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Abstract- *Acacia gummifera* is an important tree from the south-west of Morocco. It has been reported to increase the soil fertility as the other Acacia. The characterization of successful strains and the study of the potential for fixing atmospheric nitrogen was a necessary in order to succeed its establishment in the environment where it will be introduced. Growth in YMA, tolerance to stress factor (pH, salt and temperature) and resistance to metallic ions were used as phenotypic markers of isolates collected from root nodule of *A. gummifera*. Genotypic diversity was studied by amplification of polymorphic DNA and 16s RNA gene sequencing. The symbiosis effectiveness of 6 performed Rhizobium was evaluated using plant nodulation assay at two different ages in controlled condition.

The results of phenotypic characterization showed that the most of isolate are fast growing. All isolate tolerated high temperature (40°C),and a NaCl concentration that exceeds 800 mM and most of them increased under pH ranging from 7 to 10.

All six strains showed root nodules with variable number which varied between 2 and 25 nodules per plant and dry weight between 1.5 and 15mg.plant^{-1}. In addition, the statistical analysis showed that *Rhizobium* was more infective in 12-month-old Acacia. The symbiotic efficiency has shown considerable variability, the most effective symbiotic association was recorded in the strain A24 (*Rhizobium azibense* : MF769718) with 200% and an increase in the total nitrogen twice as much as control seedlings fertilized with nitrogen (KNO_3).

Keywords: acacia gummifera, nitrogen fixing, rhizobium.

I. Introduction

Nitrogen nutrition of legume plants is provided by two complementary ways: uptake of mineral nitrogen from the soil by the roots, as in all higher plants, and fixation of atmospheric nitrogen. Which is a process to these species, thanks to their symbiosis with soil bacteria. In most agricultural systems, the primary source of biologically fixed nitrogen occurs from the symbiotic interactions of legumes and soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Azorhizobium* (Alberton et al., 2006).

The use of this symbiotic interactions in agricultural and agro forestry ecosystems makes it possible to limit the use of nitrogen fertilizer to a lesser extent (Ganry & Dommergues, 1995). In soils that are deficient or low nitrogen, nitrogen-fixing woody species such as Acacia, are expected to play an important role thanks to their adaptation to hostile environmental conditions and for their positive effect on soil fertility and ecosystem productivity (Fikri Benbrahim et al., 2014). The fixing power of these trees is related to their association with nitrogen fixing bacteria of the genera Rhizobium. In fact, the efficiency of a strain is closely related to the host plant and its environment. It is for this reason that research has focused on the characterization of successful indigenous strains and a potential for fixing atmospheric nitrogen. The nitrogen-fixing potential translates a plant's ability to fix nitrogen by integrating the effect of climatic, edaphic and biological factors (Bowen et al, 1990; Trotman & Weaver, 1995). In this work, we study the comportment (growth and nitrogen nutrition) of seedlings of *Acacia gummifera*at 6 and 12 months, in association with six strains of Rhizobium. This Moroccan gum is considered as the only endemic species of Acacia in Morocco, which has interest in reforestation and thus provides a plentiful gum used in traditional medicine. This study will be preceded by tolerance tests of isolates with edaphic factors: salinity, pH and temperature.

II. Material and Method

a) Bacterial strains

All strains were isolated from *Acacia gummifera*'s nodule. Which were obtained after trapping in rhizospheric soil from Skhour Rhamna region. The (isolation and purification of the isolates were performed after several rounds of subculture on YMA medium Vincent 1970).

b) Effect of extrinsic factor

Different temperatures (4°C, 28°C, 37°C, 40°C at 50°C), pHs (3, 4, 5, 6, 7, 8, 9, 10 and 11) and salt (0, 172, 344, 517, 689, 862, 1190, 1200, 1360, 1500 mM)was studied in YMA medium.

c) Effect of heavy metals

This test was conducted to assess resistance of the isolates to the following heavy metals: $AlCl_3$ $6H_2O$, $ZnCl_2$, $CoCl_2$, $CdCl_2$, $HgCl_2$. The solution of different metals was filtered (milipore 0,22µm), sterilized and

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added to YMA agar medium in order to obtain the concentration in µg/mL. The results of each test were evaluated after one week of incubation.

d) Genotypic characterization

The genotypic characterization was based on 16sr RNA gene which was carried out within the laboratory of molecular biology and functional genomics of the National Center of Research Science and Technical Division UATRS Rabat-Morocco. PCR amplification of the isolated was performed by real time PCR using the universal primers Fd1 and RP2 (AGAGTTTGATCCTGGCTCAG,and,ACGGCTACCTTGTT ACGACTT, respectively) (Weisburg et al., 1991). The PCR reactions are carried out in a total volume of 25 µL containing the reaction buffer at 1/10 of the final volume, 0.125 µL of each primer (100 µM), 0.2 µL of the Taq polymerase (5 µL / L) and 5 µL of the DNA sample. In the negative control, the 5 µL of DNA is replaced by 5 µL of sterile H_2O. The amplifications were performed according to the following conditions: a first denaturation at95 ° C for 1 min and then cycle in each a denaturation at 95 ° C for 15 seconds, hybridization at 52 ° C for 20 seconds and a 72 ° elongation C for 15 seconds finally a final elongation at 72 ° C for 3min.

e) Sequencing

The amplicons were sequenced using the Big Dye v3.1 kit (Applied Bio systems) of the ABI 3130xl Genetic Analyzer Sequencer. The reaction consists of an introduction into a final volume of 10 µL, 0.75 to 1.5 µL of template DNA and 3.2 to 5 pmol / µL of each primer 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCCTTTRAGTTT) (Weisburg et al., 1991). The optimal conditions of the sequencer are as follows: for 25 cycles 96 °C for 1 min, 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 min.

f) Sequence analysis

The sequences obtained were analyzed with the program DNA Baser v 4.36.0 (http://www.dnabaser.com) corrected manually. The sequences were then compared to those available in the NCBI database using the BLAST program (Basic Local Alignment and Search Tool, NCBI) to determine their phylogenetic affiliation. The identification of the genus and the species was carried out as described by Drancourt et al., 2014, where > 99% similarity a strain to a species already described, between 97% and 99 similarity a strain to a genus and > 97% represents a new species. The 16S rRNA gene sequences of the selected isolates were deposited in the Gen Bank database under accession numbers (Table 3).

g) Study of the symbiotic effectiveness of different rhizobial isolates

Six rhizobial isolates were compared for their symbiotic effectiveness. For that *A. gummifera* seedlings were inoculated with 5 ml of a freshly bacterial suspension (108 bacteria / ml).The inoculation was performed after each week for 20 days andthe pots were arranged in randomized random blocks with four repetitions for each strain. The watering was done daily, once a week 30 ml of a nutrient solution of nitrogen-free was added to each plant, except for the control seedlings which receive nitrogen in the form of $KN0_3$ (0.5 g / 1) (Munns., 1968).

The experience was maintained in green houseat 28°C day/25°C night, 16hours light/8 hours dark photoperiod (Beck et al.,1993; Soma segaran and Hoben, 1994). The following parameters were measured after 6 and 12 months of culture: dry weight of the aerial part (DRW) and the root part (PSR) which was obtained after drying the sample at 70 ° C for 48 hours, number of nodules per plant (NN) and their dry weight (DRW n).

The amount of total nitrogen (N) in the whole plant was measured using the Kjeldahl method and the symbiotic efficiency (SE) was calculated by comparing each isolate with the positive control plant. (Chalk., 1998).

SE = (nitrogen content of inoculated plants / nitrogen content of non inoculated positive control plants) x 100.

h) Statistical analysis

All root and aerial dry weight, plant N concentration, and symbiotic efficiency data were subjected to analysis of variance (ANOVA) using the SPSS general linear model procedure. version 17.Averages were tested for significance using the difference of least significant means (LSD) at $p<0.05$.Pearson correlation coefficients were calculated to establish the associative relationships between isolate infection or efficacy characteristics of isolates and age of *Acacia gummifera* seedlings.

III. Results

The isolates produced translucent and transparent colonies and a high production of mucus was observed in most of them. Most colonies obtained in YMA added to bromothymol blue, have acidified the medium. According to their phenotypic identification shown in table 1. The better growth was recorded at 28 °C, although all isolates recorded average growth at 40 °C, no multiplication was recorded at temperatures exceeding 45 °C. Our isolates in their majority (83%) support concentrations in Na Cl up to 862mM and all the isolates can grow on an alkaline medium at pH between 9 to 10. However, no growth was observed at acid pH except for isolate A10 which was shown to be able to multiply at pH = 4 (Table 1).

Table 1: Phenotypic characteristic of isolatnodulating *A. gummifera*

Isolat	A24	A26	A32	A12	A10	A4
Ph 3	-	-	-	-	-	-
4	-	-	-	-	+	-
5	-	-	-	+	+	-
6	+	+	+	+	+	-
7	+	+	+	+	+	-
8	+	+	+	+	+	-
9	+	-	+	+	+	+
10	+	-	+	+	+	-
11	-	-	-	-	-	-
Temperature (°C) 4	-	+	+	-	+	-
28	+	+	+	+	+	+
37	+	+	+	+	+	+
40	+	+	+	+	+	+
45	-	+	+	-	+	+
50	-	-	-	-	-	-
Salinity (mM) 0	+	+	+	+	+	+
172	+	+	+	+	+	+
344	+	+	+	+	+	+
517	+	+	+	+	+	+
689	+	+	-	+	+	+
862	+	+	-	+	+	+
1190	-	+	-	+	+	-
1200	-	+	-	+	+	-
1360	-	-	-	+	-	-
1500	-	-	-	-	-	-

+: Growth, - : No growth

All strains tolerate different metal ions at varying concentration. From table 2and figure 1 we note a resistance to high concentrations for aluminum and zinc. However, at lower concentrations of mercury, cobalt and cadmium the bacteria were negatively affected. These metal ions are therefore the most in hibitory for the development of our isolates.

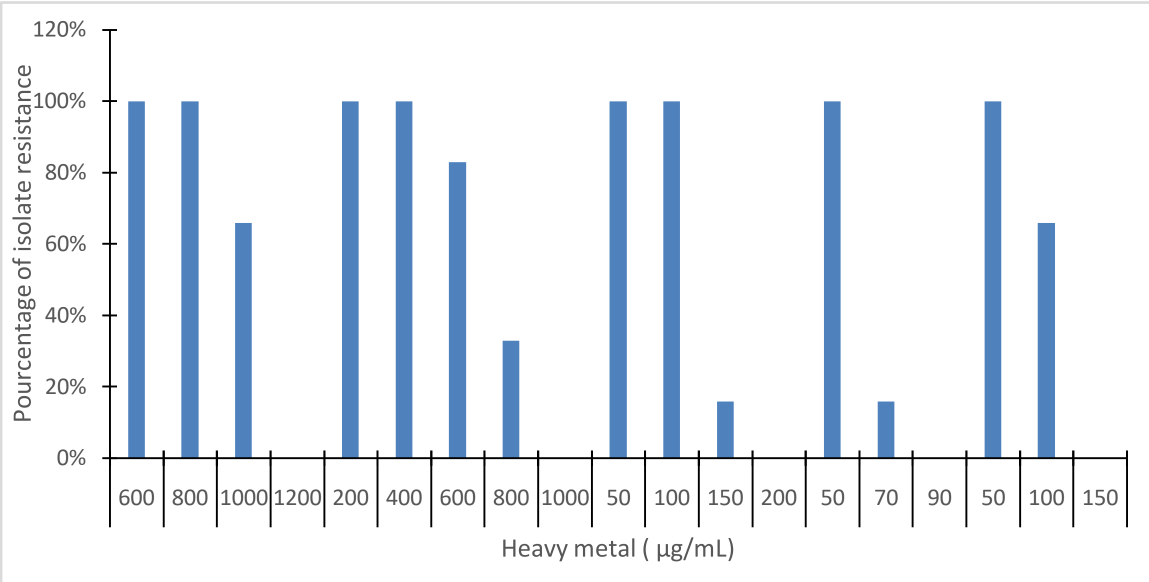

Figure 1: Effect of different concentration of heavy metals on the growth of isolates

Table 2: Resistance to heavy metals of isolates nodulating *A. gummifera*

Heavy metals	Concentration (µg/ml)	A4	A10	A12	A26	A32	A24
Al	**600**	+	+	+	+	+	+
	800	+	+	+	+	+	+
	1000	+	-	+	+	-	+
	1200	-	-	-	-	-	-
Zn	**200**	+	+	+	+	+	+
	400	+	+	+	+	+	+
	600	+	-	+	+	+	+
	800	-	-	-	+	-	+
	1000	-	-	-	-	-	-
Co	**50**	+	+	+	+	+	+
	100	+	+	+	+	+	+
	150	-	-	+	-	-	-
	200	-	-	-	-	-	-
Cd	**50**	+	+	+	+	+	+
	70	+	-	-	-	-	-
	90	-	-	-	-	-	-
Hg	**50**	+	+	+	+	+	+
	100	+	+	+	-	+	-
	150	-	-	-	-	-	-

Moreover, the comparison of the obtained sequences of the ribosomal RNA 16s gene of the most tolerant ones with those available in databases, using the BLASTn program, has indicated that the strains A10, A12 and A4 can be assigned respectively to *Rhizobium naphthalenivorans*, *Rhizobium pusense* and *Rhizobium nepotum* at 99% identity. Two strains A32 and A26 have a percentage of similarity 99% with *Rhizobium giardinii*, and A24 with 100% sequences identical to *Rhizobium azibense* (Table 3).

Table 3: Genotypic character of isolates nodulating A. gummifera

Isolates	Species	% similarity	Accession number
A24	*Rhizobium azibense*	100%	MF769718
A26	*Rhizobium giardinii*	99%	MF629731
A32	*Rhizobium giardinii*	99%	MF663789
A12	*Rhizobium pusense*	99%	MF774692
A10	*Rhizobium naphthalenivorans*	99%	MF629733
A4	*Rhizobium nepotum*	99%	MF972515

a) *Evaluation of strain'sinfectivity*

The examination of the root system of plants have shown a variability in the number of nodule. There is also a variability in the dry weight of the nodule formed during the two culture period tested (figure 2).In general, a wide significant variability in the infective capacity of the isolates adhering to plants which has 12-month old was highlighted. Furthermore the strain A4 is the most infective, with more than 25 nodule / plant and a dry weight of 15 mg. $plant^{-1}$. While the lowest infectivity was recorded in strain A32 with 2 nodule / plant and a dry weight of 2 mg. $plant^{-1}$. Also, at 6-month-old, A10 has recorded the highest dry weight and number of nodule (13mg. $plant^{-1}$ and 8 nodules/plant).

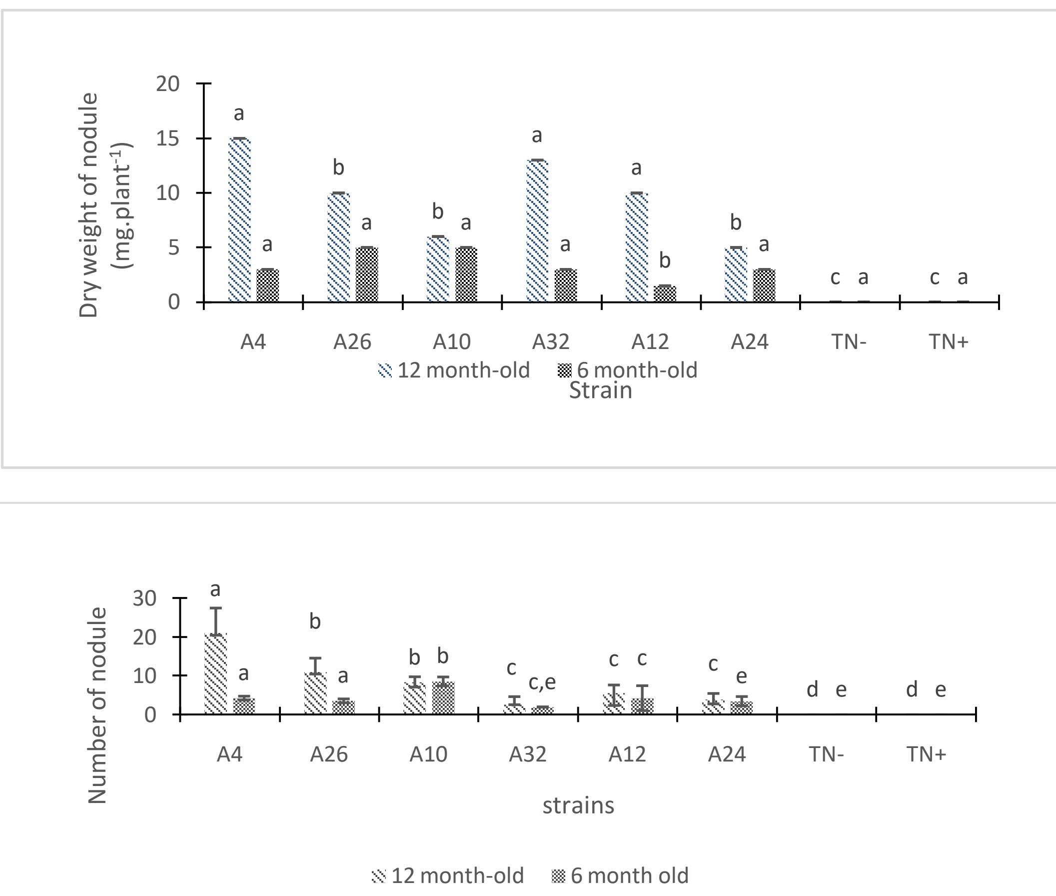

Figure 2: Number of nodule (A) and dry weight of nodule (B) collected from *Acacia gummifera* roots after 6 and 12 months of culture.

b) Evaluation of the strains'ssymbiotic efficiency

There is a large variation in the dry biomass among *A. gummifera* at 12 months-old inoculated with six different strains of Rhizobium (Table 3). The highest is recorded in those inoculated with the strain A24, which allowed to increase the aerial dry weight 3.5 times more than those obtained in positive control inoculated with KNO_3. While the strain A4 have given the lowest biomass.

For the 6-month Acacia, the statistical analysis of the variance showed that there is no significant difference between them. In addition, the 6-month-old plant's infection with the strain A10 caused the highest root biomass, while A26 had the lowest root dry matter content. The inoculation of the Acacia which have 12 months have given a higher nitrogen contents compared with those which have 6-month-old(Table 3). In particular, the total nitrogen content in the 12-month-old plants expressed in (g.plant^{-1}) shows that those inoculated with A24 have the highest content with 0.2 g / plant, which significantly exceeds that contained in Acacia fertilized with mineral nitrogen (0.1g / plant). While, the seedlings inoculated with A4 have the lowest nitrogen content. The association of *A. gummifera* of 6 months with strain A24 recorded the highest nitrogen content, which is significantly similar to that observed in plants fertilized with mineral nitrogen, while those associated with A32 have revealed the lowest nitrogen content similar to that found in uninoculated seedlings. In addition, it can be seen from the table 3 that, after 12 months of culture, fixed nitrogen is significantly higher than when they are combined with A. gummifera after 6 months of culture. Notably, 75% of strains that associate with 12-month-old acacias have been shown to be very effective with SE> 80%. However, it is important to point out that there are isolates that have shown a very low symbiotic efficiency, this is the case for example of isolate A4 which showed the least important value (SE 16%) despite the number of nodules observed. This shows that this isolate forms inefficientroot nodules.

Table 4: Effect of inoculation with six Rhizobium on dry weight of aerial and root part, total nitrogen content, fixed nitrogen and symbiotic efficiency of *Acacia gummifera* at 6 and 12 months-old

	Dry weight of aerial part(g.plant^{-1})		Dry weight of root part (g.plant^{-1})		Nt (g.plant^{-1})		Nf (g.plant^{-1})		ES (%)	
Age	**12 mois**	**6 mois**	**12 mois**	**6 mois**	**12 mois**	**6mois**	**12 mois**	**6mois**	**12 mois**	**6mois**
A4	3,4 ±1^{b,c}	1,6±0,63^{A}	2±0,56^{b}	0,42±0,09^{A}	0,09±0,008^{a}	0,02±0,01^{A}	0,06±0,01^{a}	0,01±0,007^{A}	90±91,99^{a}	24±36^{A}
A24	9,2±3,15^{a}	1,2±0,21^{A}	4,5±0,9^{a}	0,34±0,14^{A}	0,2±0,06^{b}	0,08±0,007^{B}	0,18±0,03^{b}	0,07±0,01^{B}	200±72,2^{c,b}	114±39^{B}
A10	6,75 ±1,8^{a,b,c}	2±0,1^{A}	2,3±0,57^{b}	1,5±0,48^{A}	0,14±0,01^{a}	0,03±0,01^{A}	0,12±0,01^{a}	0,02±0,005^{A}	140±96,2^{b}	42±28^{A}
A26	6,8 ±4,2^{b,c}	1±0,8^{A}	2,12±0,46^{b}	0,3±0,16^{A}	0,14±0,03^{a}	0,03±0,02^{A}	0,12±0,06^{a}	0,02±0,02^{A}	140±47,01^{b}	42±24^{A}
A12	6,6 ±0,4^{a,b}	1,5±0,35^{A}	3,87 ±0,7^{a,c}	0,31±0,35^{A}	0,1±0,03^{a}	0,05±0,02^{B}	0,08±0,007^{a}	0,04±0,01^{B}	100±41,2^{a}	71±77,5^{B}
A32	4±1,5^{c}	1,8±0,2^{A}	2,3±0,5^{b}	0,5±0,1^{A}	0,11±0,03^{a}	0,017±0,001^{A}	0,09±0,01^{a}	0,007±0,002^{C}	110±50,1^{b}	28±8,7^{A}
TN-	2,4±1^{c}	1,2±0,6^{A}	0,5±0,22^{b}	0,26±0,12^{A}	0,02±0,008^{c}	0,01±0,008^{A}				
TN+	2,6±1,1^{c}	1±0,3^{A}	0,53±0,23^{b}	0,22±0,07^{A}	0,1±0,03^{a}	0,07±0,03^{B}	0,08±0,03^{a}	0,06±0,03^{B}	100±0^{a}	100±0^{B}

Table 5: Correlation between age, number of nodules, nodule dry weight, aerial and root dry biomass, total nitrogen, fixed nitrogen and symbiotic efficiency

Variables	Number of nodule	Dry weight of nodule	Dry weight of aerial part	Dry weight of root part	Total nitrogen	Fixednitrogen	SE
Age	0, 31**	0,536**	0,738**	0,589**	0,56**	0,587***	0,52**
Number of nodule		0,659**	0,56*	0,4*	0,2	-0,1	-0,73
Dry weight of nodule			0,681*	0,5*	0,4**	0,15	0,222
Dry weight of aerial part				0,853**	0,46**	0,3	0,313
Dry weight of root part					0,3*	0,1	0,105
Total nitrogen						0,326*	0,237
Fixednitrogen							0,786**

Total nitrogen, fixed nitrogen and symbiotic efficiency

*,** : The correlation is significant at $p < 0.05$ and $p < 0.01$ respectively.

IV. Discussion

It is established that the conservation of ecosystem biodiversity depends on the composition of microbial communities of the soil. Therefore, the knowledge of the distribution and abundance of beneficial bacteria is of crucial use. In this work, we studied the characteristics and symbiotic diversity of different strains nodulating *A. gummifera*. For that we have tested the growth of isolates on YMA medium at different temperatures, pHs and salinity (Table 1) in order to select those adapted to extreme edapho-climatic conditions. Because the exposureto high temperatures may cause the symbiotic plasmid loss (Zahran et al., 2012) and the soil acidity can affect nodulation and plant growth (Habish 1970), indeed the Rhizobia subjected to salt stress can have morphological alterations causing changes in the profile of polysaccharides and extracellular lipoplysaccharides (Ventorino et al., 2013)document).Therefore, the better growth of ourisolates was recorded at a temperature of 28 ° C. These results are in perfect agreement with those found by Fikri-Benbrahim et al., 2017, showing that Rhizobia are mesophilic bacteria that multiply between 10 and 37 ° C with an optimum of 28 ° C (Fikri-Benbra him et al., 2017).The growth of our strains in pH medium between 6 and 10 is in agreement with other studies (Lebbida, 2009 ; Jourand 2004 (Fikri 2017)). Furthermore, it has been found that at a pH 5 to 5.5 the nodulation is absent in Acacia (Brock well et al., 2005).We note that our isolates in their majority support concentrations of salt up to 862mM (Table 1), These results corroborate with those found in some isolates associated with other Acacia species (Sakrouhi et al., 2016). Other studies have shown that many woody legumes such as Acacia, Prosopis and Lucaena tolerate a Na Cl concentration of 5% (Abolhasani et al., 2010).Otherwise soil contamination by metal ions affect the processes of atmospheric nitrogen fixation and nodulation of legumes. That is why the effect of heavy metals on the development of strains associated with different Acacia has been evaluated in several studies (document) (Fterich et al., 2012; Sakrouhi et al., 2016). The results presented in table 2 show that some isolates are resistant to several metal ions (Al, Zn). However, low resistance is recorded for mercury, cobalt and cadmium. These metal ions are the most inhibitory for the development of our strains, this same result was also reported by Zerhari et al., 2000. Thereafter, the most tolerant onehas been selected for 16S rRNA gene sequencing. A26 (MF629731) and A32 (MF663789) isolated from *Acacia gummifera* were found to belong to the same species *Rhizobiumgiardinii*, while A24 (MF769718) was affiliated with *Rhizobium azibense*, A12 (MF 774692) and A10 (MF 629733) has 99% homology with 16s rRNA sequence with respectively to *Rhizobium pusence* and *Rhizobium naphthalenivorans*, A4 (MF 972515) is somewhat close to *Rhizobium neptum*. The nodulation and efficiency of the strains are essential for the establishment of *Acacia gummifera* after transplanting in the fields and for maximum use of their atmospheric nitrogen fixation potential. Through this work we have studied the nitrogen-fixing potential of *Acacia gummifera* at 2 different ages inoculated with six different strains of Rhizobium previously identified. The production of nodules is an essential factor for the achievement of an efficient symbiotic relationship, their insufficient number or their absence might cancel or reduce the process of biological fixation of nitrogen. Our Rhizobialstrains assessed in this study all showed a capacity to induce nodule formation on *Acacia gummifera*. The highest amplitudes of dry nodule production were obtained in 12-month-old Acacia associated with strains A4 (*Rhizobium nepotum* MF 97515), A26 and A32 (*Rhizobium giardinii* MF663789).Gassamahas shown that Rhizobium strains become more infectious in *Acacia albida* trees that are more than 7 months old and in this case the formulation of nodules becomes continuous and increasing.Differences of nodule parameters suggest the existence of differences in efficiency between the sex strains of Rhizobium.Which is concordant with the dry matter yields results (table 3). The dry biomass of 12-month-old A.gummifera is significantly higher than that of 6 months withthe highest was produced by the strain A24(*Rhizobium azibense* MF769718).This strain recorded a number and a dry weight's nodule the least important, this indicates that *Acacia gummifera* is mobilizing less energy in the process of nodulation in favor of nitrogen fixation.The absence of correlationbetween the yield of the plant and the number of nodules (table 4) confirm that a good yield could be observed with a smaller number of nodules whereas a high number of nodules gives a low yield (ineffective nodules).These results are similar to those found by Chen et al 2004; El Akhal 2008 and Berrada, 2013. The 12-month-old seedlings accumulated more total nitrogen suggesting their better nitrogen fixation efficiency which was mostly recorded in seedlings inoculated with the A24 strain. The correlation existing between the different growth parameters comes from the accumulation of fixed biological nitrogen.

V. Conclusion

This study showed a diversity between bacteria belonging to the same generaof Rhizobium nodulating A. gummifera based on their phenotypical and genotypical characterizations. Also, it is clear from the results that inoculation with Rhizobia benefited especially plant 12 months growth and N fixation. Therefore, the introduction of native plant species such as A. gummifera associated with a managed microbial symbiont community is an effective biotechnological tool to support the recovery of desert ecosystems.

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