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Screening of stress tolerant bacterial strains possessing interesting multi-plant growth promoting traits isolated from root nodules of *Phaseolus vulgaris* L.

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ABSTRACT

Rhizobia are soil bacteria known for their ability to nodulate and fix nitrogen with legumes. Some rhizobia also show different plant growth promoting characteristics (PGP); they can therefore be used as effective bio-fertilizers. *Phaseolus vulgaris* is considered a weak nitrogen fixer; therefore, screening experiments have been designed to select stress-tolerant effective bean rhizobia that have also PGP traits. Surveys of common bean fields in Morocco lead to the isolation of 113 bacteria inhabiting root nodules. Screening tests showed that 45 isolates exhibited positive phosphate solubilization, and auxin and siderophores production as well as high salt and pH tolerance. Correspondence analysis showed that salt tolerance and phosphate solubilization were origin related. Among the screened isolates, 18 were able to renodulate common bean but only 3 were highly effective with their host plant. Interestingly, phylogenetic analysis of 16S rRNA gene showed that the nodulating isolates clustering within the *Rhizobium* genus were represented by only two isolates related to *R. miluonense* and *R. yanglingense* while the remaining strains belonged to the *Agrobacterium* genus. Our results revealed also the co-existence of several endophytic bacteria with the symbiotic strains inside the nodules. These bacteria are stress tolerant and possess potential PGP traits, which indicate that they could stimulate common bean growth and contribute to N and P plants nutrition.

1. Introduction

Common bean (*Phaseolus vulgaris* L.), is a highly appreciated grain legume worldwide and estimated as an important source of dietary proteins (22% of seed weight) for human consumption and also a major source of micronutrients such as iron, zinc and folic acid (Pennington and Young, 1990; Broughton et al., 2003). This legume crop is widely cultivated in Central and South America, Asia as well as in many countries of Africa including Morocco. Despite the ability of *P. vulgaris* to establish symbiosis with a broad variety of bacteria (Mostasso et al., 2002; García-Fraile et al., 2010), these symbiotic relationships are often not/or less effective (Michiels et al., 1998). Also, compared to other legumes, common bean is known as a weak nitrogen fixer (Hardarson (1993); Farid and Navabi (2015)). The estimated mean value of nitrogen derived from the atmosphere for common bean across different geographical regions of the world is only 39% as compared to lentil (65%), soybean (68%) and pea (65%) (Peoples et al., 2009). The low capacity of symbiotic nitrogen fixation of this legume crop was attributed to its highly promiscuous nodulation (Rodriguez-Navarro et al., 2000). Effectively, common bean can be nodulated by different native and ineffective rhizobium symbiovars and as a result bean production is mainly based on intensive nitrogen fertilization which in return prevents the optimal expression of the nodulation and the nitrogen symbiotic fixation potentials of this symbiosis. Consequently, screening of infective and effective rhizobial strains, compatible with common bean cultivars, remains the most effective way for achieving successful inoculation of this legume and increasing the contribution of symbiotic nitrogen fixation to common bean N nutrition. Besides their symbiotic nitrogen fixation potential, some strains of *Rhizobium* are able to induce plant growth promotion (PGP) and improve agricultural sustainability in many ways. In addition to nitrogen fixation, the mechanisms by which PGP rhizobia may stimulate plant growth involves improving

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mineral nutrition by phosphate solubilization (Singh et al., 2014), modulating plant hormone levels through phytohormone production such auxins (Zahir et al., 2010), increasing iron uptake by plants through the release of siderophores capable of chelating iron with a high affinity (Neilands, 1995) or in stress alleviation such as salinity, acidity or alkalinity (Inagaki et al., 2015; Habib et al., 2016).

Many reports indicate that the isolation of efficient rhizobial symbionts from local bean production sites is one of the most successful approaches for inoculation success (Mostasso et al., 2002; Hungria et al., 2003). The present study was thus designed to select efficient native stress tolerant common bean rhizobial inoculants displaying good symbiotic capacities and/or different PGP characteristics. The most performing bacteria selected in this study will be evaluated later in common bean inoculation experiments to identify successful biofertilizer strains to recommend to growers.

2. Material and methods

2.1. Sampling and isolation of bacterial strains

During the growing season of common bean, a general survey was carried out in the main production areas of this legume in Morocco. Common bean plants were collected at the flowering stage from four production areas: Skhirat, Berkane, Sidi Allal Tazi region and Gharb plain. Nodulation in the three first areas was very low or even absent in most of the sampling fields while the highest number of root nodules was found in the Gharb plain. A collection of 113 isolates was obtained from common bean nodules harvested from 9 sampling sites from different locations (Table 1).

Bacterial strains were isolated from surface-sterilized root nodules taken from freshly uprooted plants. Roots were thoroughly washed under running tap water to remove soil particles. Nodules were excised and surface sterilized using a solution of 0.1% $HgCl_2$ during 2 min then washed with sterilized distilled water under aseptic conditions. Excised nodules were then crushed and directly streaked onto solid YEM medium containing 0.0025% Congo red dye and incubated at 28 °C (Vincent, 1970). Colonies were purified by repeated streaking onto YEM agar then stored in 20% (v/v) glycerol at −80 °C.

Furthermore, irrigation water and soil samples (0–20 cm top soil layer) in each sampling site were collected and analyzed for electrical conductivity (EC).

2.2. Screening of elite strains

2.2.1. Salt and pH tolerant strains

In order to select stress tolerant strains possessing beneficial traits for plants, we adopted a screening procedure of three consecutive steps. The first one consisted of a salt tolerance test conducted on YEM agar containing one of three salts NaCl, KCl and $CaCl_2$. Purified isolates were pre-grown in liquid YEM medium up to the stationary phase, and the optical density of each suspension was normalized to OD_{600} = 0.05. Ten microliters were spotted onto agar plates containing YEM medium supplemented with one of the three salts at concentrations ranging from 100 to 700 mM. The pH of the medium was adjusted to 6.8. Tolerance to salinity was estimated by observing the growth of colonies after 7 days of incubation at 28 °C and the minimal inhibitory concentration (MIC) of each salt was recorded.

For the evaluation of strains tolerance to acidic and alkaline pH, the same procedure described above was followed using YEM medium supplemented with 25 Mm Homopipes to buffer the pH to 4 and 5 and 25 mM TAPS (N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid) to buffer the pH to 8 and 9. In both tests, all strains were compared to the control.

Table 1
Number of bacterial isolates and sites location.

Number of isolates	Sites	Latitude N	Longitude W
17	Delalha	34°50.538'	006°10.905'
7	Ouled Mesbah	34°47.766'	006°17.456'
7	Khennache	34°45.486'	006°18.842
7	Ahmiri	34°50.683'	006°12.794'
4	Merja Zerga	34°50.384'	006°14.143'
20	Tifelt	33°49.681'	006°14.297'
29	Lgnafda	34°49.650'	006°14.429'
12	Zwaka	33°46.868'	006°36.834'
10	Mghayten	34°48.092'	006°16.458'

2.2.2. Characterization of the isolates plant growth promoting (PGP) properties

In a secondary screening step, all strains were screened for their plant growth promoting traits, namely indole-3-acetic acid (IAA) production, rock phosphate solubilization and siderophores production.

AIA production by each isolate was determined by the Salkowski's method (Ehmann, 1977). Bacterial cultures were prepared following the procedure described above and then grown in liquid cultures of YEM containing 50 µg/mL of tryptophan for 24 h at 28 °C in a shaking incubator at 180 rev min^{-1}. After incubation, the culture was centrifuged at 8000 rpm for 15 min and then passed through a 0.2 µm Millipore filter. The supernatant was taken in fresh tubes and mixed with Salkowski's reagent then kept in the dark. After 25 min incubation at room temperature, the optical density (OD) was measured at 540 nm. The auxin biosynthesis was determined based on a standard graph of IAA (Gordon and Weber, 1951).

Quantitative estimation of phosphate solubilization was carried out using Erlenmeyer flasks (250 ml) containing Pikovskaya's medium (PVK) (Pikovskaya, 1948) supplemented with 0.05 g of rock phosphate as a sole P source. The rock phosphate used in this experiment was obtained phosphate mines of Khouribga (Morocco) then ground to a particle size of 0.02 mm. Bacterial cultures were prepared following the procedure described previously and the optical density was measured at 600 nm then the final cell suspension was adjusted to a uniform cell density (OD_{600} = 0.05). The cultures were incubated at 28 °C with shaking for 3 days. After incubation, the pH value of the medium was determined with a pH meter and the cultures were centrifuged at 8000 rpm for 15 min. The phosphate content of the supernatant was determined following the Vanadomolybdophosphoric Acid colorimetric Method (Tandon et al., 1968). The absorbance of the developed yellow color was measured at 400 nm. The corresponding amount of soluble phosphate was calculated from a standard curve of KH_2PO_4.

Siderophores production was determined following Schwyn and Neilands (1987) method. Bacterial cultures were performed in iron deficient medium ($g.l^{-1}$): K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; Sodium glutamate, 1.0; NaCl, 0.1; Mannitol, 10.0 and incubated at 28 °C for 7 days in a rotary shaker. Culture supernatants were obtained after centrifugation at 8000 rpm for 15 min and analyzed for the presence of siderophores by the Chrome Azurol-S (CAS) colorimetric assay (Payne, 1994). The presence of siderophores is indicated by a color change of CAS solution from blue to orange and confirmed by the measurement of the absorbance at 630 nm. The siderophores production was recorded in percentage units of siderophores calculated as [(Ar − As) × Ar−1) × 100] where 'Ar' is the absorbance of control (uninoculated media + CAS) and 'As' is the absorbance of test (culture supernatant + CAS) (Gupta et al., 2012).

Each of the screening tests was performed in three replicates and isolates giving higher performances were selected for further experiments.

2.3. Symbiotic efficiency in greenhouse pot experiment

Based on the screening procedure listed above, 45 out of 113 performing isolates were selected. The elite isolates were evaluated for

Table 2
Bacterial isolates tolerance to different pH and salinity levels.

NaCl (M)						KCl (M)				$CaCl_2$ (M)				Number of isolates tolerating all types of salt	pH			
Sampling sites	Number of isolates analyzed/site	0,17	0,34	0,51	0,64	0,17	0,34	0,51	0,64	0,17	0,34	0,51	0,64		4	5	8	9
Delalha	17	4	4	8	1	10	1	5	1	5	1	0	0	6	3	11	14	3
Ouled Mesbah	7	2	1	2	2	5	0	0	2	1	0	1	0	2	0	4	6	1
Khennach	7	2	1	4	0	6	0	1	0	2	0	0	0	2	2	1	6	0
Ahmiri	7	0	2	4	1	4	0	2	1	2	1	0	0	3	2	4	4	3
Merja Zerga	4	0	1	2	1	3	0	0	1	2	0	0	0	2	1	3	3	1
Tifelt	20	5	8	7	0	15	4	0	1	7	0	0	0	7	1	9	14	6
Lgnafda	29	14	10	4	1	12	13	2	2	5	0	0	0	5	9	9	23	4
Zwa9a	10	0	3	9	0	9	0	3	0	2	0	0	0	2	2	7	11	1
Mghayten	12	1	7	2	0	8	1	1	0	0	0	0	0	0	0	6	5	5
%		31	50	47	7	81	21	16	9	29	2	1	0	32	22	61	97	27

Data were recorded as the minimal inhibitory concentration (MIC) and presented as the number of tolerating isolates per sampling site.

their nodulation capacity and symbiotic effectiveness. These isolates were inoculated to their host plant (common bean) and grown under controlled conditions. The symbiotic effectiveness of each isolate was determined based on the number of nodules and the shoot dry weight it induced. The commercial and widely cultivated variety in Morocco, Tania, was used in this experiment. Seeds were sown in pots containing sterilized perlite and grown in a glasshouse under natural light with day/night temperatures of 27/21 °C and a photoperiod of 16 h/8h and 70% relative humidity during the day. All pots were watered twice a week with an N-free nutrient solution during the whole experiment duration. Before sowing, seeds were surface-sterilized with alcohol (95%) for 5 min followed by calcium hypochlorite (2%) for 1 min, and then washed profusely with sterilized distilled water. Seeds were germinated at 28 °C in the dark for 2 days in plates containing 1% water-agar.

Bacterial isolates were grown in YEM medium and the cells suspensions were adjusted to a concentration of 10^9 cells/ml. Each seedling in the pot received 1 ml bacterial inoculums at the sowing stage. Two non-inoculated controls were included, with and without mineral N. All pots were watered twice a week with an N-free nutrient solution. The pots were arranged in a completely randomized design with three independent replications.

Plants were harvested at the flowering stage (40 days after planting). Nodulation parameters recorded were root and shoot dry weights, nodules number and their dry weight. Data obtained were subjected to the analysis of variance.

2.4. Molecular analysis

2.4.1. DNA extraction

Nodulating isolates were identified using 16S rRNA sequence. Genomic DNA of each isolates was extracted according to the procedure of Chen and Kuo (1993). The purity and concentration of the extracted DNA were checked using a microspectrophotometer NanoDrop 2000 (NanoDrop Technologies Inc., USA). Genomic DNA was stored at −20 °C.

2.4.1.1. PCR reaction and sequence analysis of 16S rRNA gene. Partial 16S rRNA gene was amplified using the universal forward primer 41 F (5- GCTCAGATTGAACGCTGGCG-3′) and reverse primer 1488 (5-CGGTTACCTTGTTACGACTTCACC-3'). PCR amplification reaction was performed according to the followings: initial denaturation step of 5 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 1min30 s at 62 °C, 10 min at 72 °C and a final elongation step of 10 min at 72 °C. The partial length 16S rRNA amplicons were purified and sequenced by Genoscreen Inc (Lille, France). Sequences were manually corrected using Chromas LITE (version 2.1) then aligned with the GeneDOC (version 2.7) (Nicholas et al., 2017).

The phylogenetic tree was constructed with MEGA7 software using Neighbour-joining method (NJ) (Saitou et al., 1987) and following Kimura's 2-parameter model (Kimura, 1980). Bootstrap support for each node was evaluated with 1000 replicates. The obtained sequences were deposited in the GenBank database and the accession numbers of study isolates are indicated on the phylogenetic tree (Fig. 5).

2.5. Statistical analyses

Statistical analyses were performed using Xlstat software (version 2014.5.03). To determine groups that significantly differ from each other, a one way ANOVA (analysis of variance) was performed using LSD and Tukey as post hoc tests. The difference between all the comparisons made is significant at 95% confidence interval. To study the relationship between isolates level of tolerance and specific plant growth-promoting abilities or between studied properties and sampling sites of isolates, a correspondence analysis was performed as an exploratory data analysis (Benzecri, 1992). Isolates were divided into different classes based on their performance in each test. To test the independence between categorical variables, a Chi-square test of independence was performed. Results are presented as the test statistic ($\chi 2$), degrees of freedom (d.f.), and probability of equal or greater deviation (P). Spearman's correlations were performed in order to determine if any of the tested properties related with the symbiotic effectiveness of the nodulating isolates.

3. Results

3.1. Salt and pH tolerance

Isolated bacteria showed a great diversity regarding salt and pH tolerance. Their response to different concentrations of sodium, calcium and potassium chlorides were different and the salt effect appeared to be ion-specific with calcium being more toxic than sodium and potassium chlorides. All isolates tolerated 0.17M NaCl and KCl while only 3% were able to grow at the same concentration of $CaCl_2$. As the concentration of the salt increased, the bacterial growth showed a steady reduction towards 0.68M. The concentration 0.17M of KCl was the least toxic while 0.6M of $CaCl_2$ was lethal for all the isolates (Table 2). No considerable variations were observed among isolates in their sensitivity to pH; they could grow within a wide range of pH and tolerate both moderate alkalinity (pH 9) and acidity (pH 4). The bacterial growth was not much affected by alkaline pH while pH 4 reduced the growth to 22%. Moreover, the results of soil analysis of the sampling sites revealed that the values of soil EC varied between 0.1 and 0.3 mS cm^{-1}. However, the irrigation water of sampling sites revealed different levels of water salinity (EC (dS/m)) (Dlalha: 1.176; Ouled Mesbah: 0.92; Merja Zarka: 1.176; Lgnafda: 0.496; Zwaka: 1.244;

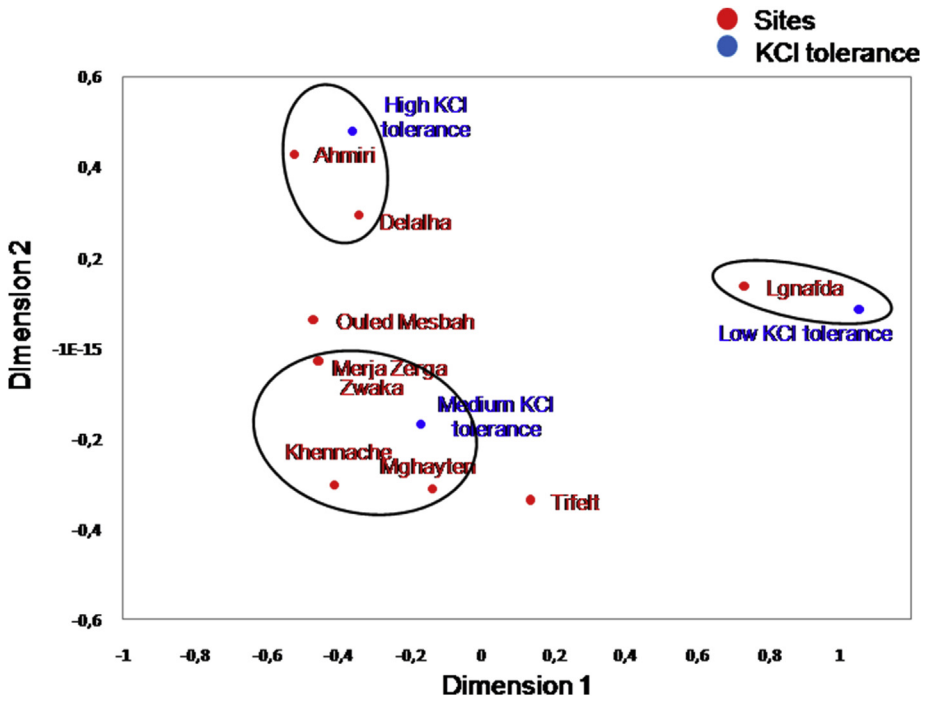

Fig. 1. CA biplot of the relationship between sampling sites and tolerance of the isolates to KCl.

Ahmiri: 1.102; Khennache: 0.707 and Mghayten: 1.03; Tifelt: 0.83).

Based on the Chi-square test of independence, the isolates ability to tolerate NaCl ($\chi 2 = 32{,}262$; d. f. = 16; $P < 0.05$) and KCl ($\chi 2 = 33.366$; d. f. = 16; $P < 0.05$) was related to the sampling sites. For instance, isolates obtained from Merja Zarka, Zwaka, Dlalha and Ahmiri were associated with high tolerance to NaCl. Furthermore and according to the water analysis results, these sites are also irrigated with highly saline water. Whereas, isolates collected from Lgnafda were associated with low tolerance to both NaCl and KCl (Fig. 1) which also correspond to the low electrical conductivity of the irrigation water of this site. The only sites that made the exception are Ouled Mesbah and Tifelt whose isolates were mostly moderately tolerant to KCl while for NaCl they were distributed into three groups, highly, medium or low tolerant (see Fig. 2).

3.2. Plant growth promoting traits

The analysis of the results revealed that at least 57% (64 isolates) of the isolated bacteria can be considered as IAA producers (ranging from 2 to 78 μg ml^{-1} IAA-equivalents). Also, 77 isolates were able to produce siderophores with a level of production ranging from 11 to 88%. On the other hand, all the isolates were able to solubilize inorganic phosphate

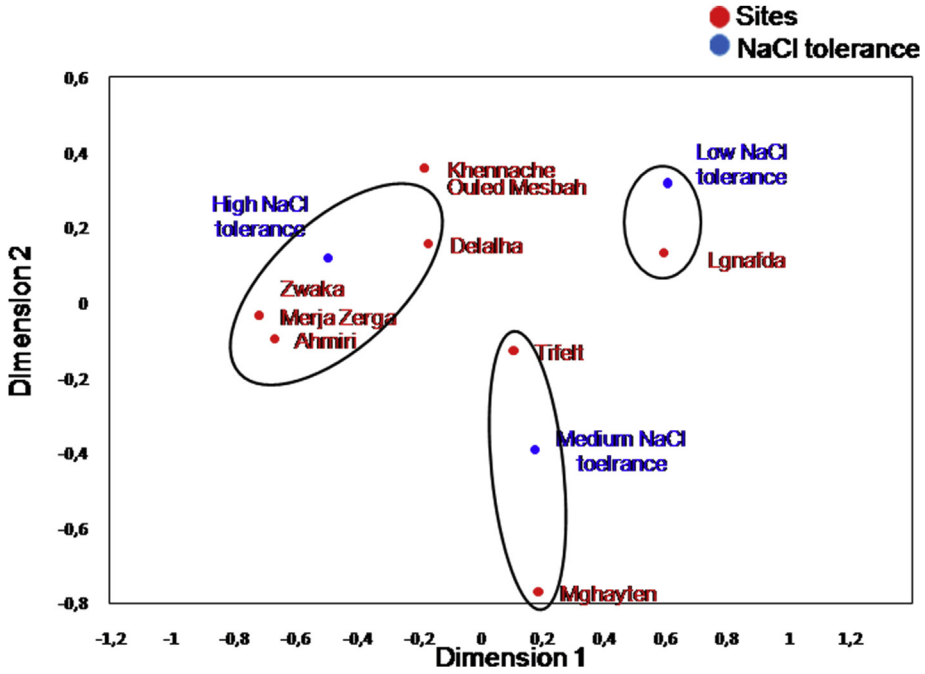

Fig. 2. CA biplot of the relationship between sampling sites and tolerance of the isolates to NaCl.

Table 3
Siderophores and IAA production and phosphate solubilization abilities of the bacterial isolates per sampling site.

Phosphate solubilization					Siderophores production			[IAA] production			Number of isolates displaying all PGP traits
Sampling sites	Number of isolates analyzed/site	Phosphate solubilizing isolates above 11 ppm	Max P (μg ml^{-1})	Min P (μg ml^{-1})	Siderophores producing isolates above 30%	Max (%)	Min (%)	[IAA] producing isolates above 10 ppm	Max (μg ml^{-1})	Min (μg ml^{-1})	
Delalha	17	5	18	8	7	77	16	5	55	9	3
Ouled Mesbah	7	3	14	8	3	88	16	3	49	5	2
Khennach	7	4	13	7	4	85	46	5	28	5	4
Ahmiri	7	4	24	8	3	83	11	4	70	5	3
Merja Zerga	4	0	11	7	4	88	53	1	57	4	2
Tifelt	20	16	25	9	15	89	33	8	73	3	11
Lgnafda	29	13	28	7	21	88	31	10	70	2	10
Zwa9a	12	5	24	7	9	80	59	5	29	3	6
Mghayten	10	4	18	9	6	78	26	4	78	3	4
%		61			81				51		45

Max P: Highest level of production per sampling site.
Min P: Lowest level of production per sampling site.

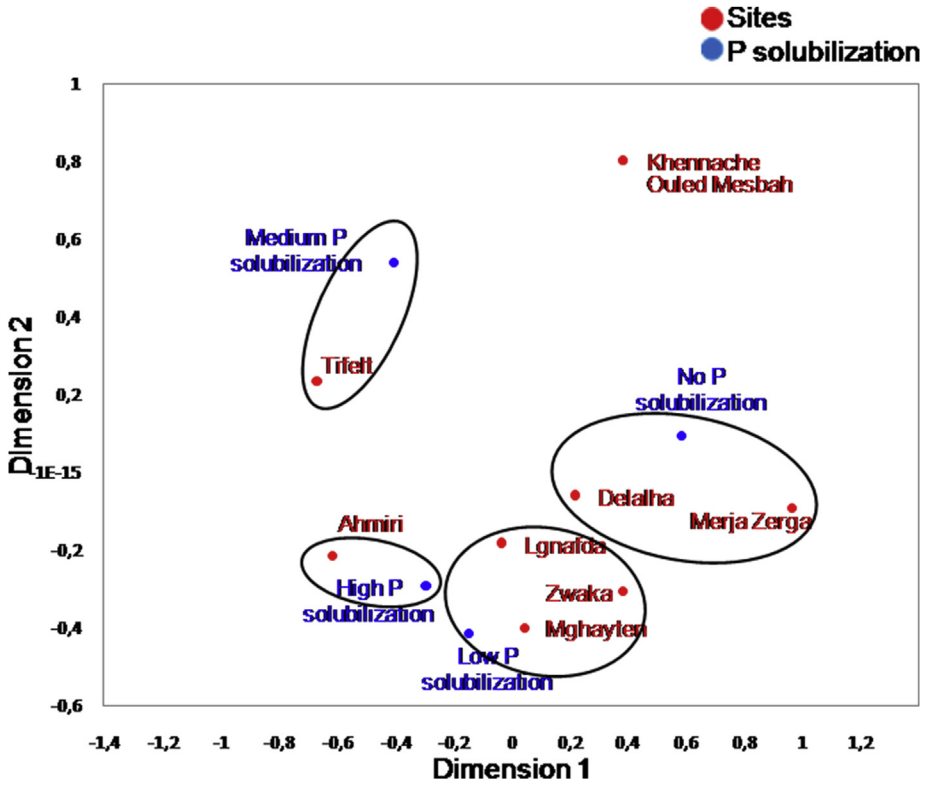

Fig. 3. CA biplot of the relationship between sites of origin and phosphorus solubilization by the isolates.

on PVK broth. The amount of soluble phosphorus (P) produced varied from 19 to 113 μg ml^{-1} (Table 3). The final pH of the medium in which isolates were grown decreased from 7 to 2.10 after incubation (data not showed). A negative correlation between P-solubilizing activity and the pH of the medium was found ($r = -0.383$, $P < 0.0001$). Furthermore, the CA biplot revealed significant relationship between the ability of an isolate to solubilize inorganic phosphate and the sampling sites ($\chi 2 = 42.798$; d. f. = 24; $P < 0.05$) (Fig. 3). The phosphate solubilization of isolates obtained from Khennache and Ouled Mesbah was either moderate or absent. In addition, a negative correlation between the isolate's ability to solubilize inorganic phosphorus and their acidic pH tolerance was found ($r = -0.777$, $P < 0.0001$).

Most of the isolates can be considered as producing siderophores, as their level of production ranged from 11 to 88% for 77 isolates out of 113. These bacteria were isolated from the different locations prospected. However, among the 113 isolate, only 45 were able to solubilize P and also produce siderophores and auxin.

The association between PGP activities and tolerance to salt and pH stresses was investigated by performing a Chi-square test of independence. No association was found among the three PGP traits tested or between these properties and pH or salt tolerance of the strains. On the other hand, isolates obtained from Ahmiri showed high KCl and NaCl tolerance as well as a high phosphorus solubilization, while isolates collected from Delalha and Merja Zarga despite their high NaCl and KCl tolerance were unable to solubilize inorganic phosphorus confirming the independency of these two properties.

Interestingly, a significant relationship between siderophores production and isolates ability to tolerate NaCl ($\chi 2 = 14.325$; d. f. = 6; $P < 0.05$) and KCl ($\chi 2 = 16.934$; d. f. = 6; $P < 0.01$) was found (Fig. 4). The CA biplot showed approximately the same distribution of variables between siderophores production and tolerance to both salts. For instance, all isolates with high NaCl and KCl tolerance were unable

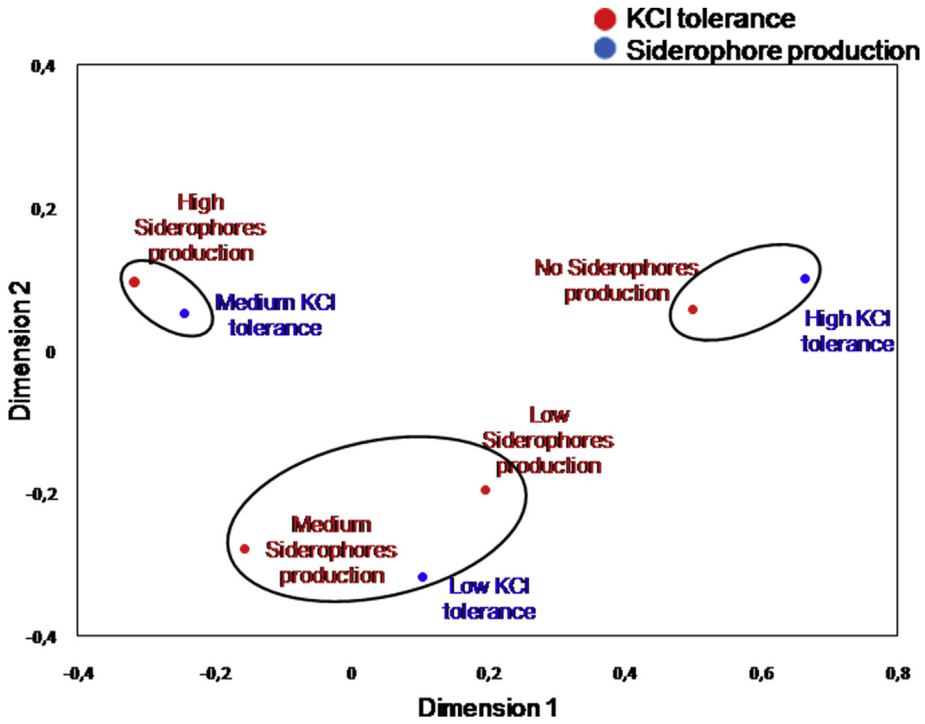

Fig. 4. CA biplot of the relationship between isolates ability to tolerate NaCl and siderophores production.

to produce siderophores whereas isolates with high siderophores production showed only moderate tolerance to both salts.

3.3. Symbiotic efficiency

The symbiotic efficiency of the elite isolates, selected for their interesting PGP activities and tolerance to pH and salt stresses was evaluated under greenhouse conditions. Of the 45 isolates tested, only 18 formed nodules. Shoot dry weight (SDW) ranged between 0.090 and 0.467 g plant^{-1}. Three of the tested isolates induced the highest values of SDW; two of them were not statistically different from the nitrogen supplemented control. Ten isolates had SDW values higher than the unfertilized control while the remainder had either similar or less SDW values than the unfertilized control, thereby showing the lowest values. However the highest number of nodules (NN) was produced by the isolate LMR 659 followed by the three isolates that showed the highest SDW values, respectively LMR 676, LMR 670 and LMR 674. The four isolates also produced the highest NDW values after LMR 659, which are not statistically different. Control plant roots never showed any nodules (Table 4).

There was a highly positive correlation between nodules dry matter and nodules number ($r^2 = 0.705$, $p < 0.0001$). Shoot dry matter was positively correlated with root dry matter ($r^2 = 0.382$, $p < 0.001$), nodules number ($r^2 = 0.341$, $p < 0.01$) and nodule dry matter ($r^2 = 0.540$, $p < 0.0001$). Root dry matter also correlated significantly with nodule dry matter ($r^2 = 0.353$, $p = p < 0.001$) but was not correlated with nodules number. Furthermore, no correlation was found between symbiotic effectiveness and isolates tolerance to pH and salts or the PGP traits studied.

3.4. Strains identification based on 16S rRNA genes

Partial sequences of 16S rRNA gene of nodulating strains were analyzed and a phylogenetic tree was built. The native rhizobial isolates that were able to re-nodulate the host plant were clustered into two main groups. The first group contained two isolates belonging to the genus *Rhizobium* namely strains LMR 671 and LMR 673; they were closely related to the type strains *R. miluonense* CCBAU 41251 and *R. yanglingense* CCBAU 71623 respectively. The second group contained 16 isolates which are clustering with the *Agrobacterium* genus. Seven isolates were close to the type strain *A. radiobacter* and two isolates were related to *A. deltaense* YIC4121 whereas the remaining formed a separate subgroup within the *Agrobacterium* genus (Fig. 5).

4. Discussion

The screening process of a 113 isolates derived from *P. vulgaris* root nodules indicated that the majority of the isolates were able to tolerate stressing levels of NaCl and KCl but were sensitive to $CaCl_2$. The same profile of tolerance was reported by Helemish and El-Gammal (1987) while investigating the growth of a strain of *R. leguminosarum* that tolerated NaCl up to 2 per cent (equivalent to 340 mM), while $CaCl_2$ was found to be more toxic. It is interesting to note that in our study some isolates were able to tolerate high levels of salt and a similar high level of tolerance was also observed by Boukhatem et al. (2016) while studying isolates obtained from root nodules of *Acacia nilotica* that were identified as belonging to different species of endophytic bacteria including a *Rhizobium* sp. Strain. In contrast with our results, Bouhmouch et al. (2001) reported that *P. vulgaris* rhizobia are poor salt tolerant with only 30 osmotolerant strains out of 150 tested (growing at NaCl concentrations above 2%). Thus, the high variability in salt tolerance observed in our study may reflect the high diversity of isolates that can be encountered in root nodules of *P. vulgaris*.

Despite the great variability in salt tolerance observed among the isolates, we noticed that KCl and NaCl tolerance was origin-related. The naturally occurring soil bacteria in non saline soils are generally expected to have lower tolerance to salt stress and vice versa. For example, Zahran (1992) indicated that the majority of *Rhizobium* strains isolated from saline soils were found to be salt-tolerant. Therefore, it is expected that salt-tolerant isolates could originate mostly from saline soils. However, in this study soil samples had a low electrical conductivity (ranging from 0.1 to 0.3 mS cm^{-1}) unlike the irrigation water which showed different levels of salinity that were in line with the levels of isolates tolerance observed. Hence, it could be said that in

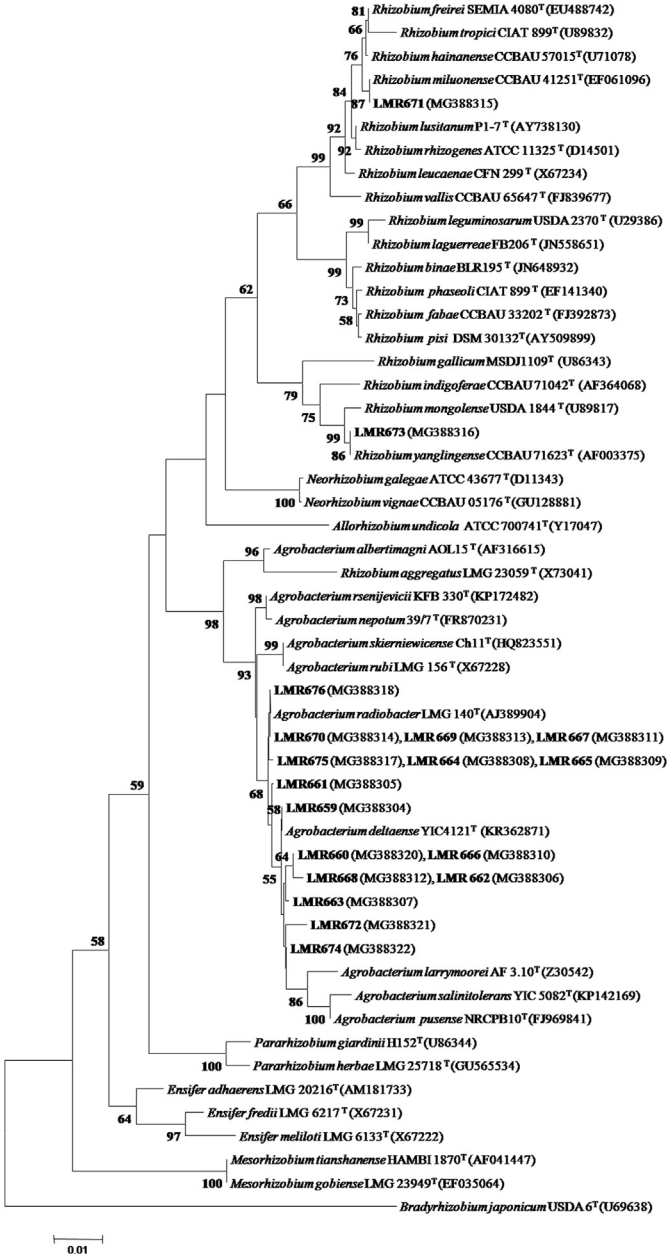

Fig. 5. Phylogenetic tree based on the 16S rRNA gene sequences (800 pb) of 18 strains and closely related species. Only bootstrap probability values greater than 50% (1.000 replicates) are indicated at the branching points. The type strains are shown by a "T" at the end of each strain code.

addition to soils conditions, the irrigation water conditions may also contribute in the selection of osmotolerant isolates. However for pH it seems that there is no relationship between the isolates tolerance and the conditions prevailing in the sampling sites.

The analysis of PGP traits among the isolates revealed that siderophores production is the most frequent followed by P. solubilization while only 51% of the bacteria tested were IAA producers. Siderophore production is an important element of the iron uptake strategy adopted by the microorganisms to best fulfill their needs under Fe-limiting conditions (Ratledge and Dover, 2000). Siderophores are also important for the biocontrol of some plant pathogens. Further investigations are needed for assessing the biocontrol potential of our strains and their suitability as an environmentally friendly alternative to hazardous pesticides (Schenk et al., 2012).

The correspondence analysis revealed an association between the

Table 4
Effect of isolates inoculation on shoot and root dry weights, nodules number and nodule dry weight of common bean grown under greenhouse conditions.

Inoculation	NN	NDW (g)	SDW (g)	RDW (g)	Relative efficiency (%)
+N	0 f	0 e	0,467 a	0,372 a	100
-N	0 f	0 e	0,149 de	0,204 de	32
LMR 676	34 c	0,036 a	0,416 ab	0,235 bc	89
LMR 670	28 cd	0,054 ab	0,343 ab	0,222cd	74
LMR 674	82 b	0,073 a	0,282 bc	0,328 ab	60
LMR 663	7 ef	0,009 de	0,221 cd	0,187 cd	47
LMR 667	17 cde	0,018 cd	0,204 cd	0,212 cd	44
LMR 659	121 a	0,064 a	0,197 de	0,142 cd	42
LMR 666	2 f	0 e	0,195 de	0,170 cd	42
LMR 662	19 cd	0,008 de	0,189 de	0,089 f	40
LMR 672	14 cd	0,027 cd	0,172 de	0,203 cd	37
LMR 665	19 cd	0,012 de	0,163 de	0,207 cd	35
LMR 660	10 de	0,034 bc	0,150 de	0,139 de	32
LMR 671	2 f	0,002 e	0,143 de	0,165 cd	31
LMR 673	9 de	0,005 e	0,137 e	0,223 cd	29
LMR 668	24 cd	0,007 de	0,129 e	0,126 ef	28
LMR 661	8 de	0,004 e	0,126 e	0,176 cd	29
LMR 675	13 de	0,009 de	0,096 e	0,090 f	21
LMR 664	7 de	0,003 e	0,093 e	0,119 ef	20
LMR 669	10 de	0,005 e	0,090 e	0,144 cd	19

Means in the same column followed by the same letter are not significantly different at the 5% probability level by LSD's test. All values represent the mean of 5 replicates.
+N: control plants non-inoculated but supplied with mineral N (30 mg of N as KNO_3 plant^{-1} week^{-1})- N: control plants non-inoculated with no mineral nitrogen added.
NN: Average number of nodules plant^{-1}, NDW: nodule dry weight plant^{-1}, RDW: dry weight of nodules plant^{-1}.
SDW: shoot dry weight plant^{-1}, Efr: relative efficiency (inoculated SDW/SDW of +N) x 100.

levels of siderophores production and NaCl and KCl tolerance. The co-existence of these two traits in the same bacteria has been reported previously by several authors (Tank and Saraf, 2010; Damodaran et al., 2013). The association found in our study may suggest a potential interaction between these two properties. However, as far as we know, the inter-connection of siderophores production and salt tolerance remains unclear.

Phosphate solubilization was the second PGP trait analyzed. The importance of this microbial activity lies in the importance of this nutrient for plants after nitrogen. However, in agricultural soils this component occurs mostly in insoluble forms (Miller et al., 2010). In this study, 113 isolates obtained from common bean nodules were tested for their capacity to solubilize rock phosphate which is considered as an interesting P source that can enhance biomass and nitrogen accumulation by legumes (Somado et al., 2006).

An association between the level of phosphate solubilization activity of the strains and sampling sites was found. This association may be due to the difference in soil properties of the sampling sites (physical and chemical properties, organic matter, and P content) or to agricultural activities. Our results suggest that isolates ability to solubilize P may be related to soil characteristics which can act as a selective pressure.

The amount of IAA produced varied between 3 and 78 mg ml^{-1}. The level of IAA production by bacteria varies among different species and strains and this production may also depend on the origin of the rhizobacteria (Sarwar and Kremer, 1995). The previous authors as well as Beneduzi et al. (2008) reported that isolates from bulk soil were less efficient auxin producers than those isolated from the rhizosphere while Mwajita et al. (2013) stated that rhizosplane bacterial population had a greater percentage of IAA producers than rhizospheric bacterial isolates. However, little is known about the level of IAA production by root nodules isolates as it is the case of the isolates obtained from common bean root nodules presented here.

Surprisingly among the 45 selected isolates only 18 were able to renodulate their plant host, which could indicate that the remaining strains are non symbiotic and may be considered as endophytic bacteria. Despite the high specificity between symbiotic partners, several non rhizobial bacterial species have been isolated from root nodules. For instance, Sturz et al. (1997) reported the isolation of bacteria other than *Rhizobium* from root nodules and Rajendran et al. (2012) isolated putative endophytes from nearly 15% of the nodules processed. According to Pandya et al. (2013), these non rhizobial strains migrate with the host-nodulating rhizobial strains during the infection process and become localized in root nodules. Most of these non-rhizobial isolates are generally nonpathogenic and it is assumed that many of these bacteria found within nodules as endophytes could be safe and effective partners for enhancing nitrogen fixation in legumes (Martínez-Hidalgo and Hirsch, 2017).

In the last few years, diversity of rhizobia isolated from *P. vulgaris* has been investigated almost worldwide showing a vast diversity among the bean nodulating bacteria (Bernal and Graham, 2001; Oliveira et al., 2011; Wei et al., 2008). Nowadays, at least 20 species of rhizobia able to nodulate common bean are identified. In Morocco, rhizobia that nodulate common bean have been assigned to the species *R. etli* and *R. tropici* as the most abundant species in five soils of the North-West of the country in addition to 3 other genotypes that were less represented (*R. gallicum*, *R. leguminosarum* and *Sinorhizobium meliloti*) (Mouhsine et al. 2007). In a second study conducted by Faghire et al. (2012), the most abundant rhizobial strains nodulating *P. vulgaris* in soils of Marrakech-Tensift-Al Haouz region were closely related to *R. etli* and *R. phaseoli* while a second group of strains was identified as *R. gallicum* sv *gallicum* and *R. tropici*.

In the present study, based on 16S rRNA sequencing, two isolates belonged to the *Rhizobium* genera where LMR 673 was related to *R. yanglingense* and LMR 671 to *R. miluonense*. These species were previously isolated from different legume root nodules and formed ineffective nodules on *Phaseolus vulgaris* in cross-nodulation tests (Gu et al., 2008; Tan et al., 2001). Similar results were obtained in our study where LMR 671 and LMR 673 exhibited a low relative efficiency of 31 and 29% respectively.

The remaining isolates of our collection were assigned to the genus *Agrobacterium*. This is not the first time where agrobacterial strains are isolated from common bean root-nodules (Aguilar et al., 2016; Mhamdi et al., 2005; Verástegui-Valdés et al., 2014) and from other legumes (Chen et al., 2000; de Lajudie et al., 1999). Their ability to nodulate legume plants could possibly be due to the acquisition of symbiotic genes via lateral gene transfer as reported in other studies (García-Fraile et al., 2010; Moulin et al., 2004). Furthermore, some *Agrobacterium* strains have been shown to carry effectively nodulating symbiosis-specific genes (e.g *nifH* and *nodA*) similar to those of other legume symbionts (Cummings et al., 2009; Rincón-Rosales et al., 2009; Youseif et al., 2014).

Among the agrobacteria group identified in our study, seven isolates were closely related to *A. radiobacter*. This species formerly considered as non-symbiotic was also reported to nodulate common bean by Ribeiro et al. (2013) who found that among the bacteria isolated from common bean four strains were affiliated within the *A. radiobacter* clade. On the other hand, strains of *A. radiobacter* has been reported previously as symbionts of different legumes such as *Acacella angustissima* (Rincón-Rosales et al., 2009), *Sesbania cannabina* (Cummings et al., 2009) and soybean (Youseif et al., 2014; Chen et al., 2000).

Interestingly, two other isolates were grouped with *A. deltaense* (YIC4121^T), a new type strain recently described by Yan et al. (2017) isolated from root nodules of *Sesbania cannabina*. Tolerance studies revealed that this strain is able to grow at a pH range of 5.0–10.0 and on YMA supplemented with up to 4% of NaCl. These characteristics are in line with those obtained in the present study as all isolates selected for the symbiotic efficiency test were tolerant to high salt concentrations (0.6M equivalent to 3.5%) and pH range (4.0–9.0).

The identification of native Moroccan agrobacterial strains that effectively nodulate common bean roots and fix N_2, is a novel and potentially valuable result which, certainly, deserves further studies. It is relevant that these nodulating strains co-exist in the nodules with several endophytic bacteria that are stress tolerant and possess a set of potential plant beneficial traits which makes them good candidates for common bean biofertilisation. In particular, it can be expected that consortia of selected strains of rhizobia and endophytic bacteria could stimulate common bean growth and contribute to N and P plants nutrition under the conditions prevailing in the main production areas.

Declarations of interest

None.

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