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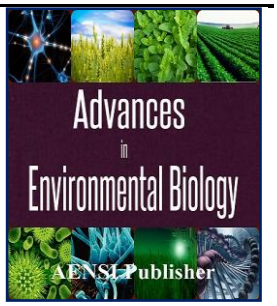

Effect of abiotic factors on the *Sinozhizobium sp* growth isolated from *Medicago sativa* cultivated on northeastern Algeria soils and the selection of the highly tolerant strains

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ABSTRACT

In order to promote the *Medicago sativa* culture in areas with difficult environmental constraints, the inoculation of the plants by adapted and compatible rhizobial strains constitutes an effective way to enhance biologically agricultural production and the soils fertility. In this context, strains of sinorhizobium native of northesatern Algeria soils were caracterized phenotypically. To include bioclimatic and edaphic variability, the strains were isolated by trapping from root nodules of *Medicago sativa* grown on soils of four different geographical sites**.** The aim of this work is to establish a strains collection specific to *Medicago sativa*, to study their capacity to grow and to survive under different abiotic stresses then to select the most efficient strains able to resist to the most unfavorable constraint of the environment. After the results of cytomorphological tests, distinctive tests and nodulation test on the original host plant, a collection of 85 sinorhizobium strains was constituted and subjected to biochemical test and at different stresses: salinity, pH, temperature and antibiotics. The search for specific enzymes (biochemical test), necessary for symbiotic relation, showed that all the strains are provided of catalase, nitrate reductase and urease. Under the abiotic factor variations effect, the results revealed that tolerance limits depend on strains and their geographical origins. Their growth profiles in the range between 1% and 10% NaCl showed that about 54% of the total isolates were tolerant to 3% of NaCl of which 5 strains were able to survive up to 10% NaCl. For their tolerance to pH, the strains exhibited a wide diversity in the range between pH 3,5 and pH10. The maximum growth rates were between pH 6 and pH 8. Concerning resistance profiles to temperature changes, the high growth rates were between 28°C and 35°C while highly thermoresistant strains were between 40 and 45°C. For antibiotics, almost the majority of the strains were sensitive to Ampicillin and to Gentamycin. The highest resistance profile was observed in the presence of Rifampin followed by Tetracycline then Chloramphenicol. The combined analysis of these phenotypic characteristics allowed us to identify high-performing strains presenting heterogeneity sufficient to allow us to select strains adapted to highly variable environmental conditions.

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INTRODUCTION

The aridity of soil and climate are the major constraints that significantly limit plant productivity on 40% of the earth's surface especially in Mediterranean regions [26]. Actually, these factors that combined with a strong multiform anthropic pressure are the major cause of the destruction of vegetation cover and a significant erosion of the biodiversity on a planetary scale. Thus, the ecosystem assessment has shown that the species extinction rate has significantly increased since the early industrial era [47, 51]. Such a loss of the biodiversity, it leads to ecosystems destabilization and biological processes modification [4]; consequently, a loss or a decrease in the density and activity of the microbial soil appears followed by a loss of the soil fertility that result from impoverishment in the organic matter and combined nitrogen, which is one of the most essential nutrients for plant live and the limiting factor for their growth and development [16].

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In order to establish the fertility of degraded soils, attention is focused on biological nitrogen fixation in recent years because it substitutes inorganic fertilizer and is environmental friendly farm inputs essential for poor resource farmers [35]. Installation of legumes would be one of the sustainable solutions and the most promising. These species are more effective to bring biologically molecular nitrogen in the soil in its organic form because they have ability to fix atmospheric nitrogen by symbiotically interaction with soil bacteria of the Rhizobium genus [43, 28], and they deposit significant amounts of nitrogen in the soil during growth [76]. Legume-Rhizobium symbiosis may provide nitrogen easy and inexpensive manner to enhance soil fertility and promote agricultural production [61]. Thereby, they allow reducing inputs of nitrogen chemical fertilizers sparing thus a large part of fossil fuels and contribute to the development of a sustainable and respectful agriculture of the environment. The symbiotic Rhizobia-legume system is the major contributor of biologically fixed nitrogen as compared with non-symbiotic nitrogen-fixing bacteria [68]. Its contribution was estimated at 1.44 × 108 metric tons of nitrogen per year globally [45, 60].

Soil microorganisms specifically bacteria called rhizobia are able to colonise the rhizosphere, infect legume roots and biologically fix nitrogen in the soil through symbiotic process [52,25] and they are responsible for almost 30% of the annual nitrogen fixation [14, 24]. Inside the root nodules, microsymbionts convert atmospheric nitrogen into ammonium, an organic form they exchange with the plant against photosynthates [71].

The leguminous plants are an important family of the angiosperms. Undoubtedly, their association with atmospheric nitrogen fixing bacteria enabling them to have growth even on the soils deficient in nitrogen would be the major factor in the origin of their great success among all plants [57]. In Algeria, ovin and bovin rearing development poses an important socio-economic problem; indeed, the animal feed more often of poor quality does not cover the ruminants' protein and energetic needs. In order to ensure an adequate feed for this livestock, it is fundamental to develop forage crops especially leguminous, that are privileged in the context of a sustainable agriculture, to introduce them in natural grasslands, in lands unsuitable for cereals or to replace unproductive fallow. Many of these species have an important and a wide agricultural and environmental application. *Medicago sativa* or alfalfa is a perennial legume resistant to overgrazing. By its deep root system, it also resists drought, improves soil structure, protects against erosion and limits nitrate losses through leaching [48, 11]. Alfalfa crop provides the major biological source of fixed nitrogen in the agricultural soils and builds up nitrogen reserves in topsoil [58]. Besides this ecological interest, *Medicago sativa* is the choice species to promote in the current agricultural context prohibiting animal meal. Indeed, it plays an important socio-economic role for ensuring an adapted feed responding to ovin and bovin's qualitative and qualitative needs. It is able to produce the high yields of the high-quality of forage with the high quality of protein and a variety of vitamins, minerals and even the biologically active molecules [12, 42, 27]. It is also a plant with many medicinal properties [74, 9, 3, 31].

Alfalfa may be naturally nodulated by bacteria of the genus *Sinorhizobium* which is in its rhizosphere. However, the natural process is affected by several abiotic constraints such as temperature changes, soil acidity, water stress and salinity which were often shown as the principal factors in determining the rhizobia diversity [53, 2]. It may reduce symbiotic efficiency [19, 10, 14] and negatively affect *Medicago sativa* production by acting on the survival and persistence of the strains saprophytic state [5, 18] or by influencing nitrogen fixing activity after the establishment of the symbiosis [44]. Under these conditions, the use of the Rhizobial strains may be an approach to improve the plant productivity in symbiosis [41]; therefore, the inoculation of culture with adequate *Rhizobium*, well adapted to harsh environmental conditions, becomes imperatively necessary [19].

The aim of this study is to isolate and to collect indigenous *Sinorhizobium meliloti* strains from different geographical areas in Northestern Algeria, to analysis their performance under some environmental constraints then to select strains for their high resistance profile.

The relationship between the phenotypic diversity and some soil properties were also evaluated.

MATERIAL AND METHODS

Study areas characteristics:

Soil samples used for trapping Rhizobia were collected from different sites located in four geographic areas in northeastern Algeria (Figure 1): Chatt (7°51′31″ East, 36°49′49″ North), El-Hadjar (7°43′60″ East, 36°48′0″ North), Heliopolis (7°27′0″ East, 36°30′0″ North) and Fetzara (7°29′11″ East, 36°47′33″ North). These soils are different in their physical and chemical characteristics, the preceding crop or the predominant flora and the bioclimatic Characteristics (Table 1, Figure 1).

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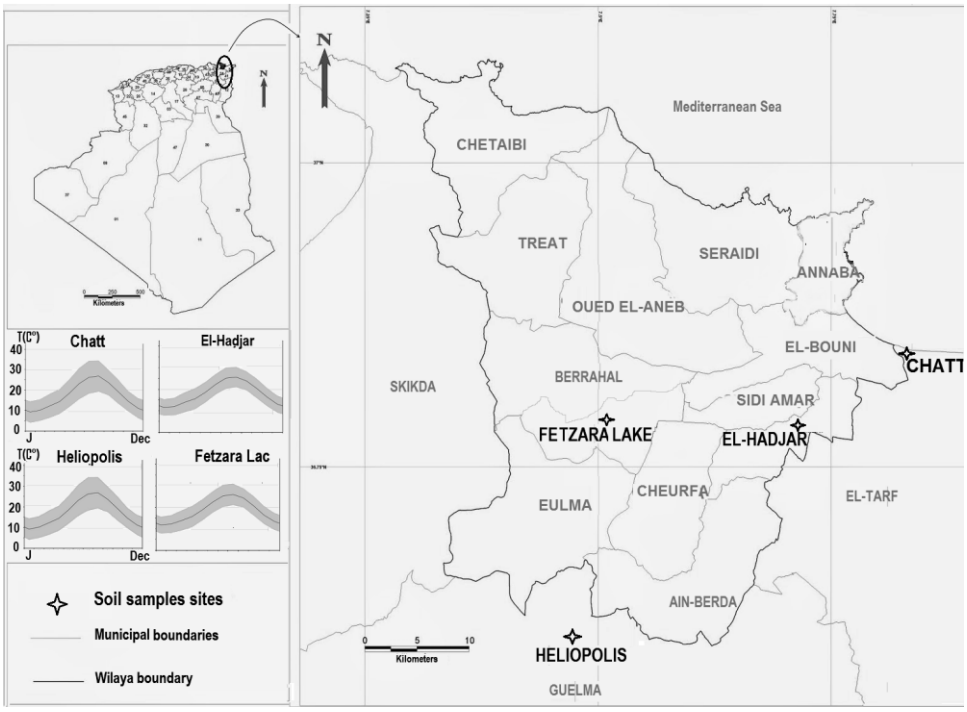

Fig. 1: Geographic location map and temperature of areas where the soil samples were collected

Table 1: Soils and areas characteristics used in this study

Site	Altitude (m)	Bioclimatic Floor	pH water	pH Kcl	EC* (mS/cm)	Texture	Organic Matter (%)	Preceding crop or predominant flora
Chatt	6m	Subhumid	7.22	6.94	2.09	Loamy-clay	2.11	Vegetables crops
El-Hadjar	10m	Subhumid	6.22	5.94	0.17	Clay-loamy	1.58	Vegetables crops
Heliopolis	224m	Semi-arid	6.35	6.03	0.09	Clay-loamy	2.06	Cereal growing
Fetzara	11m	Subhumid	7.01	6.31	3.20	Loamy-clay	2.69	Natural grassland

*EC, electrical conductivity

Soil samples collection:

For each aforementioned site, three soil samples were taken at a distance of about 35 m between each sampling point. From each emplacement, about 500 g of soil were collected from 15 to 20 cm in depth with a clean steel spatula sterilized with ethanol. The soil samples were then collected in sterile plastic bags. In the laboratory, they were thoroughly homogenized, air-dried and passed through a 2 mm sieve to remove stones and large pieces of organic matter and then aliquots were placed into plastic pots previously sterilized.

Isolation of rhizobial strains:

The isolation of Rhizobia strains was obtained by the technique of the host plant infection that involve trapping Rhizobia by legume grown in pots containing soil collected in the four aforementioned sites. Alfalfa seeds were sterilized with calcium hypochlorite 5% (w/v) for 5min. This was followed by a thorough rinsing (6-10 rinses) with sterile distilled water. The seeds were then put to swell during 5 minutes in the sterile distilled water. Finally, the seeds were aseptically disposed in Petri dishes containing water agar 1% and placed inverted in the dark at a temperature of 28°C [66]. The germination was observed after 3 days.

Five (5) pre-germinated seeds were transferred into pots in triplicate (three pots for each site) and kept in a greenhouse at 22 C° day/18 C° night with a 16-h photo-period and 50–60% relative humidity. After 2 months of culture, all the plants were carefully uprooted from pots and thoroughly rinsed with tap water to remove soil particles. The large and pink nodules were collected, rinsed and sterilized by immersion in ethanol at 95°C for 30 seconds and then in mercuric chloride at 0.1% for 2-3 minutes followed by a rinsing with sterile distilled water for ten times [69]. The sterilized nodules are placed in sterile Petri dishes separately and crushed with a sterile glass rod in the presence of sterile distilled water. A loopful of the resulting extract was streaked on YEMA (Yeast Mannitol Agar) medium surface containing Congo red in a Petri dish and incubated at 28°C [69].

After 2-5 days of incubation, single colonies having absorbed little dye were selected to check their purity by repeating streaking on the YEMA medium. After purifying and before undertaking the physiological characterization of isolates and tolerance tests to antibiotics, the purified cultures were subjected to an identification test followed by a nodulation test. Three replicates per isolate were performed for all realized tests.

The identified isolates were designated by the MS code (M: *Medicago*, S: *Sinorhizobium*) and were followed by the letter and numeric number representing the first letter of the origin region and the number of the strain, respectively.

Purification and identification test:

Among the methods used to identify the *Rhizobium* genus we based on those advocated by Vincent (1970), Beck *et al.* (1993), Somasegaran and Hoben (1994).

- *Cytomorphological characterization*

Cytological and morphological tests were carried out for the identification of bacteria [30]. The cytological tests are based on Gram stain, mobility and shape of bacteria. Gram staining was done to ensure the purity and the absence of Gram positive bacteria. The reaction of Gram staining was carried out by using a loopful of pure culture grown on Tryptone agar and stained according to the Gram's standard procedure.

- *Colony morphology*

Colony morphology includes: colonies aspect, colour, size, shape, margin, elevation, opacity, time of the appearance of the first colonies and their diameters. It was evaluated by streaking a loop of the initial inoculum on YEMA plates and incubating them in dish Petri at 28°C for 5 days [69, 64].

- *Distinctive tests*

• *Growth on YMA medium Blue Bromothymol:* This test is performed to put in evidence the speed of the growth. The cultures were streaked on YEMA medium containing Bromothymol blue (BTB). The plates were incubated at 28°C. After 48 hours, a color change of the medium was observed. The isolates were classified either as a slow grower and alkali producers when the medium turns to blue or as a fast grower when the medium turns to yellow indicating that the isolates are acid producers [69].

• *Precipitation of calcium glycerophosphate*: This test allows differentiation between both *Agrobacterium* and *Rhizobium* genus. The strains are grown on YEMA medium calcium glycerophosphate [36]. On this medium, agrobacterium growth product colonies surrounded by a brownish halo and present a slight precipitate probably composed of tricalcium phosphate (positive reaction); however, *Rhizobium* ssp don't give rise to any browning (negative reaction).

Nodulation test:

This test was performed to verify the ability of the strains to form root nodules with the host plant (*Medicago sativa*) under bacteriologically controlled conditions. It is the basic test to confirm the purification and assignment of strains to the group of rhizobia [69, 64]. In this work, *Medicago sativa* seeds were surface sterilized as described above. After germination, the seedlings were aseptically transferred in the test tubes containing the sterile Farhaeus medium. After 3 days, they were inoculated with 1 ml of bacterial suspension of each strain at log phase in order to identify the infectivity of the strains. The non inoculated plants served as negative controls. The cultures thus prepared were left in a lighted area under ambient laboratory conditions.

Forty five days after inoculation, the plants were removed from the test tubes and the presence or absence of nodules was assessed.

Biochemical characterisation:

- *Catalase activity*

Different isolates which were 48 hours old were flooded with hydrogen peroxide and observed for the liberation of the effervescence of oxygen around the bacterial colonies [33].

- *Urea Hydrolysis*

YEM broth was amended with 2% (w/v) urea and 0.012% phenol red to check the urea hydrolysis. The broth was inoculated with log phase cultures, incubated for 48 hours and observed for the production of the color [49].

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- *Growth in Presence of 8% kNO3*

The strains were tested for the ability to grow in the presence of 8% KNO3 in YEM broth for 7 days incubation period at 28°C [20].

Physiological characterization:

For the physiological characterization, the solid medium was used for all performed tests. The Petri dishes were divided into small sectors (20 squares) and each of which was inoculated with 10 µl of a freshly prepared pre-culture at the rate of one strain by sector. The cultures were incubated at 28 ° C for 72 hours. The reading was performed by a comparison with the control growth. For each abiotic factor, three replicates per strain were realized.

- *Determination of salt tolerance*

Strains tolerance to salinity was evaluated by a determination of the growth on the solid YEMA medium supplemented with NaCl at the concentrations ranging from 0% to 10%. The Petri dishes were inoculated from bacterial precultures prepared on liquid YEMA medium without salt. Bacterial growth was estimated by the importance of the diameter of the colony compared to the controls without salt.

- *Determination of pH tolerance*

The ability of the strains to grow on acid and basic pH was performed on the solid medium YEMA by adjusting the pH to 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10. All the plates were incubated at 28°C for 72 hours. The diameter of the colony is compared to the control at pH 7.

- *Determination of temperatures tolerance*

The temperature tolerance was evaluated on YEMA plates inoculated as described above and incubated at temperatures from 4 to 45°C. The control plates were incubated at 28°C.

Antibiotics resistance test:

The antibiotic resistance was detected using the disc diffusion method on a solid medium [6]. YEMA plates were inoculated with 1 ml of an exponentially growing liquid culture.

The agar surface was dried for 3-5 minutes before applying antibiotic discs. The antibiotic discs were equidistantly placed on 90 mm Petri plate using sterile forceps. The plates were incubated aerobically at 28°C for 48 hrs. A resistance to an antibiotic was detected by the absence of an inhibition zone formed around the discs [67]. The antibiotics used were Chloramphenicol (C30): 30 µg, Tetracycline (TE30): 30 µg, Rifampicine (RA30): 30 µg, Streptomycin (S10): 10 µg, Erythromycin (E15): 15 µg, Ampicillin (AM10): 10 µg, Gentamycin (CN10): 10 µg and Penicylline (P10): 10µg.

RESULTS

Cytomorphological and biochemical characterization:

A total of 85 strains of which 20 strains from the Chatt (C), 20 strains from El-Hadjar (E), 22 strains from Heliopolis (H) and 23 strains from Fetzara (F) sites were obtained from nodules of different samples of *Medicago sativa*.

The results of the morphological, cytological and biochemical characters of the strains, shown on the table 2, indicate that the isolates produce white, pink /or cream colonies of different sizes with circular homogeneous and curved shape, translucent and mucilaginous consistence on YMA-RC medium after 24 hours.The microscopic observation of different fresh bacterial suspensions showed that the isolates were gram negative, mobile and rounded tips. On YMA -BTB medium, the color of the medium was changed to yellow indicating that the isolates are acid producers and are fast growing while in YMA-Calcium Glycerophosphate medium no precipitate was formed, so the isolates show a negative effect revealing that our strains do not belong to *Agrobacterium* genus. Moreover, the nodulation test, realized under the microbiologically controlled conditions, showed the presence of nodules on all root systems which confirm that the strains are infective with their host plant (*Medicago sativa*). The biochemical characterization reveals the presence of catalase, urease and nitrate reductase in all authenticated strains.

Table 2: Cytomorphological and biochemical characteristics of isolated strains

	Characteristics								
Isolates	Gram coloration	Colonies appearance time	Colonies diameters	Colonies characteristics	YEMA+BTB reaction	Catalase	Urease	Nitrate reductase	Precipitation of calcium glycerophosphate
MSE1	-	48h	4-5	BTrM	acid	+	+	+	-
MSE2	-	96h	4-5	BTM	acid	+	+	+	-
MSE3	-	96h	4-5	BTM	acid	+	+	+	-
MSE4	-	96h	4-5	BTC	acid	+	+	+	-
MSE5	-	48h	4	PTrC	acid	+	+	+	-
MSE6	-	48h	4	PTrC	acid	+	+	+	-
MSE7	-	48h	4	PTrC	acid	+	+	+	-
MSE8	-	48h	4-5	BTrC	acid	+	+	+	-
MSE9	-	96h	4-5	BTrC	acid	+	+	+	-
MSE10	-	96h	3-4	BTC	acid	+	+	+	-
MSE11	-	96h	3-4	BTrM	acid	+	+	+	-
MSE12	-	96h	4-5	BTrM	acid	+	+	+	-
MSE13	-	96h	4-5	BTrM	acid	+	+	+	-
MSE14	-	96h	4-5	BTrM	acid	+	+	+	-
MSE15	-	96h	4-5	PTrC	acid	+	+	+	-
MSE16	-	96h	4-5	BTrC	acid	+	+	+	-
MSE17	-	96h	4-5	BTrM	acid	+	+	+	-
MSE18	-	96h	4-5	BTrM	acid	+	+	+	-
MSE19	-	48h	4-5	BTrM	acid	+	+	+	-
MSE20	-	96h	4-5	PTM	acid	+	+	+	-
MSH1	-	96h	3-4	BTrC	acid	+	+	+	-
MSH2	-	96h	3-4	BTrM	acid	+	+	+	-
MSH3	-	96h	3-4	BTrC	acid	+	+	+	-
MSH4	-	96h	3-4	BTrM	acid	+	+	+	-
MSH5	-	72h	3-4	BTrC	acid	+	+	+	-
MSH6	-	72h	3-4	BTM	acid	+	+	+	-
MSH7	-	96h	3-4	BTM	acid	+	+	+	-
MSH8	-	96h	3-4	BTM	acid	+	+	+	-
MSH9	-	96h	2-3	PTrM	acid	+	+	+	-
MSH10	-	96h	2-3	PTrM	acid	+	+	+	-
MSH11	-	96h	4-5	BTM	acid	+	+	+	-
MSH12	-	72h	3-4	BTM	acid	+	+	+	-
MSH13	-	96h	4-5	BTM	acid	+	+	+	-
MSH14	-	72h	3-4	BTM	acid	+	+	+	-
MSH15	-	96h	2-3	BTM	acid	+	+	+	-
MSH16	-	96h	2-3	PTrC	acid	+	+	+	-
MSH17	-	96h	2-3	WTrM	acid	+	+	+	-
MSH18	-	96h	2-3	WTrM	acid	+	+	+	-
MSH19	-	96h	2-3	WTrM	acid	+	+	+	-
MSH20	-	48h	2-3	PTrC	acid	+	+	+	-
MSB1	-	72h	2-3	PTrC	acid	+	+	+	-
MSB2	-	72h	2-3	PTrC	acid	+	+	+	-
MSB3	-	48h	2-3	WTrM	acid	+	+	+	-
MSB4	-	48h	2-3	WTrM	acid	+	+	+	-
MSB5	-	48h	2-3	WTrM	acid	+	+	+	-
MSB6	-	48h	2-3	PTrM	acid	+	+	+	-
MSB7	-	48h	2-3	WTrM	acid	+	+	+	-
MSB8	-	48h	2-3	PTM	acid	+	+	+	-
MSB9	-	48h	2-3	PTC	acid	+	+	+	-
MSB10	-	72h	2-3	PTrC	acid	+	+	+	-
MSB11	-	72h	2-3	PTrM	acid	+	+	+	-
MSB12	-	96h	2-3	PTM	acid	+	+	+	-
MSB13	-	96h	2-3	PTM	acid	+	+	+	-
MSB14	-	72h	2-3	WTM	acid	+	+	+	-
MSB15	-	72h	2-3	PTC	acid	+	+	+	-
MSB16	-	96h	2-3	PTC	acid	+	+	+	-
MSB17	-	96h	2-3	PTC	acid	+	+	+	-
MSB18	-	96h	2-3	PTC	acid	+	+	+	-
MSB19	-	48h	2-3	BTC	acid	+	+	+	-
MSB20	-	48h	2-3	PTrM	acid	+	+	+	-
MSB21	-	48h	2-3	PTrM	acid	+	+	+	-
MSB22	-	48h	2-3	PTrM	acid	+	+	+	-

MSF1	-	96h	2-3	BTC	acid	+	+	+	-
MSF2	-	96h	2-3	BTrC	acid	+	+	+	-
MSF3	-	72h	2-3	BTrC	acid	+	+	+	-
MSF4	-	72h	2-3	BTC	acid	+	+	+	-
MSF5	-	72h	2-3	BTrC	acid	+	+	+	-
MSF6	-	72h	2-3	BTC	acid	+	+	+	-
MSF7	-	72h	2-3	BTrM	acid	+	+	+	-
MSF8	-	72h	2-3	BTM	acid	+	+	+	-
MSF9	-	72h	2-3	BTM	acid	+	+	+	-
MSF10	-	96h	2-3	BTC	acid	+	+	+	-
MSF11	-	96h	2-3	BTC	acid	+	+	+	-
MSF12	-	96h	2-3	PTC	acid	+	+	+	-
MSF13	-	96h	2-3	BTC	acid	+	+	+	-
MSF14	-	96h	2-3	BTrM	acid	+	+	+	-
MSF15	-	72h	2-3	BTrM	acid	+	+	+	-
MSF16	-	72h	2-3	PTrM	acid	+	+	+	-
MSF17	-	96h	2-3	WTM	acid	+	+	+	-
MSF18	-	96h	2-3	BTrM	acid	+	+	+	-
MSF19	-	96h	2-3	WTrC	acid	+	+	+	-
MSF20	-	96h	2-3	BTrM	acid	+	+	+	-
MSF21	-	96h	2-3	PTM	acid	+	+	+	-
MSF22	-	96h	2-3	WTrC	acid	+	+	+	-
MSF23	-	96h	2-3	BTM	acid	+	+	+	-

B: Beige, P: Pink, W: White, T: Transparent, Tr: Translucent, M: Mucous, C: Creamy

Physiological characterization:

- *Salt tolerance*

The study of the salt tolerance shows that the salt has a deleterious effect on growth rhizobia. At 1% of NaCl, the growth percentage of the strains is optimal; it is 100% (Fig. 2). From this concentration, this percentage decreases as the salinity increases, and a wide diversity of the strains for their halotolerance is then observed in the 4 prospected sites. At 2% of NaCl, the percentage of the tolerant strains is at 70% at Chatt soil, 80% at El-Hadjar soil, 63.63% at Heliopolis soil and 82.60% at Fetzara soil.

At 3% of NaCl, an average more than 50% of the strains are able to grow normally. The concentrations of 5% and 6% of NaCl constitute the tolerance limits to the native strains of Heliopolis and El-Hadjar, respectively. The most salt-tolerant strains are those isolated from Chatt and Fetzara soils. These soils, which are highly affected by salt, justify the presence of the highly halotolerant strains of which 5 strains can even tolerate 10% of NaCl: MSC2 and MSC9 are native from Chatt soil, and MSF21, MSF20 and MSF19 are native from Fetzara soil.

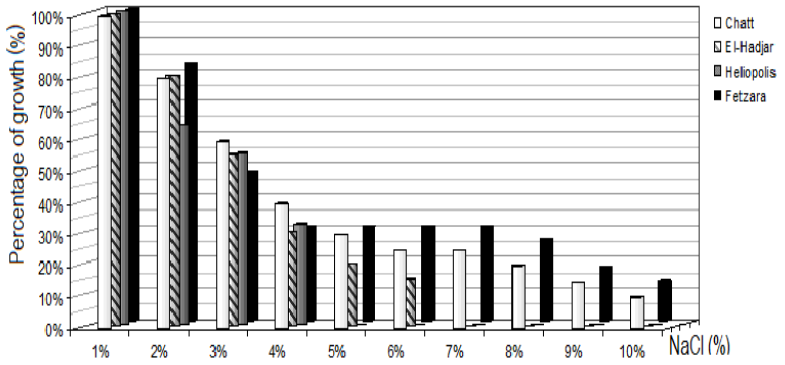

Fig. 2: Isolates growth on different concentration in Nacl

- *pH tolerance*

The results of pH tolerance observed in the different prospected areas are shown in the figure 3. In the range between pH 3.5 and pH 10, the vast majority of the strains isolated from Chatt soil are rather neutrophils. Their growth is optimum at pH 7 whereas more than 70% of the strains have sensitivity outside this value. The strains isolated from three other sites showed a wide diversity for their tolerance to pH changes. At alkaline pH, the strains are more tolerant especially those isolated from the soils of Heliopolis and Fetzara. The growth percentages recorded at pH 8 are 55%, 91% and 73.91% at El-Hadjar, Heliopolis and Fetzara soils, respectively. At pH 9, they are of the order of 50%, 68.18 and 47, 82%. At pH 10, the rates of tolerant strains vary from 20% to over 30% according to locality. Concerning acid pH, the strains originate from El-Hadjar and Heliopolis seems rather indifferent to the acidity of the medium. At pH 6, it was recorded 85% and 81.81% of resistant

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strains, respectively, against 65.2% of strains native from Fetzara soils. At pH 5, these percentages are in the order of 55%, 63.63% and 43.48%, respectively.

Strains tolerant to pH 4 were also registered which (4) strains (MSE2, MSE7, MSE15, MSE16) from El-Hadjar soil and (1) strain (MSH5) originating from Heliopolis soil were even able to survive at pH 3.5. This resistance to extremely acid pH could be in relation with pH origin of the isolates (Table 1).

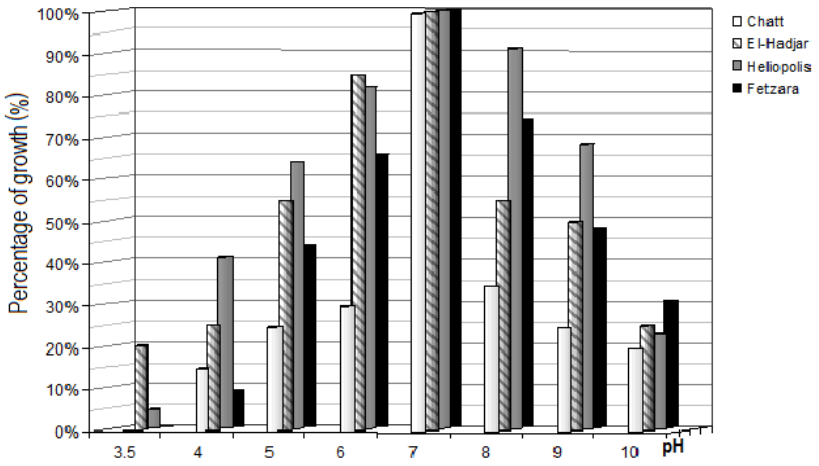

Fig. 3: Effect of pH on isolates Growth

- *Temperature tolerance*

The results indicating margins temperature tolerance of the tested strains in this study show that most of the isolates exhibit a good thermotolerance (Fig. 4). The strains isolated from Heliopolis seem to be the most indifferent and the most resistant to temperature changes. Their growth rate is optimal (100%) in the interval comprised between 4 and 35 °C. It is 81.81% and 72.72% at 40 °C and at 45 °C, respectively. The existence of highly thermotolerant strains with such a high percentage on the soils of Heliopolis could be an indication of their adaptation to the temperature of the area (Fig.1).

On the other tested sites, the maximum growth of the isolates was obtained between 28°C and 35°C. Outside these values, the percentage of the tolerant strains vary on average from 85% at 22°C to 75% at 4°C. From 35 ° C, the strains seem less tolerant; nevertheless, 65% of them exhibited a resistance at 40 ° C.

At 45 ° C, almost half of the Fetzara strains (47.82%) exhibit forever a good growth against only 20% (MSC4, MSC9, MSC18, MSC19) at Chatt soil and 25% (MSE4, MSE16, MSE17, MSE18, MSE20) at El-Hadjar soil.

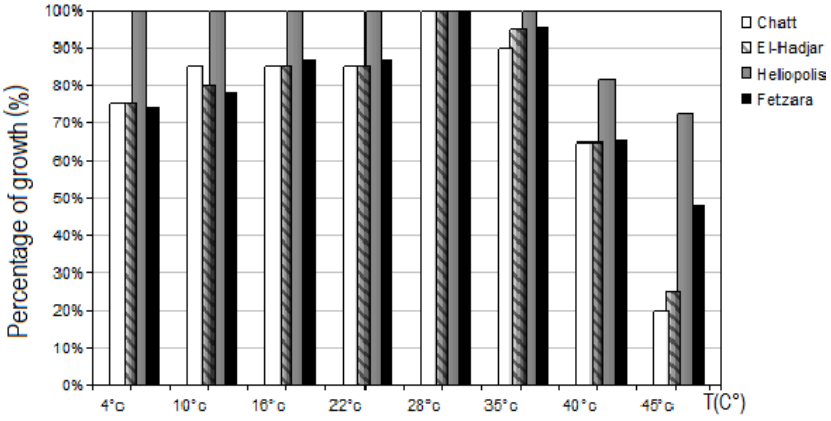

Fig. 4: Effect of temperature on isolates Growth

Intrinsic antibiotics resistance:

The evaluation of the intrinsic resistance to antibiotics of Alfalfa rhizobia indicates in figure (5) that the inhibitory effect of the antibiotics depends on their nature and on the bacterial strains. It appears from the results that AM10 and N10 have proved the most harmful antibiotics on the growth of the strains. Indeed, on a total of 85 strains, only (6) strains (MSC1, MSC19, MSF1, MSF22, MSH17, MSH21) have survived in the presence of

AM10 and (2) strains (MSC1, MSF22) in the presence of CN10. In contrast, in the vast majority, the strains have been resistant with the six (6) other tested antibiotics. The highest resistance profile was observed in the presence of Rifampin followed by Tetracycline then Chloramphenicol. Comparing the resistance of the strains in relation of their geographical origin, the results reveal that moderately resistant strains are native from Chatt and El-Hadjar soils with an average of 63.33% and 70%, respectively, whereas the most resistant strains are majority amongst those belonging to Heliopolis (77.28%) and Fetzara soil (83.38%).

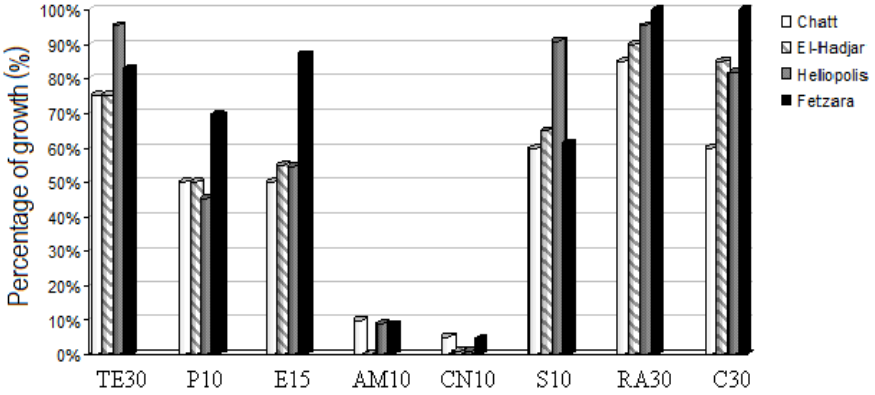

Fig. 5: Effect of antibiotics on isolates Growth

DISCUSSION

A collection of 85 *Rhizobia* strains was obtained from the nodules *of Medicago sativa* which had been grown on soils belonging to 4 different edapho-climatic characters sites. These strains were distributed as follows: 20 strains from the Chatt site, 20 strains from El-Hadjar, 22 strains from Heliopolis and 23 strains from Fetzara. The results of morphological and cytological tests supported by biochemical tests, which are realized on YMA-BTB and YMA-Calcium Glycerophosphate, show that all characters developed by our strains were consistent with the phenotypic appearance previously described in fast growing *Rhizobium* [69, 39, 63]. The nodulation test realized under the microbiologically controlled conditions authenticates the appearance of the strains to the *Rhizobia* genus, and allows identifying them as *Sinorhizobium meliloti* and/or *Sinorhizobium medicae* two species able to infect *Medicago sativa*.

The strains have all been able to reduce nitrates and nitrites and present catalasic and ureasic activities. Theses enzymes enabling the strains to resist to their environments and to infect their host plants are ecologically important for the selection.

The effects of the abiotic factors on the growth and on the survival of the isolated strains were studied. The results were submitted to comparative analysis between strains from the same site and between strains of different sites.

Regarding the effect of the salinity on the growth of the tested *Rhizobia*, an adverse effect appears beyond 1% of NaCl by a percentage of the growth that decreases when the salt concentration increases. This inversely proportional negative effect to the concentration of NaCl was also reported by other authors [34, 22, 1]. El-Sheikh (1998) reported for this factor that the osmotic effect is the main backlash of the application salt on the growth of *Rhizobia*. The results also indicate that each site presents native strains with different resistance profiles and different tolerance limits. Indeed, Jida and Assefa (2012) and Jebara *et al*. (2001) reported that the tolerance limit to NaCl for *Rhizobia* can considerably change from one species to another and even between strains of the same species. In this study, the highest tolerance limit was at 10% of NaCl. It was observed in three strains (MSF21, MSF20, MSF19) isolated from Fetzara and two strains (MSC2, MSC9) isolated from Chatt. This high tolerance to salt could be related to soil isolation highly affected by salt. In this context, Mpepereki *et al*. (1997) reported that the existence of tolerant salt strains in halomorphic sites may be an indication of their adaptation to osmotic stress due to an increase of the ion concentration and to the variation in soil moisture during dry periods. Many studies mentioned a very interesting resistance profiles in *Sinorhizobium* associated with the species of *Medicago* [21]. In other legumes, associated strains to *Lupinus sp* were able to grow at 10% of NaCl [75], and one associated strain to fenugreek was able to grow at a limit of 14% of NaCl [1]. In this regard, Boncompagni *et al.* (1999), Gouffi *et al.* (1999) and Vriezen *et al*. (2007) reported that the salt high tolerance of some rhizobial strains was associated to their ability to accumulate some protective organic osmolytes such as amino acids (proline, betaine, glutamate) or carbohydrates (trehalose, sucrose) in order to maintain the cell turgor and to limit the damage caused by salt.

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The results on the effects of the pH changes show that tested *Rhizobia* strains exhibited a wide diversity for their tolerance to pH variations for all prospected sites in the range between pH 3.5 and pH 10. On average, the majority of the isolates grow best between pH 5 and pH 9 with an optimal growth rate at pH 7. The tolerance to pH changes seems to depend on the strains; a preference to basic pH was observed to strains native of Heliopolis and Fetzara soil. Tolerant strains to pH extremely acids (pH 4 and 3.5) were also recorded. The margins of tolerance to pH were the object for many studies on different isolated rhizobial species from various legumes of which herbaceous legumes *Medicago genus* [72, 39, 62, 21], so the results depend on the rhizobial strains and the host species. Concerning alkalinity, Yadav and Vyas (1971) reported that the growth of fast growing strains was normal in the alkaline medium up to pH 10. On acidity, Brockwell *et al*. (1991) reported that *Sinorhizobium meliloti* is sensitive to acidic pHs. However, Yadav and Vyas (1973) and Elboutahiri *et al*. (2010) were able to isolate tolerant *Sinorhizobium* strains at about pH 3.5. Under these conditions of low pH, Kurchak *et al.* (2001) reported that some strains in response to acid stress would present specific genes, the act genes (acid tolerance). Many other mechanisms related to this tolerance were also reported: exclusion and expulsion of protons H + [78], polyamines accumulation [29] and exopolysaccharide production [70].

By their effects on the turgor and on the enzymes action of the bacterial cell, the extreme temperatures have adverse effects on the growth, the survival of *Rhizobia* and on their ability to fix atmospheric nitrogen. Our conducted study on the isolated *Rhizobia* submitted to temperature variations reveals that the vast majority of the strains exhibited a good growth in the temperature range between 4 and 35 ° C with a luxuriant growth between 28 and 35°C. The effect of the low temperatures on the growth of *Rhizobia* was little reported [59, 50]. In general, the bacteria are tolerant to temperatures from the order of 4°C and 5°C. However, in the stressed bacteria, the extremely low temperatures could result a jellification of the cellular water and sometimes irreversible inactivation of their enzymes from which inhibition of the expression of the "nod" genes and therefore the infection and the nodulation [77].

Concerning the high temperature, our results show an interesting growth percentages at 40 °C of which some strains have continued to survive even at 45 °C. The isolated strains from the Heliopolis soil represent the highest percentage of resistance at the mentioned temperature limits in this study. The resistance of *Sinorhizobium* to such a high temperatures was also reported by Nich *et al.* (1999), Sebbane *et al*. (2005), Elboutahiri *et al.* (2010) and Chriet *et al.* (2014), who mentioned tolerances between 42 and 45 ° C according to the host plant. For other *Rhizobia* species, many results reported a high tolerance limits in the order of 47 ° C [40] and 48.7 ° C [55]. Cloutier *et al.* (1992) indicated that not the extreme heat stress generally induces the expression of HcP (Heat shock Proteins), which provide protection key enzymes of microbial physiology. In contrast, the exposure to the extreme high temperatures can lead to a loss of the infective capacity of the bacterium [40].

The evaluation of the intrinsic resistance to the antibiotics reveal that all strains of four geographical groups have presented a remarkable sensitivity in the presence of AM and CN except some strains (MSC1, MSC19, MSF1, MSF22, MSH17, MSH21) and (MSC1, MSF22) that have made proof of resistance to these antibiotics, respectively. In contrast, in the presence of other antibiotics, each site presents native strains with different resistance profiles. The highest resistance has been observed in the presence of Rifampin followed by tetracycline then Chloramphenicol. Comparing the resistance profiles of the different strains, we find that the most resistant strains are predominantly found among the strains belonging to Fetzara and Heliopolis sites. In these sites considered as few or not treated with pesticides, the strong presence of the resistant strains to the different antibiotics could be explained by the fact that these bacteria might adapt to different emitted stimulus by various fungi and bacteria by sharing, initially, the same ecological niche with these microorganisms, present in the soil.

CONCLUSION

To the term of this work, the study of the resistance profiles to the variations of the abiotic factors revealed a wide phenotypic diversity in isolated *Sinorhizobium* in relation to their geographic origins. This allows us to select the suitable candidates for the inoculation of *Medicago sativa* according to the different envisaged conditions of soil and climate. Highly tolerant strains to salt, acid and basic pH, temperature and antibiotics were identified; they reflect the environmental stresses pressure predominant in their sites. These strains constitute biological material which should be tested in combination with *Medicago sativa* in controlled conditions to select those with the best performance symbiotic under unfavorable conditions of the environment.

REFERENCES

[1] Abdelmoumen, H., A. Filali-Maltouf, M. Neyra, A. Belabed and M.M. El Idrissi, 1999. Effect of high salts concentrations on the growth of rhizobia and responses to added osmotica. Journal of Applied Microbiology, 86: 889-898.

[2] Alexandre, A. and S. Oliveira, 2013. Response to temperature stress in rhizobia. Critical Reviews in Microbiology, 39(3): 219-228.
[3] Al-Dosari, M.S., 2012. *In vitro* and *in vivo* antioxidant activity of alfalfa (*Medicago sativa* L.) on carbon tetrachloride intoxicated rats. American Journal of Chinese Medicine, 40(4): 779-93.
[4] Balvanera, P., A.B. Pfisterer, N. Buchmann, J.S. He, T. Nakashizuka, D. Raffaelli and B. Schmid, 2006. Quantifying the evidence for biodiversity effects on ecosystem functioning and services. Ecology Letters, 9: 1146-1156.
[5] Batzli, J., W.R. Graves and P. Van Berkum, 1992. Diversity among Rhizobia effective with *Robinia pseudoacacia* L. Applied and Environmental Microbiology, 58: 2137-2143.
[6] Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a stansardized single disk method. American Journal of Clinical Pathology, 45: 493-496.
[7] Beck, D.P., L.A. Materon, F. AFANDI, 1993. Practical Rhizobium Legume Technology Manual. Technical Manual International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, Box 5466, 19, 170.
[8] Boncompagni, E., M. Osteras, M.C. Poggl and D. Le Rudulier, 1999. Occurrence of choline and glycine betaine uptake and metabolism in the family of Rhizobiaceae and their role in osmoprotection. Applied and Environmental Microbiology, 65: 2072-2077.
[9] Bora, K.S and A. Sharma, 2011. Phytochemical and pharmacological potential of *Medicago sativa*: A review. Pharmaceutical Biology, 49: 211-220.
[10] Borucki, W., and M. Sujkowska, 2008. The effects of sodium chloride salinity upon growth, nodulation, and root nodule structure of pea (*Pisum sativum* L.) plants. Acta Physiologiae Plantarum., 30: 293-301.
[11] Broderick, G.A., 2001. Maximizing utilization of alfalfa protein: The example of the lactating dairy cow. In: Delgado I., Lloveras, J. (eds.). Quality in lucerne and medics for animal production, Zaragoza, Spain, CIHEAM-IAMZ: 183-192
[12] Burris, R.H. and G.P. Roberts, 1993. Biological nitrogen fixation. Annual Review of Nutrition, 13: 317-335.
[13] Cesar, A.I., M.G. Esther, M. Daniel, L. Ruben, L. Estibaliz and G.Q. Erena, 2011. Physiological response of legume nodules to drought. Plant stress. 5: 24-31.
[14] Cheng, Q., 2008. Perspectives in biological nitrogen fixation research. Journal of Integrative Plant Biology, 50: 786-798.
[15] Cheriet D., A. Ouartsi, D. Chekireb and S. Babaarbi, 2014. Phenotypic and symbiotic characterization of rhizobia isolated from *Medicago ciliaris* L. growing in Zerizer from Algeria. African Journal of microbiology Research, 8(17): 1763-1778.
[16] Cleland, E.E., and W.S. Harpole, 2010. Nitrogen enrichment and plant communities. New York Academy of Sciences. 1195: 46–61.
[17] Cloutier, J., D. Prévost, P. Nadeau and H. Antoun, 1992. Heat and cold shock protein synthesis in artic and temperate strains of rhizobia. Applied and Environmental Microbiology, 58: 2846-2853.
[18] Cordovilla, M.P., F. Ligero and C. Lluch, 1994. The effect of salinity on N2 fixation and assimilation in *Vicia faba*. Journal of Experimental Botany, 45: 1483-1488.
[19] Deka, A.K and P. Azad, 2006. Isolation of Rhizobial strains cultural and Biocemical Characteristics. Legume Research, 29 (3): 209-212.
[20] Dreyfus, B.L., J.L. Garcia, and M. Gillis, 1988. Characterization of *Azorhizobium caulinodans* gen. nov., spp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. International Journal of Systematic Bacteriology, 38: 89-98.
[21] Elboutahiri, N., L. Thami-Alami and S.M. Udupa, 2010. Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S madicae* from draught and salt affected regions of Moroco. BMC Microbiology, 10: 1-15.
[22] El-Sheikh, E.A.E. and M. Wood, 1989. Response of chickpea and soybean rhizobia to salt: Influence of carbon source, temperature and pH. Soil Biology and Biochemistry, 21: 883–887.
[23] El-Sheikh, E.A.E., 1998. Effects of salt on rhizobia and bradyrhizobia: a review. Annals of Applied Biology, 132: 507-524.
[24] Ertl, G., 2012. The Arduous Way to the Haber - Bosch process. Zeitschrift Fur Anorganische Und Allgemeine Chemie, 638: 487-489.
[25] Eskin, N., 2012. Colonization of Zea mays by the Nitrogen Fixing Bacterium *Gluconacetobacter diazotrophicus*. M.S. thesis, Western Ontario univ., London, Canada.
[26] FAO, 1988. Programme de coopération technique. Programme de développement des productions fourragères et de l'élevage. Rapport de synthèse, p: 45.
[27] Faye, L., N. Landry, P. Lerouge, V. Gomord and L.P.Vézina, 2001. La production de protéines à usage biopharmaceutique dans les plantes. Synthèse médecine/sciences, 17(8-9): 867-77.
[28] Franche, C., K. Lindstrom, C. Elmerich, 2009. Nitrogen fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil., 321(1-2): 35-59.

[29] Fujihara, S., and T. Yoneyama, 1993. Effect of pH and osmotic stress on cellular polyamine contents in the soybean rhizobia *Rhizobium fredii* P220 and *Bradyrhizobium japonicum* A1017. Applied and Environmental Microbiology, 59: 1104-9.
[30] Gachande, B.D. and G.S. Khansole, 2011. Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn and *Bradyrhizobium japonicum* of soybean. Bioscience Discovery, 2: 1-4
[31] Gaweł, E., 2012. Chemical compositions of lucerne leaf extract (EFL) and its applications as a phytobiotic in human nutrition. Acta scientiarum polonorum. Technologia alimentaria, 11: 303-309.
[32] Gouffi, K., N. Pica, V. Pichereau and C. Blanco, 1999. Disaccharides as new class of non-accumulating osmoprotectants for *Sinorhizobium meliloti*. Applied and Environmental Microbiology, 65: 1491-1500.
[33] Graham, P.H and C.A. Parker, 1964. Diagnostic features in the characterization of root nodule bacteria of legumes. Plant Soil, 20: 383-396.
[34] Greenway, H. and R. Munns, 1980. Mechanisms of salt tolerance in nonhalophytes. Annual Review of Plant Physiology, 31*:* 149–190.
[35] Haru, A. and W. Ethiopia, 2012. Influences of Inoculation Methods and Phosphorus Levels on Nitrogen Fixation Attributes and Yield of Soybean (*Glycine max* L.). American Journal of Plant Nutrition and Fertilization Technology, 2: 45-55.
[36] Hofer, A.W., 1941. A characterization of *Bacterium radiobacter*. Journal of Bacteriology, 41: 193-224.
[37] Jebara, M., R. Mhamdi, M.E. Aouani, R. Ghrir and M. Mars, 2001. Genetic diversity of Sinorhizobium populations recovered from different Medicago varieties cultivated in Tunisian soils. Canadian Journal of Microbiology, 47(2): 139-147.
[38] Jida, M. and F. Assefa, 2012. Phenotypic diversity and plant growth promoting characteristics of Mesorhizobium species isolated from chickpea (*Cicer arietinum* L.) growing areas of Ethiopia. African Journal of Biotechnology, 11: 7483-7493.
[39] Jordan, D.C., 1984. Family III. Rhizobiacea Conn (1938). In Bergey's Manual of Systematic Bacteriology. Vol (1) (eds Krieg.N.R. and Holt, J.G) Wilhams and Wilkins Press, Baltimore, pp: 234-254.
[40] Karanja, N.K. and M. Wood, 1988. Selecting Rhizobium phaseoli strains for use with beans (*Phaseolus vulgaris L.*) in Kenya. Tolerance of high temperature and antibiotic resistance. Plant Soil, 112: 15-22.
[41] Kenenil, A., F. Assefa and P.C. Prabu, 2010. Characterization of acid and salt tolerant rhizobial strains isolated from faba bean fields of Wollo, Northern Ethiopia. Journal of Agricultural Science and Technology, 12: 365-376.
[42] Khoudi H., S. Laberge, J.M. Ferullo, R. Bazin, A. Darveau, Y. Castonguay, G. Allard, R. Lemieux and L.P. Vézina, 1999. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotechnology and Bioengineering, 64: 135-143.
[43] Kiers, E.T., R.A. Rousseau, S.A. West and R.F. Denison, 2003. Host sanctions and the legume Rhizobium mutualism. Nature, 425: 78-81.
[44] Kucuk, C. and M. Kivanc, 2008. Preliminary characterization of Rhizobium strains isolated from chickpea nodules. African Journal of Biotechnology, 7(6): 772-775.
[45] Kumar Gothwal, R., V. Kumar Nigam, M.K. Mohan, D. Sasmal and P. Ghosh, 2007. Extraction of Bulk DNA from Thar Desert Soils for Optimization of PCR-DGGE Based Microbial Community Analysis. Electronic Journal of Biotechnology, 10: 400-408.
[46] Kurchak, O.N., N.A. Provorov and B.V. Simarov, 2001. Plasmid pSym1-32 of *Rhizobium leguminosarum* bv. *viceae* Controlling Nitrogen Fixation Activity, Effectiveness of Symbiosis, Competitiveness and Acid Tolerance. Russian Journal of Genetics, 37: 1025-1031.
[47] Lawton, J.H. and R.M. May, 1995. Eds., Extinction rates. Oxford: Oxford University Press.
[48] Le Gall, A., 1993. Les grandes Iégumineuses: Situation actuelle, atouts et perspectives dans le nouveau paysage fourrager français. Fourrages, 134: 121-144.
[49] Lindstrom, K. and S. Lehtomaki, 1988. Metabolic properties, maximum growth temperature and phage sensitivity of Rhizobium *sp.* (galegae) compared with other fast growing rhizobia. FEMS Microbiology Letters, 50: 277-287.
[50] Lynch, D.H., and D.L. Smith, 1993. Soybean (*Glycine max*) nodulation and N2-fixation as affected by exposure to low root-zone temperature. Plant Physiology, 88: 212-220.
[51] MA (Millennium Ecosystem Assessment), 2005. Ecosystems and human well-being: synthesis. Synthesis, 1-24, Island Press,Washington, D.C.
[52] Mohammadi, K. and Y. Sohrabi, 2012. Bacterial Biofertilizers for Sustainable Crop Production: A Review. Journal of Agricultural and Biological Science, 7: 307-316.
[53] Moschetti, G., A.L. Peluso, A. Protopapa, M. Anastasio, O. Pepe and R. Defez, 2005. Use of nodulation pattern, stress tolerance, nodC gene amplification, RAPD-PCR and RFLP–16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar viciae. Systematic and Applied Microbiology. 28: 619-631.

[54] Mpepereki, S., F. Makonese, A.G. Wollum, 1997. Physiological characterization of indigenous rhizobial nodulation *Vigna unguiculata* in Zimbabwean soils. Symbiosis, 22: 275-292.
[55] Munevar, F., and A.G. Wollum II, 1981. Growth of *Rhizobium japonicum* strains at temperatures above 27◦C. Applied and Environmental Microbiology, 42: 272–276.
[56] Nick, G., P. de Lajudie, B. D. Eardly, S. Suomalainen, L. Paulin, X. Zhang, M. Gillis, and K. Lindström, 1999. Sinorhizobium arboris sp. nov. and Sinorhizobium kostiense sp. nov., isolated from leguminous trees in Sudan and Kenya. International Journal of Systematic Bacteriology, 49: 1359-1368.
[57] Noel, K.D., 2009. Bacteria Rhizobia. Encyclopedia of microbiology, Schaechiter M. San Diego. Marquette University, Milwaukee, W.I., USA., 3: 877-893.
[58] Peterson, T.A. and M. P. Russelle, 1991. Alfalfa and the nitrogen cycle in the Corn Belt. Journal of Soil and Water Conservation, 46: 229-235.
[59] Prévost, D., L.M. Bordeleau, S. Caudry-Reznick, H.M. Schulman and H. Antoun, 1987. Characteristics of rhizobia isolated from three legumes indigenous to the Canadian high arctic:*Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*. Plant and Soil, 98(3): 313-324.
[60] Rakash, N. and K. Rana, 2013. Food Legumes for Livelihood and Nutritional Security in North Eastern Himalayan Region: Prospects and Constraints. Indian Journal of Agricultural Sciences, 83: 899-906.
[61] Roychowdhury, R., U. Banerjee, S. Sofkova and J. Tah, 2013. Organic Farming for Crop Improvement and Sustainable Agriculture in the Era of Climate Change. OnLine Journal of Biological Sciences, 13: 50-65.
[62] Sebbane, N., M. Sahnoune, F. Zakhia, A. Willems, S. Benallaoua and P. De Lajudie, 2006. Phenotypical and genotypical characteristics of root-nodulating bacteria isolated from annual Medicago spp. in Soummam Valley (Algeria). Letters in Applied Microbiology, 42(3): 235-41.
[63] Singh, B., R. Kaur and K. Singh, 2008. Characterization of Rhizobium strain isolated from *Trigonella foenumgraecum* (Fenugreek).African Journal of Biotechnology, 7(20): 3671-3676.
[64] Somasegaran, P. and H.J. Hoben, 1985. Methods in legume-Rhizobium technology. University of Hawaii, Honolulu. pp: 128-138.
[65] Somasegaran, P. and H.J. Hoben, 1994. Handbook for Rhizobia: Methods in legume-Rhizobia technology. Springer-Verlag. New-York. pp: 450.
[66] Tillard, P. and J.J. Drevon, 1988. Nodulation and nitrogenaes activity of chickpea cultivar INRA 199 inoculated with different strains of *Rhizobium ciceri*. Agronomie, 8: 387-392.
[67] Van Berkum, P., D. Beyene, G.P. Bao, A. Campell and B.D. Eardly, 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. International Journal of Systematic Bacteriology, 48: 13-22.
[68] Venieraki, A., M. Dimou, E. Vezyri, L. Kefalogianni, N. Argyris, G. Liara, P. Pergalis, I. Chatzipavlidis and P. Katinakis, 2011. Characterization of Nitrogen-Fixing Bacteria Isolated from Field-Grown Barley, Oat and Wheat. The Journal of Microbiology, 49: 525-534.
[69] Vincent, J.M., 1970. A Manual for the Practical Study of the Root-nodule Bacteria. IBP Handbook 15. Oxford: Blackwell.
[70] Vriezen, J.A.C., F.J. Bruijn and K. Nüsslein, 2007. Responses of Rhizobia to desiccation in relation to osmotic stress oxygen temperature. Applied and Environmental Microbiology, 73: 3451-3459.
[71] Wang, D., S. Yang, F. Tang and H. Zhu, 2012a. Symbiosis specificity in the legume: rhizobial mutualism. Cellular Microbiology, 14: 334-342.
[72] Yadav, N.K. and S.R. Vyas, 1971. Response of root nodule bacteria to saline, alkaline and acid conditions. Indian Journal of Agricultural Science, 41: 875-881.
[73] Yadav, N.K. and S.R. Vyas, 1973. Salt and pH tolerance of rhizobia. Folia Microbiology, 18: 242-247.
[74] Yu, L., J. Nie, C. Cao, Y. Jin, M. Yan, F. Wang, J. Liu, Y. Xiao, Y. Liang and W. Zhang, 2010. Phosphatidic acid mediates salt stress response by regulation of MPK6 in *Arabidopsis thaliana*. New Phytologist., 188: 762–773.
[75] Zahran, H.H., L.A. Rasanen, M. Karsisto and K. Lindstrom, 1994. Alteration of lipopolysacharide and protein profiles in SDS-PAGE of Rhizobia by osmotic and heat stress. World Journal of Microbiology and Biotechnology, 10: 100-105.
[76] Zeng, X., A. Hourse and A. Tzagoloff, 2007. The Saccharomyces cerevisiae ATP22 gene codes for the mitochondrial ATPase subunit 6-specific translation factor. Genetics, 175(1): 55-63.
[77] Zhang, Z.Y., A.B. Walsh, L. Wu, D.J. McNamara, E.M. Dobrusin and W.T. Miller, 1996. Determinants of substrate recognition in the protein-tyrosine phosphatase, PTP1. Journal of Biological Chemistry, 271(10): 5386-92.
[78] Chen, H., A.E. Richardson and B.G. Rolfe, 1993a. Studies on the physiological and genetic basis of acid tolerance in *Rhizobium leguminosarum* biovar trifolii. Applied of Environmental Microbiology, 59: 1798-1804.