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Phenotypic And Symbiotic Diversity, Of Nodulating Rhizobia Associated With Bean (Vicia Faba) In West Algeria

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ABSTRACT: Forty renodulating rhizobial bacteria associated with *Vicia faba* were collected and isolated from different regions in West Algeria and characterized at phenotypic level. The analysis of their tolerance to salinity, high temperature, acid and alkaline pH, antibiotic resistance, and carbon substrates metabolism, as well as their symbiotic and cultural characteristics, highlighted a wide physiological diversity within this rhizobial population. The numerical analysis of phenotypic characteristics showed that the bacterial strains were grouped into four different clusters. Interesting stains for inoculation trials have been identified; they proved their efficiency in addition to their tolerance to 1 and 1.5M of NaCL concentrations and maximum temperatures of 40°C.

Keywords: Vicia faba, rhizobium, phenotypic diversity, efficiency.

1 Introduction

Bean (charm) is one of the most cultivated seeds worldwide [18]. In 2005, Mediterranean countries had produced 1093000 tons of beans which were equivalent to 25% of the world production. With a production of 27000 tons, Algeria occupied the 17th rank in the world and 6th rank in Africa headed by Ethiopia (51600 tons), Egypt (3500000 tons), Sudan (112000 tons), Morocco (73000 tons) and Tunisia (45000 tons) [10].Bean had played an important role in human alimentation long time agoand agronomists had noticed its role in improvement of poor soils in dry regions in Algeria [10].It may be used as a green fertilizer due to its capacity to fix nitrogen thanks to symbiotic association with rhizobial bacteria in root nodules, this capacity to fix nitrogen depends on many factors, such as the rhizobial strain efficiency, the genetic variation of the host plant and many other environmental and agronomical factors [31]. Vicia faba is one of the best nitrogen fixeramong crop legumes [29]. The quantity of fixed N2byV. Faba is estimated at about 240 to 325 kg ha-1 [38] with an efficiency rate of 66% of Ndfa (Nitrogen derived from air) [22] and responds to 80% of its needs in nitrogen .[50] For this reason, it has been incorporated in the traditional agricultural mix offering a less expensive agronomic practice by insuring a sufficient input of N for pasture production.[49]. In order to obtain an efficient symbiotic association between vegetal and bacterial partners, it is important to identify native rhizobia which prove their efficiency in association with plant host in laboratory and in greenhouses conditions [3]. The most widey characterized microsymbiont in association with this Legume was Rhizobium leguminosarum bv. viciae [23] and [37], many other fast growing rhizobia had also been characterized as Rhizobium etli and R. leguminosarum, and two new species, named Rhizobium fabae [44], Rhizobium laguerreae sp. nov [36] in addition to Rhizobium rubi, and a group of strains most closely related to Sinorhizobium meliloti in Egypt (Shamseldin et al.,2009. [36]. It should be noticed that little studies had been conducted on diversity of microsymbionts associated to bean in Algeria. In Algeria,58000 hectares meaning 44.3% of the total crop legume areais dedicated to the cultivation of this legume [27]. However, with the increase of Algerian population and reduction of areas destinated to its cultivation, the production is gradually decreasing. So, it is necessary to improve this strategic culture for the country's food security. The objective of this study is to evaluate the phenotypic and symbiotic diversity of nodulating bacteria associated to Vicia faba, in order to increase the production of this legume in Western.

MATERIALS AND METHODS

Sampling sites
Samples of the plant (Vicia faba) have been harvested in situ during raining season (November, December, January) in six Wilayas in Western Algeria: Mostaganem (FM), Oran (FO), Relizane (FZ), Mascara(FR), Telemcen (FC) and AinTémouchent (FT) (Figure I)

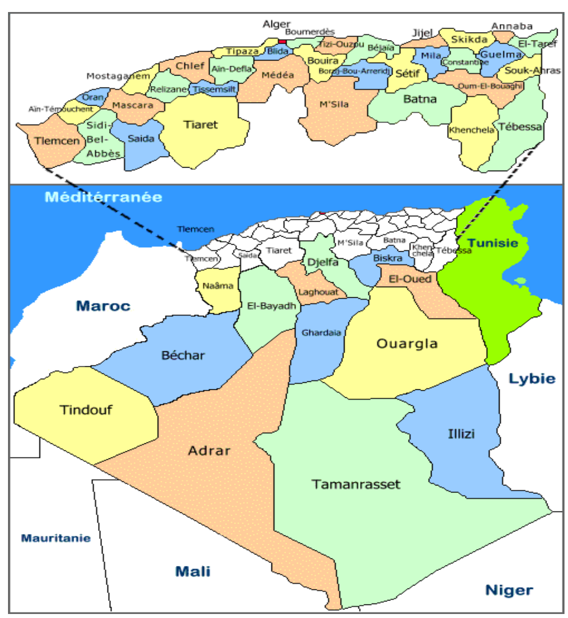

Fig 1. *Localization of nodule sampling sites from western Algeria*

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Bacterial isolation from nodules:
The harvested nodules from plants roots in naturawere immergedin sterilized distilled water for half an hour in Eppendorf tubes for soil traces elimination. Water was discarded then nodules were sterilized in surface by immersion in sodium hypochlorite at 3% (w/v) for 3 minutes. After that, they were rinsed 10 times in sterilized distilled water to eliminate disinfectant. Each nodule was crushed in a sterilized Eppendorf tube containing 2 drops of sterilized water with a sterile plastic stick. One drop of the obtained homogenate was spread on YEMA Petri dishes [47]. Nodulation test This test illustrates the ability of isolates to renodulate their host plants in controlled microbiological conditions and then demonstrates their membership to BNL (Bacteria Nodulating Legumes)[41].The Victafaba seeds used in the nodulation test were an introduced local variety (kindly provided by Centre of professional training in agriculture of Misserghin). They were disinfected in sodium hypochlorite (32°) for 1 minute, then rinsed 10 to 15 times with sterilized distilled water where they were soaked for 24h. The treated seeds were aseptically transferred into 1% water agar and then incubated for 4 days at 28°C [48].Upon seeds germination, all the obtained seedlings with 2cm fine roots length were transferred to 250ml bottles containing Fahrëus nutritive solution [15].The plants inoculation was realized 48h after plants transfer with 1ml of bacterial culture at an exponential phase of development, in triplicates. Each trial comprised a non-inoculated negative control. The plants were transferred in culture chamber. Roots have been maintained in obscurity and plants were incubated for six weeks.

Tolerance to salinity, temperature and Ph
Bacterial NaCl tolerance was performed by assessing strains growth on agar and broth YEM containing increasing concentrations of NaCl (0.3, 0.4, 0.6, 0.8, 1and 1.5M). Incubation lasted for 72 hours at 28 ° C.The evaluation of growth at different temperatures was performed by incubation of the strains inoculated on agar and broth YEM, at different temperatures of 5, 10, 15, 35, 40 and 45 ° C (Lupwayi and Haque, 1994) **[25]** for 72 hours.YEM agar was prepared and adjusted to different pH 4, 5 and 9. The inoculated plates were incubated at 28 ° C for72hours.

Absorption of Congo red
Typical colonies of rhizobia absorb weakly the Congo red stain **22] and [35]** compared to contaminants or non-fixing bacteria. The test consisted in streaking isolates in YEMA containing 0.0025% Congo red and incubated in the dark at 28 ° C.

Growth on YEM containing Bromothymol blue
Each of the tested strains was cultured on Petri dishes containing YEM agar supplemented with bromothymol blue which is a colored pH indicator. After five days of incubation at 28 ° C, it can be noticed if the rhizobial strain was acidifying (yellow color) or alkalinizing (blue color). It could be assumed thatfast and slow growing rhizobia causedmedia acidification and alkalinization respectively **[23].**

Resistance to antibiotics
The principle is based on the observation of bacterial growth in the presence of antibiotics diffused from impregnated discs deposited onYEMA. The used antibiotics were ampicillin(10 µg), Nalidixic acid (30µg), Streptomycin (10µg), Erythromycin (15µg), Tetracycline (30µg), Chloramphenicol (30µg),rifampcin (30µg).

Carbon substrates assimilation
The carbon substrate assimilation was determined on Hugh and Leifsonmedium **[20]**. pH indicator: bromocresol purple at 0.2% was used. 100 ml of media with a concentration of 10% for each sugar (manitol, sorbitol, cellobiose, saccharose, maltose, galactose, raffinose and glucose) were prepared. Sugar metabolism causedenvironment acidification resulting in the shift of pH indicator to yellow.

Numerical analysis
Efficiency test results were submitted to statistical analysis ANOVA, difference between means .On the other hand, results of the phenotypic and physiological characterization are codified in binary form and phenotypic dendrogram was constructed by the software STATISTICA7 based on 34 characters.

RESULTS AND DISCUSSION
The bean has been cultivated for more than 600 years in 57 countries around the world **[16].** Regarding the fact that *Faba bean* had been cultivated for centuries in Algeria, it was of a great interest to determine the diversity of rhizobia forming nitrogen-fixing symbioses with this important crop. This study provides the first phenotypic characterization of rhizobia nodulating *Vicia faba* in western Algeria. A total of 140 strains were isolated from this legume root nodules in six wilayas in West of Algeria. The forty isolates are Gram negative staining; presented for the majority a mucous aspect after 2-3 days of incubation, their colonies aspects, in addition to their ability to didn't absorb Congo red and acidifying media indicated probably their membership to fast growing rhizobia group **[23].** In addition to their ability to renodulate their host plant, which is in agreement with a study made by **[2]** on *Faba bean* rhizobia isolated from Northern Shoa confirmed that all isolates were acid producing. All the features cited above are characteristic of fast-growing rhizobia **[44]**, including the group of *Rhizobium leguminosarum* bv.*viciae* **[46]; [23] and [38].**Two characteristics, the infectiveness (the ability to form nodules) and symbiotic efficiency (ability to fix nitrogen), are commonly used to assess the ecological and evolutionary relationship between rhizobia and host **[8]**. Strains of rhizobia tend to be more effective on the host from which they were trapped than other species **[31]**.The obtained results from the nodulation test showed that 30 out of 40 isolates renodulated their host plant. Nodules are all located at the secondary roots level of bean with the same shape and a size of 2 to 3 mm. All of the rhizobial isolates were found to induce nodulation, and hence were authenticated as rhizobia **[17] and [12].** On the basis of host plant specificity for infection and nodulation, these species were generally assumed to be *R. leguminosarum* bv. Viciae **[4]**. To compare the efficiency of the infective strains, we measured the stems height (Figure 3), nodules number (Figure 2) and aerial part biomass of

the harvested plants (Figure 4).According to the number of nodules formed, FM24 strain was the most efficient (30 nodules recorded), it was originated from Mostaganem region; followed by FR36, FR8.3 and FR14.2 strains from the wilaya of Mascara, FT16 from AinTémouchent and FZ7.1 from the wilaya of Relizane with 19, 19 , 23,17 and 24 nodules recorded respectively. FM24.5 and FR26.4.2 strains are those which showed the lowest number of nodules (Figure 2) Regarding the plant's height of the stem, *Vicia faba* inoculated with FM24 strain originated from the wilaya of Mostaganem presented the best development with a height of over 90 cm followed by FM19.4 , FZ28 .4 and FR14.2 with about 79 cm high, while the size of the control was 52 cm (Figure 3).All strains increased aboveground biomass plants compared to the control apart from the two strains FR31.4 and FM20.4 originated from Mascara and Mostaganem respectively. Inoculation significantly increased nodule number plant^{-1} ($P<0.05$) as compared to the control. The most efficient strain is the FM24 issued from Mostaganem region; it had the highest number of nodules and induced the better growth of the host plant revealed by the stem height. Renodulating strains showed significant effects in dry weight of plant shoot and root parts comparing to the control, many studies have shown that the dry weight is a good indicator of the effectiveness because there is a relationship between the production of dry matter and the ability of legumes to fix nitrogen **[38] and [33]**. Several researchers had studied growth of *Vicia faba* inoculated with different strains of rhizobia to select the better symbiotic couples Rhizobia-*Vicia Faba* with the highest symbiotic performance **[3]**. The obtained results of this study suggest that efficient strains are worthy of a new series of field trials to select effective and competitive strains *in natura*.

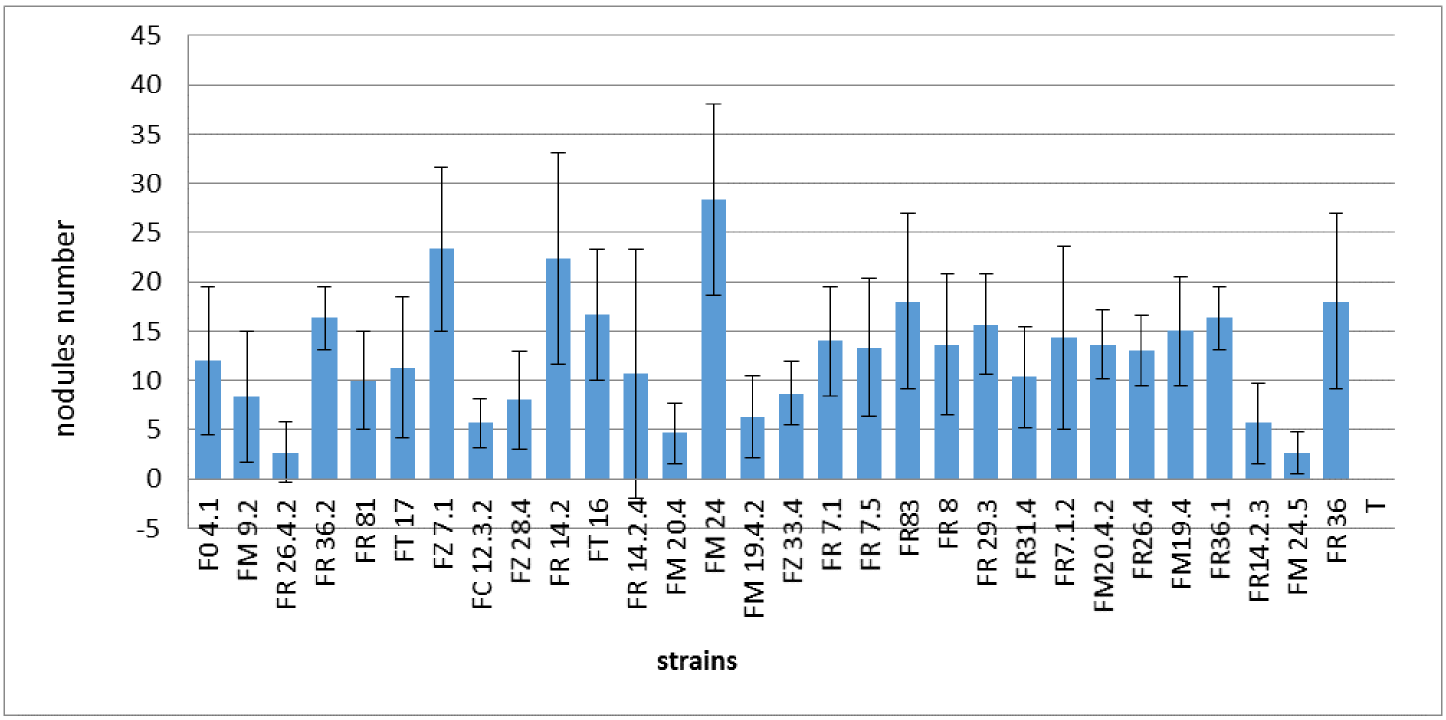

Fig 2. *Efficiency of isolated rhizobial strains associated to Vicia faba estimated by nodules number after six weeks of growth under controlled conditions.*

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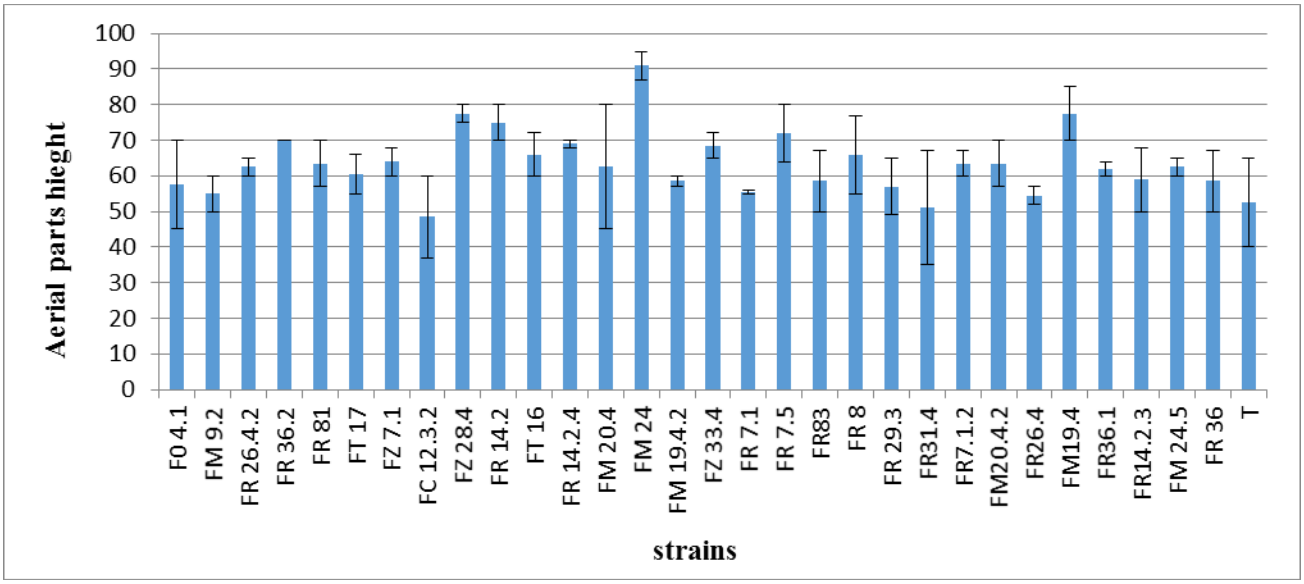

Fig 3. *Aerial parts height of inoculated plants and no inoculated control after 6 weeks of growth under controlled conditions.*

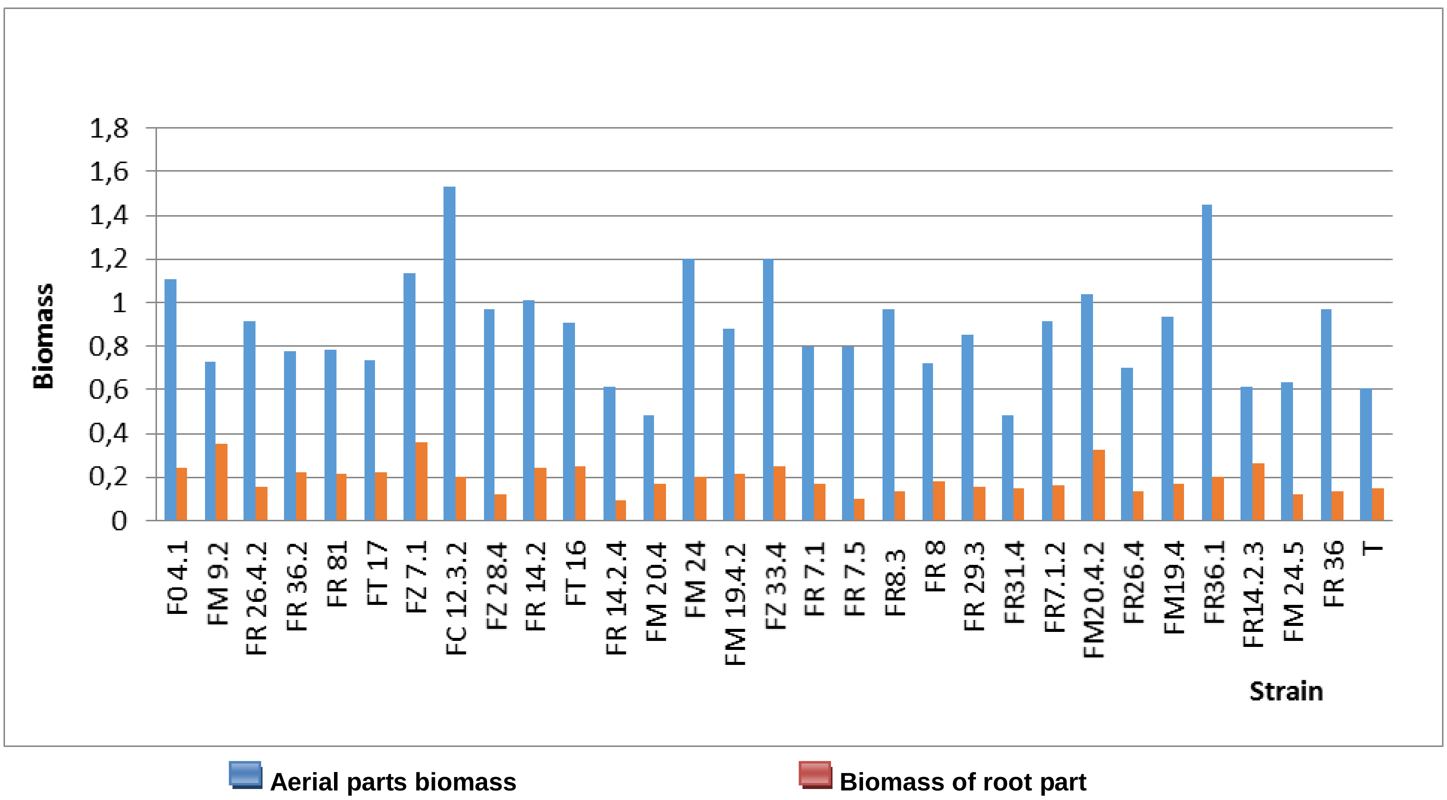

Fig 4. *Hoot and root parts biomass of inoculated plants and no inoculated control after 6 weeks of growth under controlled* Conditions

The results of physiological analysis revealed that Most strains were mesophilic and grew at temperatures ranged from 15 to 40 °C excepted for two strains: FC12.3.2 FR14.2.3 originated respectively from Tlemcen and Mascara, both showed good growth on solid YEM at 10 °C. The results of physiological analysis revealed that most of the studied strains were able to tolerate temperatures of 35 °C, with optimal growth at 30 °C for all strains, but some of them were also able to grow at 5 °C (Table 1). The results of this test are consistent with previous studies **[3] and[25]**which showed that rhizobia were mesophilic and could grow at temperatures between 10 and 37 °C with an optimum growth temperature of 28 °C.The obtained results showed that the strains exhibited a wide growth variation on

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different salt concentration. NaCl tolerance of these strains was represented in Table (1) showing that all the strains have a good growth on YEMA supplemented with 0.6MNaCl and half of them can tolerate the presence of 1MNaCl.It has been reported that salt concentration could decrease the efficiency of the symbiosis rhizobia / legume symbiotic inhibiting early events such as chemotaxis, colonization of roots and interfering in the formation of nodules **[20]**. Thirty percent of the strains were tolerant to 1 M NaCl.**[5];[1] and [51]** isolated *Viciafaba* associated strains that could tolerate up to 5M NaCl. At the opposite,**[37]and [30]** have shown that the strains isolated from *Viciafaba* nodules in Egypt and Italy were only able to grow at 0.1 and 0.2 M NaCl.The isolates were able to tolerate a wide pH range from moderately alkaline to acidic (pH 11-4) (Table 1). 60 % of the strains were able to growth at an alkaline pH from 9 to 11. Tolerance to acidity is variable depending on the strain tested: 90 % of the strains could grow at pH 5 and 56% of the strains were able to tolerate a pH of 4.These results were in agreement with those of [**1] and [3]** who reported tolerance of a few strains associated with *Viciafaba* to pH ranging from 5 to 9 and. Moreover, **[19] and [23]** indicated that rhizobia could tolerate pH from 4.5 to 9. **[5]** had shown that the strains isolated from food and fodder legumes growing in Fez regions were also able to tolerate a pH range from 4.8 to 8.8. The conducted antibiogram showed that 70 % of the strains presented resistance to tetracycline, erythromycin, rifamycin. 56% were resistance to ampicillin, streptomycin and chloramphenicol and only 36 % of the strains were resistant to nalidixique acid (Table 1), it was reported that fast -growing strains are more susceptible to antibiotics than slow-growing ones**[23] and[14]**. In this study, the antibiotic resistance of the isolated strains showed a high level of resistance to streptomycin, ampicillin, Erythromycin, chloramphenicol and to Nalidixic acid. Our results confirmed those of **[24];[7] and [3].** All tested strains grew well on Hugh and Leifsonmedium supplemented with glucose and mannitol. 40-63 % used sorbitol, cellobiose and maltose substrates, and 20% metabolized sucrose and only FR14.2 and FR712 were growing in the presence of galactose and FR83 could grow in the presence of rafinose (Table 1). A significant number of works dealt with this metabolism such as work of **[11]** which state that for *Rhizobium meliloti,* the following classification can be established based on growth fast: mannitol and glucose then gluconate; on another hand for *Rhizobium leguminosarum*bv. *trifoli* growth order is mannitol, glucose, arabinose then gluconate .

Table 1. *Physiological and biochemical characterization of renodulating strains*

	F0.4.1		**FR.26.4.2**	**FR36.2**	**FR8.1**	**FT17**	**FZ7.1**	**FC12.3.2**	**FZ28.4**	**FR14.2**	**FT16**	**FR14.2.4**	**FM20.4**	**FM24**	**FM19.4.2**	**FZ33.4**	**FR7.1**	**FR7.5**	**FR8.3**	**FR8**	**FR29.3**	**FR31.4**	**FR7.1.2**	**FM20.4.2**	**FR26.4**	**FM19.4**	**FR36.1**	**FR14.2.3**	**FM24.5**	**FR 36**
Temperature																														
5°C	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	-	+
10°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	-	+
15°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
40°C	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	-	+	-
Salinity tolerance																														
0,1M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0,3M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0,5M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0,6M	**+**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	**+**	+	+	+	+	+	+	+
0,7M	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
0,8M	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
1 M	-	-	+	-	-	+	+	-	+	+	+	+	+	-	-	+	-	+	+	-	+	-	-	+	-	-	-	+	-	+
pH tolerance																														
5	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
9	+	-	-	+	-	+	+	-	-	-	+	-	-	+		+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	-	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-	-
4	+	-	-	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	+	+	-	+	+	+	+	-	+	-	+	+

BTB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
RG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotic resistance																														
Am	±	+	±	+	+	+	+	+	+	+	-	+	±	+	+	±	±	±	±	+	±	+	+	±	±	-	+	+	±	+
C	-	+	+	+	+	+	-	±	-	+	-	±	-	+	+	±	±	+	±	+	-	+	+	+	+	-	+	+	±	+
E	±	+	+	+	+	+	+	+	-	+	±	+	+	+	+	±	±	-	+	±	+	+	+	+	+	±	+	+	±	+
TE	-	±	-	-	-	-	-	-	-	-	±	-	+	±	-	-	±	-	±	±	+	-	-	-	-	-	-	+	±	-
Na	+	+	-	+	±	+	+	+	-	-	±	±	-	+	-	±	±	±	-	-	-	-	-	+	+	-	+	+	±	+
RA	-	-	-	-	+	±	-	-	-	-	-	-	+	-	-	+	-	±	-	-	+	-	-	±	+	-	-	-	-	±
S	-	+-	+-	+	+-	+	+	+	+	+	+	+-	-	+-	+	+	+	+	+-	+-	-	-	+	+	+-	+-	+-	+	+	+
Carbohydrate assimilation																														
Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	+	-	+	-	+	+	+	+
Cellobiose	-	-	+	+	+	+	+	-	-	+	+	-	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+
Saccha-rose	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Maltose	+	-	+	-	+	+	+	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-
Galactose	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Raffinose	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: Good growth, -: no growth, + - : Intermediate growth, BTB: bromothymol blue, RG: Congo red
Am: Ampicillin, Na: Nalidixic acid, S: Streptomycin, E: Erythromycin ,TE: Tetracycline, C: Chloramphenicol, RA: Rifampc

Numerical Analysis

Physiological and biochemical studies are the basis for detailed polyphasic taxonomy, but they cannot be used alone in the taxonomic analysis. However, the results of carbon metabolism, salinity tolerance and maximum growth temperature may provide guidance on the taxonomic position of the strains.The analysis of 34 phenotypic traits by the Statistica 7 software had established a dendrogram (Figure 5). This phenodendrogram showed four distinct clusters and the presence of two separate strains FC12.3.2 and the FT16 which didn't belong to either of the two clusters.The obtained dendrogram showed four separate clusters. The first includes the isolated strain FM24.5 from Mostaganem and FR71 strain isolated from Mascara. Indeed the two strains were resistant to acidic pH, extreme temperature, and concentration of 0.8 M NaCl, they presented a close antibiotic resistance profiles, the only difference between both of them is that the strain FR7.1 used more mannitol, sorbitol and glucose comparatively to the other one which used maltose and cellobiose. The second group is formed by the FM24 and FM9.2 strains isolated from Mostaganem. They can tolerate NaCl concentration of 0.7 M, grew at 10 °C and 35 °C and exhibited good growth at pH 5.They were resistant to rifamycin(30µg), both strains used mannitoland glucose, in addition FM24 used cellobiose. In this group, FM24 strain used as inoculum for *Vicia faba* under controlled conditions in hydroponic conditions was the most effective. The third cluster is formed by two strains FR29.3 and FM20.4 which were characterized by high halotolerancereaching1 M NaCl, could grow at 40 °C, and were sensitive to pH 4. The sensitivity of these strains to various antibiotics was identical.The fourth cluster includes FR14.2.3, FR31.4, FZ28.4, FZ7.1, FR14.2, FR7.1.2, FM19.4.2, FR36.2 strains. They could grow at 5 °C, were sensitive to alkaline pH but had varying tolerance to acidic pH. They showed good uptake of glucose and mannitol except for FR14.2.3, FR31.4, FR36.2 strains. These latest used cellobiose and sorbitol more. The distinctive phenotypic traits are useful for describing new species *Ensifer saheli* and *E. terangae* **[13]** and *E. medicae* **[3]** ; *Ensifer numidicus* and *Ensifer garamanticus* **[29]**; *Rhizobium leguminosarum* bv. Viciae **[23]**;*Rhizobium fabae* **[44]** and for the current identification. These are simple tests that allow the comparison with the old data. In this study, four phenotypic clusters were identified on the basis of 34 characteristics of growth which suggests the presence of interesting diversity among BNL associated with *Vicia faba* in the western Algerian soil

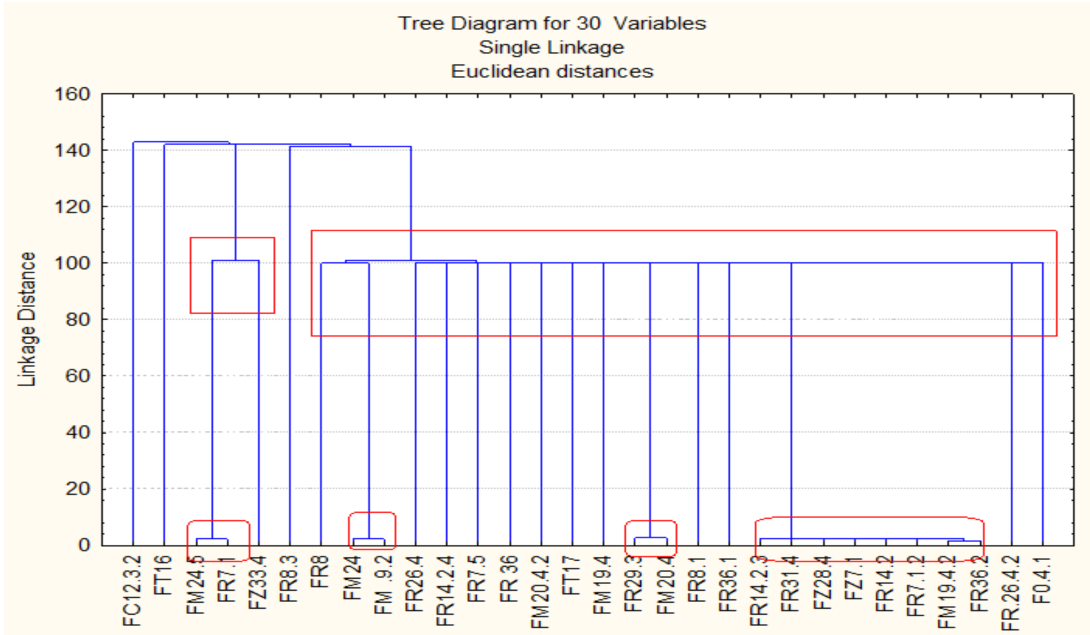

Fig 5. Dendrogram of phenotypic characters using numerical analysis of 34 phenotypic traits

CONCLUSION

The present study shows physiological and symbiotic diversity of rhizobial population on *Faba bean* from Northern Algeria. Some of the strains showed remarkable physiological characteristics such as resistance to antibiotics, high salt tolerance and survival at low pH. In addition, some of these isolates scored highest percentage in effectiveness of symbiotic nitrogen fixation, which may qualify them as good candidates for inoculation approaches under adverse environmental conditions. The results also demonstrate that selection of adapted strains under stress conditions in agar culture is possible and pure culture evaluation may be a useful tool in searching for rhizobial strains better suited for soil environments where high NaCl and pH constitute a limitation for symbiotic nitrogen fixation. To verify the taxonomic position of these strains, this study should be completed by a genotypic characterization including sequencing taxonomic genes of interest.

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