ISOLATION AND EVALUATION OF THE EFFECT OF SOME ENVIRONMENTAL STRESSES ON RHIZOBIA GROWTH ISOLATED FROM LOCAL POPULATIONS OF TWO ANNUAL *Medicago* SPECIES

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Abstract. The objective of the present study is to evaluate the tolerance of isolate rhizobial strains nodulating *Medicago laciniata* and *Medicago minima* to high temperature, salinity and pH. Nodules collected in ten local populations of *Medicago laciniata* and *Medicago minima*, collected from Djelfa regions (Algerian central steppe), were used for the isolation of the rhizobia strains. Nineteen strains were isolated and purified in YEM medium containing Congo red. Strains were tested for their tolerance to high temperatures (30, 32, 35, 40 and 40°C), to different concentrations of NaCl (200, 400, 600 and 800 mM) and to different levels of pH (5.5, 6.5, 7.5, 8.5 and 9.5). The tolerance assessment of strains to the main environmental stresses has allowed us to identify some strains with high tolerance. Results showed that some strains can tolerate high temperatures (40°C) and high concentrations of NaCl (800mM). The strains were also able to grow at pH 5.5 to 9.5.

Keywords: environmental stresses; *Medicago laciniata*; *Medicago minima*; rhizobia; tolerance.

INTRODUCTION

Biological fixation of molecular nitrogen from the atmosphere is one of the main sources of nitrogen pool enhancement in agricultural soils [4]. N requirement is the most central feature for plant production [2]. Consequently, nitrogen deficiency may exert pronounced adverse effects on crop development and yield [36].

N is one of the main components of organic compounds such as amino acids, proteins, and nucleic acids [15]. Low N leads to developmental responses such as stunted growth, early flowering, reduced tillering in cereals, shoot branching and smaller leaves due to decreased cell division and expansion [9].

Symbiotic nitrogen fixation by rhizobia in root nodules of grain and forage legumes provides substantial economic and environmental benefits. Symbiotic nitrogen fixation is dependent on the host plant genotype, the rhizobia strain and the interaction of these symbionts with the pedoclimatic factors and the environmental conditions [11].The symbiosis is based on specific recognition of signal moleucles produced by bacteria and its plant parteners. Process of symbiosis results in biological nitrogen fixation in which atmospheric nitrogen (N2) is converted into ammonia (NH3) which is subsequently available for plants, and in turn plant provide nutients to the bacteria [23].

Symbioses have major environmental and agricultural importance since it is responsible for most of the biological atmospheric nitrogen fixation on earth [35]. Symbiosis represents one of the most productive nitrogen-fixing systems and effectively renders the host plants to be more or less independent of other nitrogen sources [8]. This advantage offers great potential for increasing yields, improving soil fertility and reducing the cost of agricultural production while protecting the environment [5].

However, The harsh environmental condition may have depressive effect on the step involved in legume-Rhizobium symbiosissuch as molecular signaling, infection process, nodule development and function, resulting in low nitrogen fixation and crop yield [24]. Thus, knowledge about the diversity in natural population pertaining to different stresses is necessary before the selection and application of the tolerant strains of rhizobia for biological nitrogen fixation [11].

Thanks to their ability to establish symbioses with atmospheric nitrogen-fixing bacteria at the root level (Rhizobia), legumes are of considerable agronomic importance. On the one hand, they constitute an important source of protein for human and animal food without the need for nitrogen fertilizers, and on the other hand, they enrich the soil with assimilable nitrogen [27]. In the world, legumes rank as the second most significant food source, following cereals. Another viable, pricey meat substitute [32].

Among legumes reliable to promote pastoral zones that produce forage and restore destroyed pasture land especially in arid and semi-arid areas, the genus *Medicago* L. (Fabaceae) constitutes an important genetic resource [19]. Medics are excellent candidates for pastures and cover crops in sustainable agriculture systems, such as pastures and cover crops [10]. They grown as regenerating pasture in the agro-pastoral Mediterranean systems or cereal farming systems are an important feed resource not only as green forage throughout the growing season but also as stubbles and pods in summer and early autumn [29]. They express high levels of N-fixation and protein production per hectare [21] and have the ability to increase soil fertility and structure when grown in rotation [20].

During the 1980s, *Medicago* cultivars were selected in Australia and tested for introduction in Algeria. However, these introductions had major difficulties in adapting to local conditions. They exhibited low productivity, sensitivity to abiotic stress (drought and salinity) and a low degree of nodulation [26]. Thus, the

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investigation of local cultivars adapted to Algerian conditions, will allow us to identify and select effective medic rhizobia in order to improve fodder production and to ensure food security as well as the sustainability of agricultural practices.

The objective of our study is to determine the capacity of strains, isolated from *M. laciniata* and *M. minima* populations of the Algerian steppe, to grow under environmental stress such as salinity and extremes of temperature and pH, in order that rhizobia can be used to increase the productivity of medics plants and the corresponding improvement in feed production in arid and semi-arid areas, ensuring food security and the sustainability of agricultural practices.

MATERIALS AND METHODS

Bacteria isolation and purification

The bacteria were isolated from nodules of ten local populations of *Medicago laciniata* and *Medicago minima* collected from Djelfa regions (Algerian central steppe) by the National Institute of Agronomic Research of Algeria (INRAA) (Fig. 1).

Isolation of bacteria was performed according to Vincent method [33]. Nodule surface was previously sterilized by immersion in ethanol at 95° for 30 seconds, then in a solution of $HgCl_2$ (0.1%) for 3 minutes, followed by rinsing in six successive baths of sterile water. The nodule was crushed in 0.5 mL of sterile NaCl solution at 8.5% in order to release the bacteria. For each dilution, we inoculated three Petri dishes containing YEM medium. The colonies were obtained after 4-5 days of incubation at 28°C and purified by successive subcultures on the YMA medium. Isolates were maintained on YMA slants at 4°C.

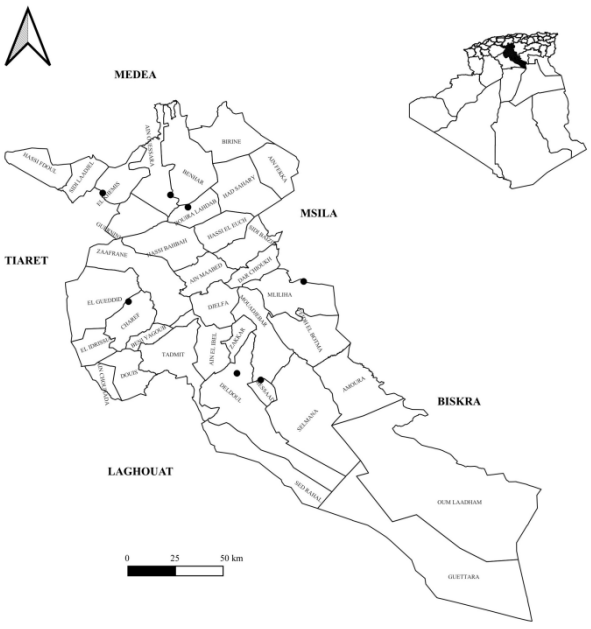

Figure 1. Geographic localization of the collection sites of *Medicago minima* and *Medicago laciniata* populations.

Nodulation test

The ability to induce the formation of nodules on the root of the host legume is a basic criterion in the characterization of rhizobia [12]. Isolates extracted from nodules can only be recognized as rhizobia after having allowed the induction of nodules on their host plants [18]. Seeds of *M. laciniata* and *M. minima* populations were sterilized for 30 seconds in 95% ethanol and followed by $HgCl_2$ bath for 3 minutes. They were rinsed six times with sterile distilled water and scarified. After germination on water agar for 72h, uncontaminated seeds were transferred aseptically to tubes containing Fahraeus medium at the rate of one seedling per tube. Three plants were inoculated with 1mL of the bacterial isolate suspension (approximately 10^8 cells/mL). Plants were observed for nodule formation during 4-6 weeks. Nodulation was observed by the existence of nodules.

Generation time and physiological characteristics

The identified strains were subjected to a series of specific tests the rhizobia characterization. Generation time of the isolated strains was determined by inoculation in 100 mL of YM broth into 250 mL of Erlenmeyer flasks. Cultures were incubated in a gyratory shaker at 200 rpm at 28°C. Growth was checked by measuring the optical density at 600 nm every 2h using UV-1601 spectrophotometer Shimadzu. The generation time was deduced from the exponential phase of growth curves. It could be defined as time needed for a strain to reach logarithmic growth phase.

For physiological characterisation, the isolates growth ability was evaluated on Yeast Mannitol Agar (YMA) plates under different conditions of temperature (30, 32, 35, 40 and 44°C), NaCl concentrations (200, 400, 600 and 800 mM) and adjusted pH (5.5, 6.5, 7.5, 8.5 and 9.5). Growth was measured as above indicated. Isolates were incubated for 2-4 days at 28°C. Three replicates per treatment were executed.

A cluster analysis of physiological variables related to strains performance under different abiotic stress was carried out. A dendrogram, based on ward method, was produced showing the relationships among strains tested using agglomerative hierarchical clustering (AHC) using the statistical software XLSTAT version 2020.

RESULTS

Nineteen isolates were obtained from root nodules of *M. laciniata* and *M. minima* populations (Table 1). According to the results obtained, 26.31% of the strains presented a generation time less than 4h, while 73.68% have a generation time ranged between 4 and 5h. Strains MB61 (3h 35mn) and MB62 (3h 39mn) exhibited the fastest generation time (Table 2).

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Table 1. Rhizoial isolates designation and geographical origin of host population

Isolates	Host population	Geographical origin
MB11	*M. minima*	Mliliha
MB12	*M. minima*	Mliliha
MB31	*M. laciniata*	Messaad
MB32	*M. laciniata*	Messaad
MB33	*M. laciniata*	Messaad
MB34	*M. laciniata*	Messaad
MB35	*M. laciniata*	Messaad
MB41	*M. minima*	Charef
MB42	*M. minima*	Charef
MB61	*M. laciniata*	Mliliha
MB62	*M. laciniata*	Mliliha
MB71	*M. minima*	Ain Oussera
MB72	*M. minima*	Ain Oussera
MB91	*M. laciniata*	Oued Touil
MB92	*M. laciniata*	Oued Touil
MB101	*M. minima*	Oued Touil
MB121	*M. laciniata*	Bouiret Lahdab
MB131	*M. minima*	Messaad
MB151	*M.laciniata*	Deldoul

Table 2. Generation time of rhizoial isolates from *M. laciniata* and *M. minima* populations.

Isolates	Generation time
MB11	4h 36
MB12	4h 38
MB31	3h 58
MB32	3h 54
MB33	3h 56
MB34	4h 07
MB35	4h 17
MB41	4h 32
MB42	4h 35
MB61	3h 35
MB62	3h 39
MB71	4h 06
MB72	4h 01
MB91	4h 08
MB92	4h 11
MB101	4h 01
MB121	4h 10
MB131	4h 09
MB151	4h 03

Results of physiological characteristics are given in Table 3. The 19 isolates exhibited displayed tolerance response to salinity, temperature and pH. All of the strains obtained grew well at 20°C to 32°C, at concentrations of 200 and 400 mM NaCl and in pH values ranging from pH 5.5 to 7.5. Results showed also that some isolates presented good growth at temperature 35-40°C, at 600-800 mM NaCl concentration and in pH 8.5-9.5.

There was a varied response of the19 isolates for tolerance to temperatures. The Cluster analysis allowed the formation of 3 strains groups, which are delimited at a similarity rate of 92% (Fig. 2). Group I consisted of isolates showed good growth between 25-35°C. Group II grouped isolates that grew at temperature of 20°C. Group III includes 5 isolates with good growth at temperature 20°-40°C.

Results indicate a wide response variation of strains tolerance to different NaCl concentrations. The cluster analysis revealed the formation of 3 groups with a similarity rate of 84% (Fig. 3). Group I grouped 10 strains, that sensitive to salinity. Group III composed by those that grew at 600 mM. Group II consists of only one strain (MB42) with good growth at 800 mM.

Tested strains vary widely in their tolerance to pH. The cluster analysis showed that the 19 isolates were grouped into three groups, which are delimited at a similarity rate of 91% (Fig. 4). Group I consists of isolates that exhibited low growth between pH 5.5 and 7.5 and no growth above pH 8.5. Group II composed by 3 isolates (MB11, MB12, MB151), with good growth at pH 5.5-9.5. Group III grouped isolates that have a preference for acid pH.

Table 3. Tolerance response of rhizobial isolates to different temperatures, NaCl concentrations, and pH.

Isolates	20°C	25°C	30°C	32°C	35°C	40°C	200mM	400mM	600Mm	800mM	pH5.5	pH6.5	pH7.5	pH8.5	pH9.5
MB11	+++	+++	+++	+++	+++	+++	+++	+++	-	-	++	++	+++	+++	++
MB12	+++	+++	+++	+++	+++	+++	+++	+++	++	-	++	++	+++	+++	+++
MB31	++	+++	+++	+++	+++	+++	+++	+++	-	-	+	+	+	+	-
MB32	++	+++	+++	+++	++	-	+++	+++	++	-	+	+	+	-	-
MB33	++	+++	+++	+++	-	-	+++	++	+	-	++	++	+	-	-
MB34	++	+++	+++	+++	-	-	+++	++	+	-	+	+	+	-	-
MB35	++	+++	+++	+++	+++	-	+++	+++	++	-	++	++	+	-	-
MB41	++	+++	+++	+++	+	+	+++	+++	++	-	++	+++	+++	+++	-
MB42	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	++	++	+
MB61	++	+++	+++	+++	++	++	+++	+++	-	-	+++	+++	+++	+	+
MB62	+++	+++	+++	+++	+++	+++	+++	+++	_	-	+++	+++	+++	++	+
MB71	++	+++	+++	+++	+	-	+++	+++	++	-	+++	+++	++	+	+
MB72	++	+++	+++	+++	+++	+++	+++	+++	++	-	+++	+++	+++	++	+
MB91	++	+++	+++	+++	-	-	+++	++	-	-	+	+	+	-	-
MB92	++	+++	+++	+++	++	++	+++	++	-	-	++	+++	+++	+++	+
MB101	++	+++	+++	+++	+	+	+++	+++	-	-	+++	+++	+++	+	-
MB121	++	+++	+++	+++	+	+	+++	+++	++	-	+	+	+	-	-
MB131	+++	+++	+++	+++	+	+	+++	+++	-	-	++	+++	+++	+	-
MB151	++	+++	+++	+++	-	-	+++	+++	++	-	++	+++	+++	+++	++

Growth of isolates was estimated in comparison with the control treatment: −, no growth; + weak growth (10-30%); ++, good growth (30-80%); +++, very good growth (similar to the control treatment).

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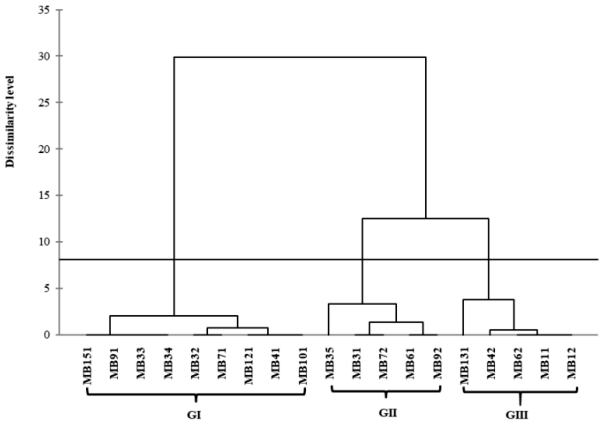

Figure 2. Dendrogram showing relationships among rhizobial isolates for high temperatures tolerance.

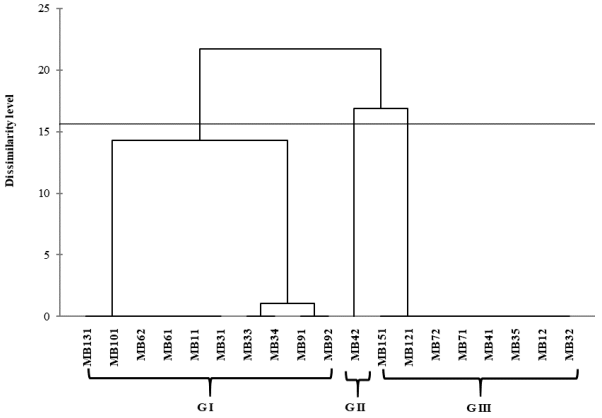

Figure 3. Dendrogram showing relationships among rhizobial isolates for NaCl concentrations.

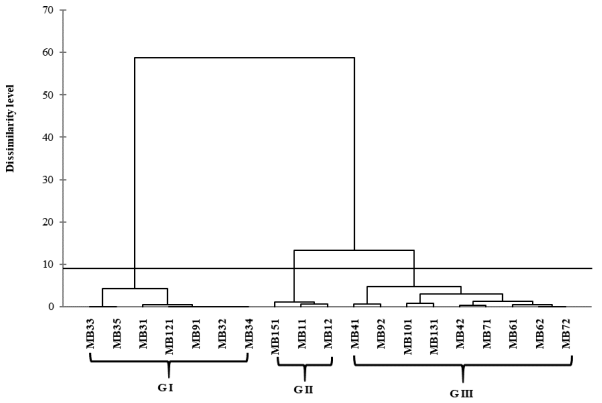

Figure 4. Dendrogram showing relationships among rhizobial isolates for pH.

DISCUSSION

Generation time could be defined as time needed for a strain to reach logarithmic growth phase. This important parameter is prerequisite for any study of rhizobia [23]. The generation times for all strains were between 3 and 5 hours. Similar results were obtained by Chebouti *et al*. [6] and Rome *et al*. [30], who reported that the mean generation times range from 3 and 5h in strains isolated from annual *Medicago* species. According to Elkan [13], who reported that fast growing bacteria have less than 6h in selective broth medium, strains in this study presented properties of fast-growing rhizobia. According to El-Hilali [12], fast-growing strains are of great practical importance, especially in inoculums production strategies.

Legume-rhizobia symbiosis is affected by factors such as changes in temperatures, antibiotic resistance, pH, and soil salinity, which restrict symbiotic nitrogen fixation, and the strains capable of tolerating the extreme conditions would survive efficiently [22].

Temperature is a major environmental factor which affects the survival and establishment of rhizobia both in culture and in the soil [34].The higher temperatures are detrimental to rhizobia and host and may inhibit the adherence of rhizobia to the root hair and formation of root hair and infection thread [28]. In this study, 100% of the isolates obtained grew well at 20°C to 32°C, 78.94% of the isolates grew at 35°C and 63.15% at 40°C. At 44°C, no growth was noticed in all isolates. For most rhizobia, Graham [17] reported that the optimum temperature range for growth in culture is 28 to 31°C. However, some isolates obtained from *M. truncatula* [6], *M. ciliaris* [7] and *M. minima* [3] are able to grow at 44°C, 45°C and 41°C respectively. According to Yadav and Nehra [34], a few strains of rhizobia are capable of tolerating high temperature for a short duration, but none of these could tolerate high temperature for prolonged periods.

Salinity is an important stress for rhizobia, because it inhibits persistence and development [16]. Salt tolerance range of rhizobia is variable depending on strain and type of salt [14]. Tolerance response to different NaCl concentration showed variation between strains. All strains tested presented a well and identical growth at concentrations of 200 and 400 mM NaCl. At 600 mM, 57.89% of isolates continued to grow. However, only MB42 strain was able to grow at 800 mM. Our results are in accordance with Merabet *et al.* [26], and confirm that isolates from *Medicago* species have normal growth in the presence of 200 or 400 mM NaCl. Bekki *et al*. [1] noted that 800 mM NaCl is the maximum growth inhibitory concentration of *S. meliloti* strains. According to Maâtallah *et al.* [25], Natural habitat of the strains performs a selection pressure for tolerance to salinity.

In the present work, wide range of variability was observed for the tested strains with different sensitivity to pH. All isolates grow normally in pH values ranging from pH 5.5 to 7.5. At pH 8.5 and pH 9.5, 68.42% and 47.43% were able to grew, respectively. Sebbane *et al.* [31] reported that isolates from *M. arabica, M. polymorpha, M. minima* and *M. orbicularis* are tolerant to pH 5-9. Rome *et al*. [30] indicated that strains of annual *Medicago* L. species are inhibited by pH values of less than ph 5. According to Kajic *et al*. [24], alkaline or acidic agricultural soil has a great influence on the survival or multiplication of rhizobia and can affect both symbiosis partners.

In conclusion, large variability response was observed for strains tolerance to high temperatures, Salinity and pH. This variability could offer us possibilities to select tolerant strains, which can fix

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atmospheric nitrogen in symbiotic association with *Medicago* populations and for successful nodulation in order to improve forage production and regenerating degraded steppe rangeland in Algeria.

The results have allowed us to identify some strains with interesting physiological characteristics such as high temperature tolerance (40°C), high salt tolerance (800 mM) and survival at pH values 5.5 to 9.5 pH values. However, strain MB42 was distinguished by good growth at 800 mM, good tolerance to temperatures of 35°C and 40°C, and seems to prefer acidic pH (5.5-6.5). While, strains MB11, MB12 and MB151 exhibited good growth at pH 9.5.This kind of phenotypic diversity observed in the rhizobia populations could offer selective advantages in survival and adaptation to these harsh environments [11]. Results obtained provide new perspectives that could contribute to the development of more effective inoculants in limiting agroecological contexts with the aim of improving forage production and regenerating degraded rangelands in the Algerian steppes.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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