NODULATION STUDY AND ASSESSMENT OF RHIZOBIA STRAINS ISOLATED FROM LOCAL POPULATIONS OF *Medicago truncatula* FOR THEIR TOLERANCE TO SOME ENVIRONMENTAL STRESSES

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Abstract. The aims of the present research were to study the nodulation in local populations of *Medicago truncatula* collected from different steppe regions of Djelfa (Algeria), isolate rhizobial strains nodulating *M. truncatula* to evaluate their tolerance to some environmental stress. Five *M. truncatula* populations were evaluated for their nodulation and growth. Nodules collected were used for the isolation of the rhizobia strains. Twelve strains were isolated and purified in YEM medium containing Congo red. Strains were tested for their tolerance to high temperature (30, 32, 35, 40 and 44 °C), to different concentraions 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). Results of variance analysis showed significant differences, nodules number and nodules and root biomass between *M. truncatula* populations, but aerial biomass did not exhibit significant diferences. For physiological characterization of rhizobia, the assessment of the tolerance of all strains to salinity, pH and high temperatures showed some strains with high tolerance to NaCl (600 mM) and high temperatures (>40 °C). The strains were also able to grow over a fairly wide pH range from 4.5 to 9.5.

Key words: *Medicago truncatula*; nodulation; rhizobia; temperature; salinity; pH.

INTRODUCTION

Forages occupy a fundamental place in the agricultural context. The development of fodder resources is the most logical and rational form for producing milk and meat for human consumption, and also an important element for development and protection of territories [19].

The insufficiency of fodder and pastoral production constitutes a great obstacle to the development of animal husbandry in Algeria. Most of the feed for the livestock is provided by natural environments. These latter are subject to continuous degradation caused by overgrazing and irrational exploitation of rangelands, illegal and uncontrolled clearing and the influence of climatic hazards particularly drought, which consequently leads to a significant reduction plant cover.

To make up for the production or availability shortfalls of natural vegetation, the plant breeding must offer new fodder species or varieties, persistent and hardy [35]. Among crops that 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 essential genetic resource [22].

Annual medics are of great importance in Mediterranean pastures, and in South-Western Australian and South American rangelands [7]. They are excellent candidates for pastures and cover crops in sustainable agriculture systems, such as pastures and cover crops [15]. Annual medic pastures that produce high levels of good quality forage are used extensively throughout dryland farming regions of the world [40]. Medics grown as regenerating pasture in the agro-pastoral Mediterranean systems or cereal farming systems are an important feed resource as green forage throughout the growing season and as stubbles and pods in summer and early autumn [33]. They show a high potential for seed and forage production, and self-regeneration ability [34] and express high N-fixation and protein production per hectare [24].

M. truncatula is closely related to many economically important legumes and therefore its investigation is of high relevance for agriculture [10]. *M. truncatula* is a diploid and autogamous plant having a relatively small genome. Preliminary molecular analysis suggests that allelic heterozygosity is minimal compared with the cross-fertilisilng tetraploid alfalfa [3]. *M. truncatula* was identified as being a suitable model legume because its diploidity (2n = 16), self-pollinating species with a small genome and easy to create transgenic plants [11]. The *M. truncatula* genome sequence provides significant opportunities to expand alfalfa's genomic toolbox [42].

Rhizobia-legume (RL) 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 [10] and is one of the effective methods to improve plant growth and productivity [14]. The symbiosis between legumes and rhizobia is a classic mutualistic relationship. In return for carbohydrates provided by the host legume, the rhizobia supply nitrogen to the legume [28]. This symbiosis provides the necessary nitrogen for plant growth and contributes to soil nitrogen status [20].

Symbiotic fixation is influenced by genetic factors of bacteria and plant and is very sensitive to environmental factors [32]. The main factors limiting soil biological activity are water deficit, salinity, high temperatures, extreme pH and nutrient deficiencies. The frequent interactions between these constraints affect the growth and survival capacity of microorganisms in arid soils [8]. Thus, the knowledge about diversity in natural populations facing different stresses is necessary before selecting and applying the

tolerant strains of rhizobia for biological nitrogen fixation [16].

The objectives of this research are to study the nodulation in local populations of *Medicago truncatula* collected from different steppe regions of Djelfa (Algeria), and to determine the degree of variability among strains in response to salinity, temperature and pH.

MATERIALS AND METHODS

Evaluation of nodulation

Five local populations of *Medicago truncatula* collected from steppe region of Djelfa in 2008 by the National Institute of Agronomic Research of Algeria (INRAA) were used. The origin sites of these populations are: Ain Oussera (MtAO) (35°17'08''N; 2°57'37''E), Bouiret Lahdab (MtBL) (34°15'N; 3°19'E), M'liliha (MtMli) (34°48'89''N; 3°48'94''E), Charef (MtCh) (34°40'31''N; 2°43'13''E) and Oued Touil (MtOT) (35°16'43''N; 2°33'23''E). The latitude and longitude of each site were taken using a portable Global Positioning System (GPS) receiver.

The present work was conducted at Baraki experimental station of INRAA. The trial was conducted during the 2019/2020 cropping season. Scarified seeds were sown in pots at a rate of five seeds per pot containing 2/3 clay soil and 1/3 peat under a glass greenhouse. At emergence, we left two plants per pot. Pots were arranged in a completely randomized design with six replications. At flowering, twelve plants were harvested and used to assess growth and nodulation. Fresh weight of aerial part **(AFW)** and root weight **(RW)** were measured, while nodulation was assessed by recording the number and fresh weight of nodules **(NN, NFW).** Collected nodules were used for the isolation of rhizobia strains.

Phenotypical characterization of bacteria

Bactreia isolation

Isolation of bacteria was performed according to of Vincent method [38]. The surface of the nodule 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.

Nodulation test

Seeds of *M. truncatula* populations were sterilized for 30 seconds in 95% ethanol and followed by HgCl2 bath for 3 minutes. They were rinsed six times with sterile distilled water and scarified. After germination on water agar for 72 h, uncontaminated seeds were transferred aseptically to tubes containing Fahraeus medium at the rate of one seedling per tube. Three plants were inoculated with 1 mL of the bacterial isolatre suspension (approximately 10^8 cells/mL). Plants were observed for nodule formation during 4-6 weeks. Nodulation was observed by the existance of nodules.

Generation time and physiological characteristics

For this study, 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 in a spectrofotometer Shumadzu UV-1601. 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 caracterisation, Rhizobium strains were evaluated on Yeast Mannitol Agar (YMA) plates for 2-4 days under different conditions of temperature (30, 32, 35, 40 and 44 °C), NaCl (200, 400, 600 and 800 mM) and pH (5.5, 6.5, 7.5, 8.5 and 9.5). Growth was measured as above indicated.

Statistical analysis

Data of *M. truncatula* populations biomass were statistically analysed by analysis of variance using R software version 3.6.1. Multiple comparison of means was performed using the LSD test at a 5% probability level.

A cluster analysis of physiological variables related to strains performance under different abiotic stress was carried out. A dendrogram was produced showing the relationships between different strains tested according to the Unweighted Pair Group Method using Arithmetic Average (UPGMA).

RESULTS

Variance analysis showed highly significant differences between populations for root weight, number of nodules and weight of nodules, except for fresh weight of aerial part, for which differences were not significant (Table 1). Average root weight was 19.99 g, and varied from a minimum of 15.53g for Bouiret Lahdab population to a maximum of 25.33g for Mliliha population. Concerning nodulation traits, the largest number of nodules was recorded by Charef population while the lowest value was observed in Ain Oussera population. The highest nodules weight was registered for Charef population with 179.8 mg while the population of Ain Oussera presented the lowest weight (52.0 g).

Twelve strains, which were obtained from root nodules of five *M. truncatula* populations, were studied. The mean generation times for all strains were less than five hours (Table 2). Strain MB51 exhibited the fastest generation time (3h 32mn). More than half

(58.33%) of the strains have a generation time less than four hours while 41.66% have a generation time between 4 and 5 h.

The results of physiological tests are reported in Table 3. There was a varied response of the isolates tested for tolerance to high temperatures. Results indicated that all the isolates were able to grow from 30° to 35 °C. At 40°C, only MB21, MB111and MB143 strains could grow, and MB143 strain was able to grow even at 44 °C. Results of salinity tolerance show a high diversity among the isolates tested. We noticed that all the strains showed averaged growth at concentrations of 200 and 400 mM. However, strains MB81, MB111, MB141, MB143, MB145 and MB146 were able to grow at concentrations up to 600 mM. According to the results of the pH test, we observed a wide pH tolerance variability. All isolates grew well at pH ranging from 6.5 to 9.5. However, MB81, MB111, MB141, MB145 and MB146 were able to grow even at acid pH.

Numerical analysis of the physiological characteristics showed that, at a similarity coefficient of 87%, the tested strains can be grouped into three groups (Fig. 1). Cluster I includes strains from Mliliha population (MB81), from Oued Touil population (MB111) and Charef population (MB141, MB145, MB146). These strains were able to grow at pH 5.5 and at a concentration of 600 mM NaCl. Cluster II consists of two strains from Bouiret Lahdab population (MB21, MB22), two from Ain Oussera population (MB51, MB52) and two from Charef population (MB142, MB144). Unlike the first group, these strains did not grow at ph 5.5 and at a concentration of 600 mM NaCl. Cluster III includes only the strain MB143 from Charef population that was able to grow at 44° C and at 600 mM.

Table1. Mean values of growth and nodulation traits of local populations of *M. truncatula*

Populations/traits	AFW (g)	RW (g)	NN	NFW (mg)
MtAO	31.6±3.45	21.6±2.15 [b]	52.0±10.94 [b]	52.0±14.89 [b]
MtBL	32.2±4.20	15.5±0.83 [c]	84.0±16.80 [a]	150.0±27.82a
MtCh	28.6±3.63	16.8±2.06 [c]	106.0±5.24 [a]	179.8±59.19 [a]
MtMli	31.8±3.86	25.3±4.64 [a]	59.0±512.55 [b]	77.3±44.81 [b]
MtOT	30.9±4.65	20.6±1.86 [b]	58.0±5.11 [b]	90.3±30.11 [b]
General Mean	31.0	20.0	71	109.9
p-value	4.741	0.001	0.001	0.001
LSD	0.547	3.130	13.46	45.79

MtAO: Population from Ain Oussera; **MtBL:** Population from Bouiret Lahdab; **MtCh:** Population from Charef; **MtMli:** Population from Mliliha; **MtOT:** Population from Oued Touil; **LSD:** Least Significant Difference; A**FA:** Fresh weight of aerial part; **RW:** Root weight; **NN:** Number of nodules; **NFW:** nodules fresh weight.

Table 2. Strains used in this study and generation time

Strains	Host population	Generation time
MB21	MtBL	3h 40mn
MB22	MtBL	4h 50mn
MB51	MtAO	3h 32mn
MB52	MtAO	3h 56mn
MB81	MtMli	3h 45mn
MB111	MtOT	3h 53mn
MB141	MtCh	4h 20mn
MB142	MtCh	4h 54mn
MB143	MtCh	4h 15mn
MB144	MtCh	3h 44mn
MB145	MtCh	3h 45mn
MB146	MtCh	4h 53mn

Table 3. Temperature, NaCl and pH tolerance of twelve isolate

		Isolates											
		MB21	**MB22**	**MB51**	**MB52**	**MB81**	**MB111**	**MB141**	**MB142**	**MB143**	**MB144**	**MB145**	**MB146**
Temp. (°C)	30	+	+	+	+	+	+	+	+	+	+	+	+
	32	+	+	+	+	+	+	+	+	+	+	+	+
	35	+	+	+	+	+	+	+	+	+	+	+	+
	40	+	-	-	-	-	+	-	-	+	-	-	-
	44	-	-	-	-	-	-	-	-	+	-	-	-
NaCl (mM)	200	+	+	+	+	+	+	+	+	+	+	+	+
	400	+	+	+	+	+	+	+	+	+	+	+	+
	600	-	-	-	-	+	+	+	-	+	-	+	+
	800	-	-	-	-	-	-	-	-	-	-	-	-
pH	5.5	-	-	-	-	+	+	+	-	-	-	+	+
	6.5	+	+	+	+	+	+	+	+	+	+	+	+
	7.5	+	+	+	+	+	+	+	+	+	+	+	+
	8.5	+	+	+	+	+	+	+	+	+	+	+	+
	9.5	+	+	+	+	+	+	+	+	+	+	+	+

+: growth or positive reaction; -: no growth or negative reaction

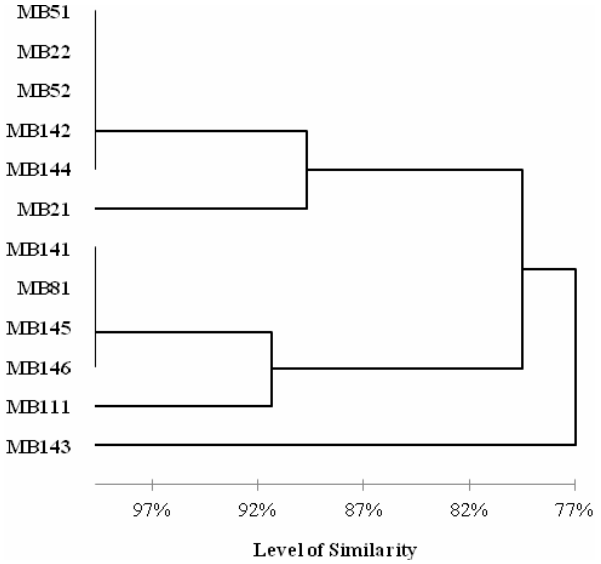

Figure 1. UPGMA dendrogram showing relationships among twelve strains isolated in local populations of *M. truncatula* based on physiologic characteristics

DISCUSSION

Genetic variability of steppe *M. truncatula* populations

We noted the existence of substantial genetic variability in the studied populations of *Medicago truncatula*. Populations showed highly significant differences for nodulation traits. *M. truncatula* populations displayed a nodules number of 52-106 and a nodules weight of 52.0-179.8 g. These differences might be caused by the genetically and environmentally characteristics [2]. Miloud [31] reported great variability in the nodulation and its effects on the weight of the aerial parts, the roots and the nitrogen content in *Medicago truncatula*. When nodulation takes place, the number of nodules set up within the root system of the legume is proportional to the nitrogen requirements of the plant for its growth [39]. According to Denton *et al*. [13], it is likely that the genetic structure and physiology of the rhizobial population present at a site may be as important to the successful outcomes of inoculation, as is the size of the population.

Rhizobiums isolated from steppe *M truncatula* nodules are fast growing strains

A total of twelve strains were identified and were characterized. The generation time importance is prerequisite for any study of rhizobia [26]. The mean generation times of the tested strains are between 3 h to 5 h. Our results are in accordance with Rome *et al*. [35], who indicate that the mean generation times range from 3 and 5 h in strains obtained from several annual species of *Medicago*. According to Elkan [18], the slow growing bacteria have mean generation time greater than 6 hours and fast growing bacteria have less than 6 h in selective broth medium. On the basis of their generation times strains isolated from the five populations of *M. truncatula* were found to be fast growing. Sebbane *et al*. [37] noted among the ten new obtained *Medicago*-rhizobia, eight isolates are related to fast-growing rhizobia and two to slow or intermediate-growers. Howieson and McInnes [23] reported that most legumes in the Mediterranean area appear to be nodulated by fast-growing bacteria. According to El-Hilali [17], the fast growth of strains is of great practical importance, especially in inoculums production strategies.

Rhizobium strains isolated from steppe *M truncatula* nodules are tolerant to high T[a], salinity and acidity

The 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 [25].

Temperature is factor that strongly influences survival of rhizobia [5]. It plays a critical role in the exchange of molecular signals between rhizobia and their host, thus reducing nodulating [27]. In this study, 100% of the isolates obtained grew well at 35 °C. At 40 °C, three strains could grow and only one strain was able to grow at 44 °C. In some species of annual *Medicago* L., strains isolated in *Medicago minima* were able to grow at 41 °C [6] and those from root nodules of *Medicago ciliaris* L. could grow at 45 °C [9]. Dekak *et al*. [12] noted a thermotolerance variability among rhizobia isolates from wild legumes (*Genista microcephala* and *Argyrolobium uniflorum*), which were able to grow up to 45 °C. A few strains of rhizobia capable of tolerating high temperature for a short duration, but none of these could tolerate high temperature for prolonged periods [41].

Salinity is an important stress for rhizobia, because it inhibits persistence and development [21]. But Rhizobia show marked variation in salt tolerance [1]. In the present study, salinity tolerance up to 600 mM NaCl was noted among strains isolated from *Medicago truncatula* populations. Above this concentration no growth was reported in all isolates. Merabet *et al*. [29] indicate that all bacterial isolates from *M. ciliaris* and *M. plymorpha* had normal growth in the presence of 200 or 400 mM NaCl. Twenty-six isolates tolerated 600 mM NaCl and fifteen strains grew on concentrations up to 800 mM NaCl. Bekki *et al*. [4] noted that 800 mM NaCl is the maximum growth inhibitory concentration of *S. meliloti* strains. According to ElBoutahiri *et al*. [16], *S. meliloti* isolates, which were sampled from the highly salt-affected areas of southern Morocco, had greater tolerance to salt than others, indicating that saline soils naturally select strains more tolerant to salinity, and results in higher recovery of salinity-tolerant strains.

In general, the optimum pH for rhizobial growth was reported to be between 6 and 7 [30]. The tested strains vary widely in their pH tolerance. All the isolates tested have a preference for alkaline pH.

However, 42 % of the strains were able to grow in acid pH value (pH 5.5). Elboutahiri *et al*. [16] indicated that the alfalfa rhizobia are acid-sensitive and most isolates only tolerated acidity of pH 5.5-6.0. According to Rome *et al*. [36], isolates obtained from annual *Medicago* L. species are inhibited by pH values of less than 5.0.

We can conclude that there is a high genetic diversity in both nodulation traits among local population of *M. truncatula* and response of the strains to different environmental factors. The results of the assessment of the tolerance of all strains to the main stress factors, salinity, pH and high temperatures, have allowed us to identify some strains with high tolerance to NaCl (600 mM) and high temperatures (>40 °C). The strains were also able to grow over a fairly wide pH range from 4.5 to 9.5. Further studies, including other annual *Medicago* species, are needed in order to identify new strains with survival ability, exhibiting efficient symbiotic characteristics with their host plants in extreme conditions of drought, salinity and acidity. These associations might improve nodulation, plant growth and Nitrogen fixation under difficult conditions prevailing in arid and semi-arid regions. Thus, use of molecular techniques, such 16S rDNA sequencing, will also allow us to identify and select efficient medic rhizobia in order to improving forage production and regenerating degraded steppe rangeland in Algeria.

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