, adding 40 ml of a nutrient solution without nitrogen to each pot (Vincent, 1970). Results are shown in Table 1. A non-inoculated common bean was used as a control to assess both nodule number and biomass and shoot and root biomass.

Physiologic and biochemical tests. Other than biochemical substrate assimilation, all tests were conducted in triplicate on 24 cm² YEM agar plates divided into 20 equal squares. Each square was spot-inoculated with 10 µl of the strain culture previously used as the liquid medium in the exponential phase ($\pm 10^9$ cells·mL⁻¹). The inoculated plates were incubated at 28 °C for six days at which time the colony growth was visually monitored. The classification of the strains (from very sensitive to tolerant) was based on measuring the bacterial colonies diameters cultivated under different conditions and compared to the colony diameters under optimal conditions. Those ratios were compared according the following arbitrary scale: lower than 0.5= very sensitive; between 0.5

and 0.99= sensitive; between 1.0 and 1.5 = moderately tolerant; greater than 1.5 = tolerant.

- **Salt tolerance.** Tolerance to sodium chloride (NaCl) was determined by measuring colony

growth of the rhizobia strains incubated at 28 °C for six days on YEM solid medium supplemented with 25, 50, 75 and 100 mM NaCl. The negative control was YEM without NaCl.

Table 1. Origin of rhizobia strains used for common bean plant nodulation test

N°	Reference	Origin	Isolation year	N°	Reference	Origin	Isolation year
1	CIAT899	International Center for Tropical Agriculture	1899	29	KHT3.96	Nabeul-Tunisia	1996
2	Alia1	Bizerte-Tunisia	1996	30	Ar3	Ariana-Tunisia	2008
3	Alia2.96	Bizerte-Tunisia	1996	31	Ar1	Ariana-Tunisia	2008
4	Tinja	Bizerte-Tunisia	1993	32	Ar6	Ariana-Tunisia	2008
5	Ar02	Ariana-Tunisia	2002	33	Ar4	Ariana-Tunisia	2008
6	Ar05	Ariana-Tunisia	2005	34	Ar2	Ariana-Tunisia	2008
7	P.Ar.09	Ariana-Tunisia	2009	35	S1	Ariana-Tunisia	1994
8	P.Bj	Beja-Tunisia	2009	36	J1.96	Bizerte-Tunisia	1996
9	P.OM.09	Oued Meliz-Tunisia	2009	37	J2.96	Bizerte-Tunisia	1996
10	P.Ps. 09	Phosphate Gafsa-Tunisia	2009	38	J3.96	Bizerte-Tunisia	1996
11	CB	Cap bon-Tunisia	1993	39	J1.92	Bizerte-Tunisia	1996
12	P.Tb.09	Teboursek-Tunisia	2009	40	J3.92	Bizerte-Tunisia	1992
13	SOM	Maroc	1998	41	J4.92	Bizerte-Tunisia	1992
14	D4.007	INRA Montpellier	2007	42	S3	Ariana-Tunisia	1994
15	D4.002	INRA Montpellier	2002	43	S7	Ariana-Tunisia	1994
16	KHS1	INRA Montpellier	-	44	S9	Ariana-Tunisia	1994
17	KHS2	INRA Montpellier	-	45	S11	Ariana-Tunisia	1994
18	GB.92	INRA Montpellier	1992	46	Raf .Raf	Bizerte-Tunisia	1992
19	GB.258	INRA Montpellier	1992	47	Ras.JB	Bizerte-Tunisia	1992
20	KH28	INRA Montpellier	-	48	Soudan1.2	Nabeul-Tunisia	2007
21	Fr1.97	Fernana-Tunisia	1997	49	Soudan2.2	Nabeul-Tunisia	2007
22	OM	Oued Méliz-Tunisia	2001	50	D2.2	Bizerte-Tunisia	2002
23	Mat.9	Mateur-Tunisia	1994	51	D3.2	Bizerte-Tunisia	2002
24	Zaar	Mateur-Tunisia	1996	52	Artn1	Ariana-Tunisia	2002
25	ZG.96	Zaghouan-Tunisia	1996	53	Ic.208	Ariana-Tunisia	2002
26	B155	CIRAD	-	54	12.a3	Ariana-Tunisia	2002
27	S10	Ariana-Tunisia	1994	55	YH15	Ariana-Tunisia	2002
28	KHT1.96	Nabeul-Tunisia	1996				

- **Ability of rhizobia to tolerate pH variation.** YEM agar medium with pH adjusted to 4, 5, 6, 7, 8 or 9 using NaOH or HCl was used to test the ability of the 55 strains to grow in acidic or alkaline media.

- **Tolerance of rhizobia to temperature variation.** YEM agar plates were inoculated and incubated at 10 to 40 °C to study colony growth, determined by measuring their change in diameter.

- **Lime-scale concentration.** The ability of the 55 rhizobia strains to tolerate limestone was evaluated on YEM agar supplemented with Congo red, containing 0.05 mM, 0.10 mM, 0.15 mM, and 0.20 mM CaCO₃. Samples were incubated at 28 °C, and colony diameters were measured after six days. Each treatment was repeated four times.

- **Biochemical substrate assimilation.** API galleries (API 20E; BioMerieux) were used to test *Rhizobium* strains' assimilation and fermentation of carbohydrates and enzymatic activity, according to Kersters and Deley (1984). Strain inoculants were grown on YEM agar plate cultures, and then the bacteria were washed and re-suspended in 100 mL of a Bergersen solution (Vincent, 1970). Strain galleries were incubated at 28 °C, and results were recorded after 2–3 days.

Harvest and data analysis. Plants in pots were inoculated, under greenhouse conditions, with each *Rhizobium* bacteria strain. For each treatment, four plants were harvested in their early flowering stage. Nodules were removed from the roots, and plants were separated into roots and shoots before drying at 70 °C in an oven for 72

hours. Dry weights were measured, and shoot samples were individually ground to determine the N content based on the Kjeldahl method. The complete root system with nodules of the four plants inoculated with the same strain was pooled to conduct field trials. The shoots and roots of each plant were gently rinsed and placed in paper bags. Symbiotic parameters were measured, including the nodule dry weight (NDW), nodule number, shoot and root dry weight (SDW, RDW), and nitrogen content per plant during the flowering stage.

Statistical analysis. The SPSS statistical program (v. 20) was used for ANOVA, and Duncan's multiple range test for mean comparisons.

RESULTS

Nodulation test

- **Nodule number and biomass.** A high level of variability was detected among the nodule number and dry weight of the 55 tested rhizobia strains (Table 2). Strain Ar02 produced the most nodules (134) and the highest dry weight (0.0375 g per plant). Allia02.96 was the second most productive, with 89 nodules and 0.02 g per plant of dry weight. Although the nodules of most nodulating strains were approximately the same size, examining the proportionality between the number and the weight of the nodules revealed that strain Allia02.96 had smaller nodules than other strains.

- **Common bean shoot and root biomass production.** The results of biomass production assays are shown in Table 3. Rhizobia strain inoculation caused the shoot dry weight to increase. Specifically, the strains P.Ps, Alia2.96, Ar02, and Ar05 produced the greatest shoot dry biomass weight, from 0.505 to 0.437 g per plant.

The root dry weight was also affected by rhizobia inoculation. Plants inoculated with strains S3, S11, Soudan2.2, B155, D2.2, Tinja, S9, Ic.208, and Alia1 exhibited enhanced root growth. Root biomass varied from 0.267 to 0.17 g per plant. The remaining strains were not significantly different from the control in root biomass production.

Some rhizobia strains enhanced both root and shoot weight. This balance of growth between the shoot and root obtained using many of these rhizobia indicates that specific strains are

promising bacteria for enhancing growth in common bean plants.

- **Nitrogen content.** Nitrogen content in common bean shoots inoculated with rhizobia strains varied between 2.85% and 1.19% (Table 4). We distinguished up to seven rhizobia strain classes of shoot nitrogen content in the common bean. The most important class of rhizobia strains with regard to nitrogen content included J3.96 (2.85 % N), J3.92 (2.85 %), and KHT1.96 (2.83 %).

Physiological testing of *Rhizobium* strains. Rhizobial bacteria showed significant growth variability in culture salinity of 25 to 100 mM NaCl, and exhibited diverse behaviour under pH variation, with most of the strains tested tolerating basic media (pH 8 and 9). There were also highly significant growth differences between strains by incubation temperature; only KHS1 and KHT1.96 strains tolerated low temperatures (10 °C), but most grew at higher temperatures, with two strains, P.OM.09 and Ras.JB, showing thermophile behaviour. At 10 °C, growth of Mat.9 and B155 strains was totally inhibited. On the other hand, most strains (60 %) were sensitive in the presence of CaCO₃, although D2.2, D3.2, 12.a3, Ar4, KHT3.96, S7, ZG.96, Zaar, Mat.9, KHS2 and GB.258 exhibited good growth at the whole range of calcium concentrations (0.05 M to 0.20 mM)

Biochemical characterization of rhizobia. The outcomes of different biochemical tests are shown in Figure 1, which illustrates three distinct groups. The first group was formed by 28 rhizobia strains that can be classified into two subgroups. The first subgroup includes a large number of bacteria unable to metabolize IND substrates. The strains of the second subgroup were unable to metabolize IND, H₂S, VP, or urea. The strains of the second group were not able to assimilate H₂S, INO, RHA, ARA, IND, SOR, or LCD. The final strain group could not assimilate RHA, INO, SOR, or ARo.

DISCUSSION

The outcome of the nodule biomass analyses is consistent with the study of Abdi et al. (2014). Furthermore, Voisin et al. (2003) showed that an increase in nodule dry weight was associated with enhanced symbiotic efficiency during nodule growth. In addition, Graham et al. (2004) reported

that nodule dry weight is a good indicator of symbiotic efficiency and thus an important tool in strain evaluation. However, Koskey et al. (2018) discussed other nitrogen-fixing bacteria, recently identified as beta and gamma Proteobacteria, that form symbiotic relationships with legumes. All *Rhizobium* strains showed similar ratios of the number of nodules to the weight of the nodules, except strain Allia02.96, which presented smaller nodules than other strains. Most studied strains had almost the same size nodules, and some strains were non-nodulating. Biomass assays

revealed that rhizobia strain inoculation caused shoot dry weight to increase.

Nitrogen content in common bean shoots and shoot and root dry weight production was also affected by rhizobia inoculation. Some strains enhanced both shoot and root weight. Some rhizobia strains have been reported to enhance phytohormone production and mineral uptake and reduce the toxic effects of metals, thereby indirectly promoting plant growth and development (Karthik et al., 2017) in polluted agricultural soils.

Table 2. Nodulation assessment (number and biomass) on common bean inoculated root with different rhizobia strains

Rhizobia strains	Nodule number	Nodule dry weight (g·pl ⁻¹)	Rhizobia strains	Nodule Number	Nodule dry weight (g·pl ⁻¹)
Ar02	134 a	0.0375 a	Artn1	0j	0i
Alia2.96	89 b	0.020 c-e	Ar3	0j	0i
J3.92	66.75 c	0.022 cd	Ar1	0j	0i
P.Ar.09	66 cd	0.025 bc	Ar6	0j	0i
P.Ps	62 cd	0.030 ab	Ar4	0j	0i
CIAT899	54.75 c-e*	0.010 f-h	Ar2	0j	0i
P.Bj	51.75 c-f	0.020 c-e	B155	0j	0i
Tinja	50.75 c-f	0.017 d-f	CB	0j	0i
P.OM.09	51 c-f	0.020 c-e	D4.007	0j	0i
Ar05	50 c-f	0.017 d-f	D4.002	0j	0i
GB.92	47.75 c-g	0.020 c-e	D2.2	0j	0i
Ras. JB	44.75 d-g	0.015 d-g	D3.2	0j	0i
GB.258	43.75 d-g	0.017 d-f	Fr1.97	0j	0i
KHT1.96	38 e-h	0.010 f-h	YH15	0j	0i
J1.92	37 e-i	0.012 e-h	Ic.208	0j	0i
J2.96	32.5 f-i	0.012 e-h	12a3	0j	0i
KHS1	28.25 g-i	0.012 e-h	J1.96	0j	0i
OM	17 h-j	0.010 fgh	J3.96	0j	0i
P.Tb	15 ij	0.005 hi	J4.92	0j	0i
KHS2	5.2 5j	0.007 g-i	KHT3.96	0j	0i
KH28	4.5 j	0.000 i	Mat.94	0j	0i
S7	3 j	0.002 c-e	Raf. Raf	0j	0i
Zaar	2.75 j	0.002 i	S1	0j	0i
Soudan12	2 j	0.000 i	S3	0j	0i
SOM	1.75 j	0.000 i	S9	0j	0i
S11	1 j	0.001 i	S10	0j	0i
ZG.96	0.25 j	0.000 i	Soudan22	0j	0i
Alia1	0 j	0.000 i	Control	0j	0i

Data are the means of four replicates harvested at flowering stage. Means with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$). *More than two letters are represented by the first and last letter

Table 3. Shoot and root dry weight of common bean inoculated with different rhizobia strains

Rhizobia strains	Shoot dry weight (g·pl ⁻¹)	Root dry weight (g·pl ⁻¹)	Rhizobia Strains	Shoot dry weight (g·pl ⁻¹)	Root dry weight (g·pl ⁻¹)
P.Ps	0.505 a	0.145 a-d	Ar3	0.355 a-d	0.162 cd
Alia2.96	0.465 ab	0.13 b-d	Artn1	0.352 a-d	0.13 a-d
Ar02	0.445 a-c	0.097 cd	Ras JB	0.352 a-d	0.14 b-d
Ar05	0.437 a-c	0.0197 a-c	Ar6	0.352 a-d	0.117 cd
GB.92	0.425 a-d	0.12 cd	Ar4	0.351 a-d	0.099 cd
Tinja	0.42 a-d	0.18 a-d	Ar2	0.35 a-d	0.095 cd
P.Bj	0.407 a-d	0.115 b-d	Ic.208	0.350 a-d	0.17 a-d
CIAT899	0.405 a-d	0.112 b-d	J3.96	0.346 d	0.115 b-d
P.Ar.09	0.4 a-d	0.13 b-d	OM	0.345 b-d	0.0137 b-d
J1.92	0.395 d	0.117 b-d	J2.96	0.345 b-d	0.107 cd
YH15	0.392 b-d	0.14 cd	P.OM.09	0.342 b-d	0.14 b-d
P.Tb	0.390 a-d	0.155 a-d	KHS2	0.32 b-d	0.137 b-d
J4.92	0.387 d	0.102 cd	KHT3.96	0.312 a-d	0.12 b-d
Alia1	0.385 d	0.17 c	D2.2	0.312 a-d	0.185 a-d
J3.92	0.382 a-d	0.112 b-d	Soudan1.2	0.31 b-d	0.105 cd
KH28	0.375 a-d	0.102 cd	D4.007	0.307 b-d	0.145 a-d
12a3	0.372 b-d	0.125 a-d	D3.2	0.307 b-d	0.157 a-d
Raf Raf	0.37 d	0.122 d	Ar1	0.305 a-d	0.145 cd
S1	0.366 a-d	0.120 b-d	Fr1.97	0.297 d	0.095 cd
S3	0.365 a-d	0.267 cd	S9	0.297 d	0.177 cd
B155	0.365 d	0.19 cd	ZG.96	0.295 cd	0.122 b-d
SOM	0.362 a-d	0.14 b-d	Zaar	0.287 cd	0.09 cd
GB.258	0.362 a-d	0.07 d	J1.96	0.277 d	0.107 cd
S11	0.362 a-d	0.227 b-d	Control	0.277 d	0.1 cd
KHT1.96	0.357 a-d	0.142 a-d	S10	0.275 d	0.13 cd
S7	0.357 a-d	0.137 b-d	KHS1	0.272 d	0.152 a-d
Soudan2.2	0.357 a-d	0.2 cd	CB	0.267 d	0.112 cd
D4.002	0.355 d	0.16 cd	Mat.94	0.22 d	0.117 cd

Data are the means of four replicates harvested at flowering stage. Means with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$)

Table 4. Nitrogen content of common bean plants inoculated with various *Rhizobium* strains

Rhizobia strains	Nitrogen content (%)
J3.96; J3.92; KHT1.96	2.85a ; 2.85a ; 2.83a
GB.258	2.73ab
S10; J2.96; Tinja	2.48b ; 2.47b ; 2.44b
Ras.JB ; Ar05	2.34bc ; 2.33bc
J1.92 ; OM ; Alia2.96 ; KHS2 ; GB.92 ; S1	2.14c ; 2.09c ; 2.08c ; 2.02c ; 2.01c ; 2.01c
P.Bj; CIAT899; D4.002; Ar6; YH15; Ar4;	1.96cd ; 1.94cd ; 1.94cd ; 1.94cd ; 1.94cd ; 1.92cd ; 1.92cd ; 1.91cd
P.Ps; Ar02; Ar1; Soudan1.2	; 1.89cd ; 1.89cd
Control; SOM; D2.2; KHS1	1.85d ; 1.85d ; 1.82d ; 1.80d
S7; Fr1.97 ; P.Tb ; S3; J1.96; P.Ar.09;	1.73de ; 1.71de ; 1.67de ; 1.67de ; 1.66de; 1.66de; 1.66de; 1.66de
KH28; S11	
CB ; Artn1 ; Mat.94 ; KHT3.96	1.63e ; 1.63e ; 1.61e ; 1.6e
P.OM.09; S9; Ar3; Ar2; Raf .Raf; D4.007;	1.56ef ; 1.56ef ; 1.55ef ; 1.53ef ; 1.49ef ; 1.49ef ; 1.47ef ; 1.46ef ;
D3.2; B155, Ic.208;	1.45ef
12.a3; Zaar; ZG.96; J4.92	1.34f ; 1.32f ; 1.32f ; 1.27f
Soudan2.2	1.25fg
Alia1	1.19g

Data are the means of four replicates harvested at flowering stage. Means with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$)

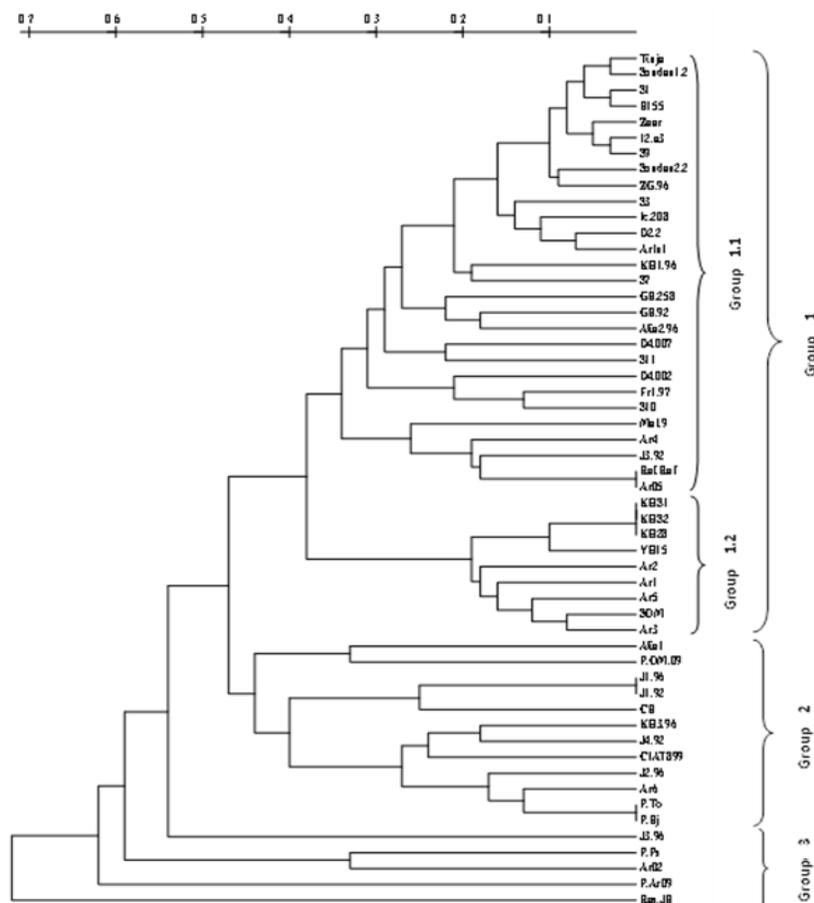


Figure 1. Rhizobia strains distribution according to their capability to assimilate various biochemical substrates

The *Rhizobium* strains demonstrated diverse capabilities of metabolizing specific carbohydrate and organic acid substrates and three large groups were formed as shown in Figure 1. In that sense, Diange and Lee (2013) found that strains of *R. sultae*, *R. etli*, *R. gallium*, and *R. indigolferae* did not react with various carbon substrates. They also demonstrate that strains of *R. yanglingense* and *R. monogolense* have a lower use of such substrates. However, the latter strain assimilated D-melibiose, L-arabinose, and L-rhamnose and had negative fermentation reactions with inositol, D-sucrose, D-mannitol, and amygdalin. Van Rossum et al. (1994) believed that rapidly growing strains preferred disaccharides, hexoses, organic acids, and pentoses. The same outcomes have been observed by Lindström and Lehtomaki (1988) regarding *Rhizobium galegae* strains. Jordan (1984) believed that slowly growing strains rarely used monosaccharides, but variably used

disaccharides. The outcome confirmed the observations of Xu (1995) for *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense*. The development of rhizobia in the soil is highly dependent on the flavonoid compounds, which may be used as a primary carbon source by slow-growing and fast-growing strains. Bradyrhizobiacea demonstrates greater use of these compounds (Parke and Ornston, 1984). Urea hydrolyzation and nitrate reduction are critical elements in choosing a specific strain. Sherwood et al. (1984) believed that a higher amount of soil nitrate would hinder the symbiotic efficiency of rhizobia within the root surface.

The degree of salt tolerance in the culture medium varied between strains. These results confirm those of Aouni et al. (1986), who showed that bacteria collected from nodules of plants of the genera *Phaseolus*, *Vicia*, *Pisum*, *Trifolium*, *Cicer*, and *Glycine* could tolerate up to 3 g·L⁻¹ of

NaCl (51 mM) and are unable to grow at 11 g·L⁻¹ (186 mM). This result is confirmed by Jebara et al. (2001), who showed that the bean variety Batt477 inoculated and hydroponically grown with indigenous strains from Tunisia were particularly tolerant to salinity. Similar results were observed by El Sheikh and Wood (1989). El Boutari (2009) reported that *R. sulae* strains tolerated salt between 170 and 850 mM NaCl. However, Squartini et al. (2002) found a lower tolerance range, ranging from 290 to 548 mM NaCl. Much higher salinity tolerance levels (1190–1700 mM NaCl) have been reported for rhizobial strains nodulating lupine (Raza et al., 2001).

The *Rhizobium* strains studied exhibited diverse behaviours with respect to pH variation in the medium. These results are confirmed by Maâtallah et al. (2001), who examined strains nodulating chickpeas that exhibited great variability in their pH tolerance, and found that 90 to 100 % of the isolates could grow in media with pH between 6 and 8; this trend could be related to the pH value ranges of soils from which the tested isolates were collected.

Graham (1998) found variability in acid tolerance for strains of *R. tropici* UMR 1999 and PRF 81 and recommended them for *P. vulgaris* in acidic soils of Brazil. According to Jordan (1984), rhizobia can tolerate pH values ranging from 4.5 to 9. Notably, our studied strains demonstrated rapid growth rates, generally tolerated alkalinity, and were sensitive to acidity. Zablotowicz and Focht (1981) reported that slow-growing strains that nodulate *Vigna unguiculata* were more sensitive to alkaline pH than fast-growing ones. Other studies have shown that fast-growing rhizobia are more sensitive to acidity than the previously mentioned bradyrhizobia (Van Rossum et al., 1994).

There were notorious differences in bacterial colony growth depending on incubation temperature. Most strains were influenced by high temperatures (40 °C), except P.OM.09 and Ras.JB strains that demonstrated thermophile behaviour. Graham (1992) reported that rhizobia are mesophilic bacteria that can grow at temperatures between 10 and 37 °C and that most strains grow at an optimum temperature of 28 °C. However, some studies have shown strains that tolerate extreme temperatures, such as those which nodulate certain legumes in arctic regions

(Lipsanen and Lindström, 1989) or those isolated in the hot and dry environment of the Sahel savannah in Africa, such as strains of *R. phaseoli*, that could tolerate temperatures of 45 to 47 °C (Karanja and Wood, 1988). Also, strains of *R. leguminosarum* isolated from lentil plants in the Nile Valley, and rhizobia of leguminous trees can tolerate temperatures of 40 °C (Moawad and Beck, 1991, De Lajudie et al., 1998). Bradyrhizobia have been reported to be more thermotolerant than rapidly growing strains (Munevar and Wollum, 1981). Tolerance at 48.7 °C has been reported for some strains of *Bradyrhizobium japonicum* (Munevar and Wollum, 1981). Almost all rhizobial strains in the current study did not tolerate low temperature (10 °C), except for KHS1 and KHT1.96. At such a temperature the growth of Mat.9 and B155 strains was completely inhibited, which, on the contrary, tolerated temperatures of 40 °C. Low temperatures have been reported to be among the main factors limiting nitrogen fixation by rhizobia in very humid or cold regions (Zhang and Smith, 1996), and high temperatures have been observed to produce a similar effect (Hungria and Franco, 1993). However, high temperatures inhibited strain multiplication, can reduce the number of cells below the level required for good nodulation, can lead to the loss of the symbiotic plasmid and, consequently, the bacteria's infective ability. Somasegaran and Hoben (1984) showed a gradual decline in the persistence of a population of *Rhizobium* strains incubated at 37 °C over eight weeks.

Rhizobia can maintain good performance when used in an environment with climatic conditions similar to their site of origin. However, several authors have reported no correlation between the temperature of the isolation site and the tolerance of strains to heat stress (Mohamed et al., 2000).

Exposure to CaCO₃ resulted in significant variability in the growth of these bacteria in culture media supplemented with calcium (Table 5). While some *Rhizobium* strains exhibited good growth in calcium concentrations up to 2 M, strains sensitive to minimal calcium were identified. EL-Hamdaoui et al. (2003) indicated that high calcium levels enhanced rhizobial growth and adsorption to roots and promoted nodulation, leading to an increase in salt tolerance for nodulated legumes.

Kouki et al. Diversity of selected rhizobia that nodulate the common bean

Table 5. Effect of abiotic constraints on the growth of the rhizobia strains

Rhizobia strain	pH					NaCl (mM)				CaCO ₃ (mM)				T°C					
	4	5	6	7	8	9	25	50	75	100	0.05	0.10	0.15	0.20	10	20	30	40	
CIAT899	--	-	-	++	++	+	-	-	-	-	--	-	-	-	--	-	++	-	
Alia1	--	-	-	+	+	+	-	-	-	--	--	--	--	--	-	+	++	-	
Alia2.96	--	-	-	++	++	++	-	-	-	-	-	-	-	-	--	+	-	-	
Tinja	--	+	-	-	-	+	--	-	-	-	-	--	--	-	--	-	++	-	
Ar02	-	-	+	+	++	+	-	-	+	-	-	--	--	--	--	+	++	+	
Ar05	--	-	-	+	++	++	-	-	-	-	--	--	--	--	--	+	++	+	
P.Ar.09	--	-	-	+	+	-	-	--	-	-	-	-	-	--	-	+	-	-	
P.Bj	--	-	-	+	+	+	-	--	-	-	--	-	-	--	--	-	+	+	
P.OM.09	--	+	+	+	+	+	-	-	-	-	-	-	-	-	--	+	+	+	
P.Ps. 09	--	+	+	+	+	+	-	-	-	-	-	-	-	-	--	-	++	-	
CB	--	-	-	+	++	++	-	-	+	-	-	-	-	-	-	-	-	-	
P.Tb.09	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	++	++	+
SOM	-	-	-	+	+	+	--	-	+	-	-	+	-	-	-	-	-	++	+
D4.007	+	-	+	++	++	++	+	-	+	++	+	-	+	+	-	+	+	+	
D4.002	--	+	-	-	+	++	+	+	++	+	+	-	-	-	-	+	+	+	
KHS1	--	-	+	+	-	-	-	-	-	--	--	-	-	+	+	++	++	-	
KHS2	--	-	-	++	++	+	-	-	-	++	+	+	+	+	-	+	++	-	
GB.92	--	-	-	+	+	+	-	-	-	-	--	-	--	-	--	++	-	-	
GB.258	--	-	--	+	+	-	--	--	-	-	++	++	++	++	--	--	-	-	
KH28	-	--	-	+	+	-	-	-	-	-	-	-	-	-	-	++	++	+	
Fr1.97	--	-	-	+	+	-	-	-	+	-	-	-	-	-	-	++	++	-	
OM	--	-	-	+	++	++	-	+	+	+	+	+	+	-	--	+	-	-	
Mat.9	--	-	-	+	+	+	+	+	++	++	+	+	+	+	--	+	+	+	
Zaar	--	--	+	+	++	+	+	+	+	+	+	+	+	+	--	-	+	-	
ZG.96	--	-	-	+	+	++	+	+	+	+	+	+	+	+	--	++	+	+	
B155	--	-	-	+	+	+	-	-	-	-	-	-	--	--	--	+	-	-	
S10	--	--	-	+	+	+	--	-	-	--	-	-	-	-	-	-	++	+	
KHT1.96	--	--	--	-	+	-	-	-	-	-	--	--	--	--	+	+	++	+	
KHT3.96	--	+	++	+	-	-	+	++	++	++	+	+	+	+	--	--	+	-	
Ar3	--	-	-	++	++	+	-	-	+	+	-	-	-	-	-	++	++	+	
Ar1	--	+	+	+	++	++	+	-	+	+	-	+	+	-	-	+	++	-	
Ar6	--	--	-	-	-	+	--	-	-	--	--	--	--	--	--	+	++	-	
Ar4	--	--	+	++	++	-	+	++	++	++	++	++	++	+	--	-	+	-	
Ar2	-	--	--	-	-	-	-	--	--	-	--	--	--	--	--	-	-	-	
S1	--	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	++	+	
J1.96	--	-	-	++	++	++	-	-	-	-	--	--	--	--	-	+	+	-	
J2.96	--	-	-	++	+	+	-	+	+	+	-	-	-	-	--	-	+	--	
J3.96	--	-	-	++	+	+	-	-	-	+	-	-	-	-	-	+	++	-	
J1.92	--	-	+	++	++	++	-	+	++	++	-	-	-	-	-	-	+	-	
J3.92	--	-	-	+	+	+	-	--	-	-	-	--	--	-	-	+	+	+	
J4.92	--	-	-	-	-	-	--	--	-	-	--	--	--	--	--	+	++	-	
S3	--	--	-	-	-	-	--	--	-	-	--	--	--	--	-	-	-	-	
S7	--	--	-	+	-	-	--	--	-	-	+	++	+	+	-	+	++	-	
S9	--	--	+	+	+	+	-	-	+	+	-	-	--	-	--	--	+	-	
S11	--	-	-	+	++	++	-	+	-	--	-	-	-	-	-	-	-	-	
Raf .Raf	--	--	-	-	++	++	-	-	-	+	+	+	+	-	-	-	++	+	
Ras.JB	--	--	--	--	--	--	--	--	--	-	--	--	--	--	--	+	+	++	
Soudan1.2	--	--	-	++	+	-	-	+	+	++	-	-	-	+	--	-	-	-	
Soudan2.2	--	-	-	+	+	+	-	+	+	+	+	+	+	-	--	+	++	+	
D2.2	--	--	+	++	+	+	+	+	++	++	++	++	++	+	--	-	-	-	
D3.2	--	--	-	++	+	+	-	-	-	-	++	++	++	++	--	-	-	-	
Artn1	--	--	-	-	-	-	-	-	-	-	--	--	--	+	--	+	++	+	
Ic.208	--	--	-	+	++	++	+	+	-	-	-	-	-	-	--	+	++	-	
12.a3	--	-	-	-	-	+	+	+	-	--	+	+	+	+	--	--	+	--	
YH15	--	--	-	-	+	++	-	++	++	+	-	-	-	-	-	+	-	-	

-- Very Sensitive, - Sensitive, + Moderately Tolerant, ++ Tolerant

Changes in temperatures, metal toxicity, pH and soil salinity are among the main factors restricting symbiotic nitrogen fixation in legume-rhizobia symbiosis, where only those strains capable of tolerating extreme conditions survive (Berrada et al., 2012). The production of extracellular polysaccharides by most isolates in this study could indicate their versatility to withstand physiological stress due to those restricting factors (Karthik et al., 2017).

CONCLUSIONS

Rhizobium strains showed significant variation in their symbiotic capabilities while responding to various controlled conditions and abiotic constraints, showing variability in nodulating potential (nodule number and weight), shoot and root biomass production. Some of them exhibited tolerance to pH, salt, CaCO₃ and temperature, and finally, capability to metabolize specific biochemical substrates. Tunisian strains Ar02 and Allia02.96 were the most nodulating rhizobia and highest dry weight strains. They also demonstrated efficient production of the shoot and root biomass in the common bean. Other *Rhizobium* strains, including J3.96, J3.92, and KHT1.96, demonstrated substantial nitrogen content.

The studied strains exhibited diverse behaviours with respect to the variation in the pH, with most of the strains tolerating basic media. Also, Rhizobial bacteria showed growth variability in culture salinity of 25 to 100 mM NaCl. Regarding temperatures, almost no strain tolerated low temperatures, but most grew at higher temperatures, with two strains showing thermophile behavior. At the lowest temperature, the growth of some strains was totally inhibited, but they showed notorious growth at very high temperatures. Furthermore, some *Rhizobium* strains exhibited good growth in a medium containing up to 2 M calcium, but some strains could not tolerate even low calcium concentrations. The rhizobia strains also demonstrated diversity in their capability to metabolize specific biochemical substrates, such as carbohydrates and organic acids.

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