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Physiological and Symbiotic Characteristics of Rhizobia Isolated from *Medicago Polymorpha* L and Trigonella Stellate L Growing in semi-arid Regions of Libya

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Abstract: Physiological and symbiotic characteristics of 17 indigenous rhizobial isolates obtained from root nodules of wild legumes, *(Trigonella stellate* L. and *Medicago polymorpha* L.) that growing in different locations of Libya (northeast and northwest) and their cross-nodulation with cultivated *Medicago sativa* were studied. The results showed that the isolates were effective in their symbiosis with cultivated *Medicago sativa* L. A numerical taxonomic analysis performed on 56 non-symbiotic characteristics showed that at similarity level of 87%, the isolates formed four distinguished groups and three isolates remained separate. The results showed high variability among the isolates in their tolerance to various temperatures, and the majority of rhizobia isolates were sensitive to high acidity and are not able to grow at pH of 4.5. The tested isolates demonstrated a wide diversity in their tolerance to salinity, from sensitive unable to grow at 1% (w/v) to resistant to high salinity on a medium containing 4% (w/v) of NaCl. All isolates forming the four groups, including reference strains, were sensitive to $CuCl_2.2H_2O$, but they grew in medium containing $HgCl_2$. The isolates forming the four groups varied in their resistance to salts. The rhizobial isolates revealed a great diversity in their ability to react with antibiotic from sensitive to resistant.

Keywords: Libya, *Trigonella Stellata*, *Medicago Polymorpha*, Rhizobia, Physiological, Symbiotic, Characteristics

1. Introduction

In the last two decades several studies on rhizobia from wild legumes were published [1-7]. They showed high tolerance to adverse conditions such as extreme temperatures and salt stress. Some even formed effective symbiosis with some cultivated crops and forage legumes such as *Vicia faba, Pisium sativum, Vigna sinensis* and *Medicago sativa* [1]. Some isolates from spontaneous tree legumes even formed more effective symbiosis on cultivated legumes than their original hosts [8]. Furthermore, these bacteria may have specific traits such as tolerance to environmental stress that can improve other bacteria [1].

Medicago polymorpha L. and *Trigonella stellata* L are spontaneous herbaceous legumes that belong to family Fabaceae. They are widely distributed in semiarid region of the Mediterranean. like many herbaceous legumes in arid and semi-arid regions of Mediterranean, these two spontaneous legumes are economically and ecologically more important i.e. they are used as a fodder, slowdown soil erosion, restore the ecosystem and increase soil fertility due to their presumable ability to form association with a group of rhizobia. Thus, they play an important role in the reclamation of degraded lands [5, 9]. *M. polymorpha* and *Trigonella stellata* which belong to one cross-nodulating group, establishing a symbiotic association with members of the genus *Ensifer* (formerly *Sinorhizobium*).

The symbiosis between rhizobia and legumes is affected by various environmental stresses, especially acidity, temperature and salinity [1]. The ability of rhizobia to persist and fix atmospheric nitrogen is dependent on leguminous host plant and the prevailing environmental conditions. Like

many medics, *M. poymorpha* is adapted to moderately acidic and saline soils and form sybmiosis with rhizobia in the genera *Sinorhizobium* . However, some researchers [10, 11] recovered *Rhizobium* and *Agrobacterium* from this plant. Some medics such as *M. Littralis* and cultivated *Medicago sativa* growing in alkaline and neutral soils or cultivated in arid and salt affected soils were associated with *S. meliloti* [10, 12]. Rhizobia isolated from *M. polymorpha* growing in a saline soil were highly tolerant to sodium chloride salinity as high as 800 Mm [11]. Rhizobial isolates from *Trigonella* are less studied than those of *Medicago* species. Few reports [13] indicated that the microsymbiont of *Trigonella stellata* indigeous to Negev Desert highlands was similar to *M. sativa,* nodulating with *Ensifer meliloti*. Geographical Location and soil properties do affect the symbiosis between legumes and rhizobia [1], as will as their diversity [14-21].

Libya is extended over the arid and semi-arid regions where natural forests are scarce and its landscape is littered with scattered shrubs and herbaceous plants. Here the native rhizobium is associated with wild herbaceous legumes such as *M. polymorpha* and *T. stellate*, but there is limited information on their symbiotic and physiological properties. Therefore, this study was intended to select native isolates that may be able to withstand the local environmental conditions of Libya and highly fix –nitrogen with cultivated forage legumes such as *M. sativa.*

2. Materials and Methods

2.1. Collection of Samples

Samples of root nodules were collected from different locations in the semi-arid areas of Libya i.e. Ras Alhilal (northeast), Gerian and Nalot regions in Jabal nfosa (northwest). Also the seeds used for plant tests were collected from the same regions

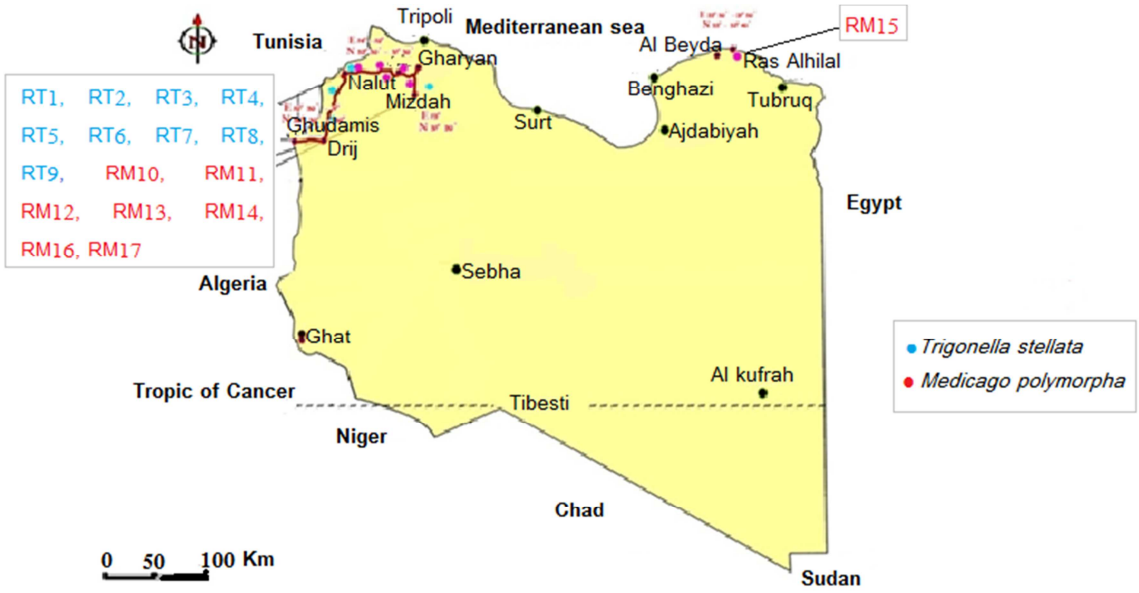

Figure 1. *Map of Libya showing the locations of collection of root nodules.*

2.2. Rhizobial Isolates

Seventeen indigenous rhizobial isolates were obtained from root nodules of wild legumes, *Trigonella stellata* and *Medicago polymorpha*), in addition to seven reference rhizobial strains (Table 1). The reference strains were obtained from the laboratory of Symbiosis Tropical and Mediterranean (STM).

Table 1. *Reference strains, their hosts and geographical origin.*

Isolates	ORS no	STM no	Other names	Host plant	Geographical origin
R.leguminosa-rum bv viciae	-	237	LMG 14904 T	*Vicia spp.*	Senegal
R. galegae	668	-	HAMB 1540 Ll	*Galega orientalis*	Finland
M. loti	664	-	NZP 2213, LM	*Lotus corniculatus*	New Zealand
M. ciceri	2738	-	UPMCa 7 T, L	*Cicer arietinum*	Spain
S. saheli	609	-	LMG 8309 t2	*Sesbania cannabina*	Senegal
Al. acaciae	1009	-	LMG 7834	*Acacia laeta*	Senegal
B. japonicum	127	-	LMG 8321-U	*Faidherbia albida*	Senegal

2.3. Isolation and Authentication of Root-nodule Bacteria

All isolates were tested for their ability to produce nodules on their respective host plants from which they were originally isolated and on cultivated *Medicago sativa*. Seeds of *Trigonella stellata* and *Medicago sativa* were surface sterilized by (0.2 % $HgCl_2$) for 5 minutes, rinsed several times with distilled water and germinated in darkness on plates of 1% water agar at 28°C.

After germination at room temperature, seedlings were aseptically transferred to test tubes containing sterilized Jensen medium [22]. After seven days, each tube was inoculated with a drop of log-phase (containing 10^6 cells/ml) of each rhizobial isolate. Three replications were made for each isolate; uninoculted tubes were also included and served as negative controls. Then, all tubes were transferred to wooden boxes and placed in the growth chamber. After four weeks of growth, the plants were examined for nodulation. Isolates that failed to form nodules were neglected. The ability of some isolates from *T. stellata* to fix nitrogen on cultivated *Medicago sativa* was tested after harvesting and oven dried at 70Ċ for one week.

Data were subjected to analysis of variance procedures using SPSS program.

2.4. Cultural Characteristics

2.4.1. Time of Colony Development

Log phase culture for each rhizobial isolate was streaked on YEM agar. The inoculated plates were incubated at 28°C and daily inspected until separate colonies developed.

2.4.2. Acid or Alkali Production

The ability of the rhizobial isolates to change the standard growth medium (YEMA) to an acidic or alkaline state was conducted as described [23-24].

2.5. Physiological and Biochemical Characteristics

2.5.1. Temperature Test

Temperature tests were carried out on YEM agar plates. All plates containing tested isolates were incubated at 38, 40, 42, 44, 46, 48 and 49.5°C for seven days.

2.5.2. pH Tolerance

Growth of thetested isolates on at acidic and alkaline pH was performed as described by [25], but on YEMA. The media were adjusted with 0.1 N HCl to acidic and with NaOH to alkaline pH that ranged from pH: 4.5, 5, 5.5, 8.5 to 9 before being autoclaved.

2.5.3. Salt Tolerance

Tolerance to salinity was determined on YEM agar plates containing 1 ,2, 3,3.5, 4,4.5 and 5 % (w/v) NaCl.

2.5.4. Resistance to Heavy Metals

Resistance to heavy metals was determined as described [26] on solid YEMA medium containing either of the following salts of heavy metals (µg/ ml): $CuCl_2.2H_2O$ (100), $Pb(CH_3COO)_2$ (500), $ZnCl_2$ (100) , $CdCl_2 . 2H_2 O$ (20) , $AlCl_3.6H_2O$ (500), and $HgCl_2$ (5).

2.5.5. Intrinsic Antibiotics Resistance

The test of intrinsic antibiotics resistance was determined using eight antibiotic discs (Oxoid) on the solid YEMA medium. The following antibiotics were used: Penicillin (6µg), Streptomycin (10µg), Erythromycin (15µg), Tetracycline (30µg), Vancomycin (5µg), Chloramphenicol (10 µg), Colistin (10 µg), Rifampicin (5 µg). The plates were inspected for the presence or absence of inhibition zone around the discs.

2.6. Numerical Taxonomy

Physiological similarity among the tested isolates were determined by UPGM linkage clustering analysis using STATISTCA program

3. Results

3.1. Symbiotic Traits

The tested isolates and their site of isolation are listed in Table 2. It also showed the symbiotic traits and groups formed by numerical analysis.

Table 2. *Rhizobial isolates, symbiotic traits (cross -nodulation) and groups resulted from numerical analysis.*

Isolates	Site of isolation	Host plant	Clusters	Symbiotic traits		
				M. sativa	*T. stellata*	*M. polymorpha*
RM13	Gerian region	*M. polymorpha*	C	E	-	E
RM12	Gerian region	*M. polymorpha*	C	E	-	E
RT8	Gerian region	*T. stellata*	C	E	E	-
RM10	Nalot region	*M. polymorpha*	D	E	-	E
RM17	Gerian region	*M. polymorpha*	D	E	-	E
RM15	RasAlhilal	*M. polymorpha*	D	E	-	E
RT7	Gerian region	*T. stellata*	D	E	E	-
RT5	Nalot region	*T. stellata*	D	E	E	-
RT6	Nalot region	*T. stellata*	D	E	E	-
RT3	Gerian region	*T. stellata*	D	E	E	-
RM11	Nalot region	*M. polymorpha*	D	E	-	E
RM16	Nalot region	*M. polymorpha*	D	E	-	E
RT4	Nalot region	*T. stellata*	D	E	E	-
RT9	Gerian region	*T. stellata*	D	E	E	-
RT2	Gerian region	*T. stellata*	D	E	E	-
RT1	Gerian region	*T. stellata*	Separate	E	E	-
RM14	Gerian region	*M. polymorpha*	Separate	E	-	E

*E = effective and (-) not effected

The authentication test confirmed that all the tested isolates were rhizobia. All isolates from *T. stellata* and *M. polymorpha* form effective symbiosis with their respective host plants (Table 2). Observation of plant shape and

statistical analysis (Tables 3 and 4) showed that the isolates seemed to be effective in their symbiosis with cultivated *Medicago sativa*. The results of statistical analysis indicated that there were no significant differences in the number of nodules of *M.sativa* plants which was inoculated by the wild *M.polymorpha* isolates *;* with the exception of RM16 the same statement was true for the shoot dry weight (table 3). However, Data in Table 3 also showed that there were significant ($p \leq 0.05$) differences in nodules dry weight of *M. sativa* plants which was inoculated by the wild *M. polymorpha* isolates between (RM10, RM12, RM15, and RM16) and the control

Table 3. *Means of nodules number, nodules dry weight and shoot dry weight of M. sativa inoculated by the wild M. polymorpha isolates.*

Rhizobial isolates	nodule number	Nodule dry weight (g)	shoot dry weight (g)
Control	0.000 a	.000 a0	.002 a0
RM10	15.66 a	.012 c0	.010 a 0
RM11	25.66 a	.008 abc0	.011 a 0
RM12	21.66 a	.009 bc0	.015 a 0
RM13	12.33 a	.002 ab0	.016 a 0
RM14	17.66 a	.004 abc0	.012 a 0
RM15	16.00 a	.010 bc0	.021 a 0
RM16	18.33 a	.021 d0	.070 b 0
RM17	25.33 a	.007 abc0	.008 a 0

Means followed by same letter(s) in columns are not significantly different according to Duncan's Multiple Range Test at 5% level of significance.

Table 4. *Means of nodule number, nodule dry weight (g) and plant dry weight(g) of M. sativa inoculated by the wild T. stellata isolates.*

Rhizobial isolates	Nodule number	Nodule dry weight (g)	shoot dry weight (g)
Control	0.00 a	.0000 a	.002 a0
RT1	37.3 c	.0520 a	.012 bc0
RT2	38.66 c	.0100 a	.013 bc0
RT3	23.33 bc	.0080 a	.013 bc0
RT4	28.33 bc	.0080 a	.008 ab0
RT5	18.66 a	.0370 a	.007 ab0
RT6	26.33 bc	0.013 a	.015 bc0
RT7	14.33 a	.0050 a	.019 c0
RT8	8.00 a	.0090 a	.010 abc0
RT9	24.66 bc	.0150 a	.011 bc0

Means followed by same letter(s) in each column are not significantly different according to Duncan's Multiple Range Test at 5% level of significance.

Data in Table 3 showed that there were significant ($p \leq 0.05$) differences in the number of nodules of *M .sative* plants which was inoculated by *T. stellata* isolates except for RT5, RT7 and RT8. Whereas Data showed that there were no significant differences in the dry weights of nodules of M. sativa plants which were inoculated by the wild T. stellata isolates . the Data also indicated significant differences in shoots dry weights of *M. sativa* plants which were inoculated by the wild *T. stellata* isolates with excepting of (RT4, RT5and RT8)

3.2. Numerical Taxonomy

A numerical taxonomic analysis performed on 56 non-symbiotic characteristics in STATICA showed that at similarity level of 87%, with the exception of RT1 , RM14 isolates and reference strain *B.japonicum* all other isolates formed four distinct groups designated as , GA, GB, GC and GD (Figure 1). The composition of each group is presented in Table 2. Which also showed symbiotic traits on *M. sativa* plant. The physiological characteristics of each group were shown in Table 5.

Table 5. *Results of physiological tests for the groups formed by numerical analysis.*

Characteristic	Groups and their reactions						
	A (n=3)	B (n=3)	C (n=3)	D (n=12)	RT1	RM14	*B. japonicum*
First colony formed							
1-5 days	(100)	(100)	(100)	(100)	(100)	(100)	(0)
6-10 *days*	(0)	(0)	(0)	(0)	(0)	(0)	(100)
Acid production	(100)	(100)	(100)	(100)	(0)	(100)	(0)
Alkali production	(0)	(0)	(0)	(0)	(100)	(0)	(100)
Growth at							
38C	(100)	(67)	(100)	(100)	(0)	(100)	(0)
40 Ċ	(100)	(33)	(67)	(100)	(0)	(100)	(0)
42C	(100	(0)	(33)	(100)	(0)	(100)	(0)
44C	(0)	(0)	(0)	(100)	(0)	(100)	(0)
46C	(0)	(0)	(0)	(25)	(0)	(100)	(0)
48 C	(0)	(0)	(0)	(0)	(0)	(100)	(0)
49.5 *C*	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Growth at pH							

Characteristic	Groups and their reactions						
	A (n=3)	B (n=3)	C (n=3)	D (n=12)	RT1	RM14	*B. japonicum*
pH 4.5	(67)	(100)	(100)	(42)	(0)	(100)	(0)
pH 5	(100)	(100)	(100)	(100)	(0)	(100)	(0)
pH 5.5	(100)	(100)	(100)	(100)	(0)	(100)	(0)
pH 8.5	(100)	(100)	(100)	(100)	(100)	(100)	(100)
pH 9	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Salinity tolerance							
1 %	(100)	(100)	(67)	(100)	(0)	(100)	(0)
2 %	(100)	(100)	(33)	(100)	(0)	(100)	(0)
3 %	(100)	(100)	(0)	(100)	(0)	(100)	(0)
3.5 %	(100)	(100)	(0)	(92)	(0)	(100)	(0)
4 %	(100)	(100)	(0)	(58)	(0)	(100)	(0)
4.5 %	(67)	(0)	(0)	(33)	(0)	(100)	(0)
5 %	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Heavy metals resistance							
$CuCl_2.2H_2O$	(0)	(0)	(0)	(0)	(0)	(0)	(0)
$AlCl_3.6H_2O$	(67)	(0)	(0)	(0)	(0)	(0)	(0)
$CdCl_2.2H_2O$	(100)	(0)	(0)	(0)	(0)	(0)	(0)
$ZnCl_2$	(100)	(0)	(33)	(100)	(0)	(0)	(0)
Pb $(CH_3COO)_2$	(67)	(67)	(100)	(100)	(100)	(0)	(0)
$HgCl_2$	(100)	(100)	(100)	(100)	(100)	(100)	(100)
Antibiotic resistance							
Penicillin	(33)	(0)	(100)	(100)	(0)	(100)	(0)
Streptomycin	(33)	(33)	(0)	(42)	(0)	(0)	(0)
Erythromycin	(0)	(100)	(0)	(100)	(0)	(100)	(0)
Tetracycline	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Vancomycin	(33)	(0)	(67)	(50)	(0)	(0)	(0)
Chloramphenicol	(33)	(0)	(0)	(50)	(0)	(0)	(0)
Colistin	(67)	(67)	(100)	(0)	(100)	(100)	(100)
Rifampicin	(0)	(0)	(33)	(0)	(100)	(0)	(100)

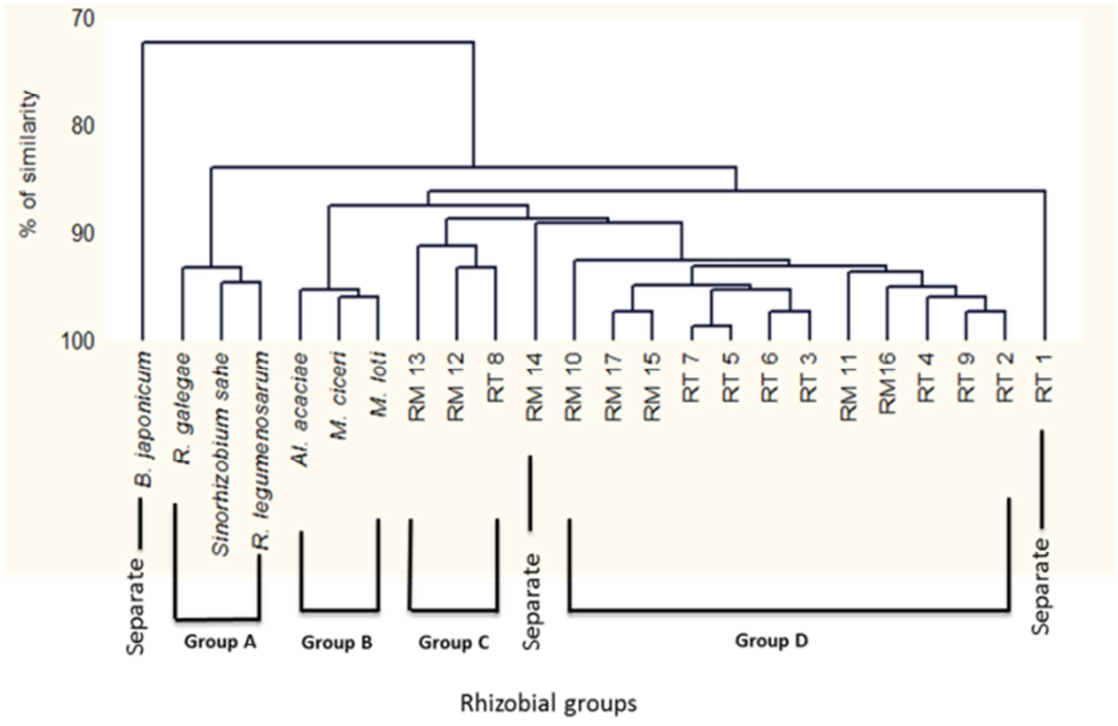

Figure 1. *Dendrogram showing the relationship between the tested rhizobial isolates.*

3.3. Growth Characteristics

The *T. stellata* formed white large colonies (> 2 mm after 48 hour) with a large gum production, changed the growth medium to acidic, but some isolates (RT1), showed red or rosy color colonies, and one isolate (RT8) showed yellowish color, whereas *M. polymorpha* isolates were homogenous and similar to the majority of *T. stellata* isolates with a large size and white color (> 3 mm after 48 hour). Also, results of the growth characteristics showed that with the exception of

(RT1) isolate and (*B. japonicum*) reference strain, which were slow growers and alkali producer, all other isolates and the reference strains forming the four groups were fast growers and acid producers.

3.4. Physiological Tests

The physiological tests showed high variability among the isolates in their tolerance to various temperatures. The isolates that were tolerant to high temperatures, grew at *44*°C. Separate strain RM14 showed the greatest tolerance to high temperature of 48°C.

3.4.1. pH Tolerance

The majority of rhizobial isolates were sensitive to high acidity, and were not able to grow at pH 4.5. With the exception of the separate isolate RT1, all other isolates forming the four groups grew at pH values of 5 -5.5, whereas all isolates forming the four groups were able to grow in the medium with high pH values of 8.5- 9.

3.4.2. Salinity Tolerance

Tolerance assay to several NaCl concentrations showed that the tested isolates showed a wide diversity in their tolerance to NaCl salinity. The sensitive isolates such RT1 was unable to grow at 1% (w/v) of NaCl , while the tolerant isolates grew on the medium containing 4% (w/v) of NaCl. Table 5, with the exception of the reference strain (*B . japonicum)*, all other isolates forming the four groups grew at 1% NaCl, whereas some isolates grew even at 4% (w/v) of NaCl .

3.4.3. Heavy Metals Resistance

A test of the isolates for their ability to grow in the medium containing different salts of heavy metals, showed that all isolates forming the four groups including reference strains were sensitive to $CuCl_2$. $2H_2O$. table 5. However all isolates grew in the medium containing $HgCl_2$. The isolates forming the four groups varied in their resistance to the remaining salts i.e. $CdCl_2$. $2H_2O$, $ZnCl_2$ and Pb $(CH_3COO)_2$

3.4.4. Antibiotic Resistance

Rhizobial isolates showed a wide diversity in their ability to react with antibiotics ranging from sensitive to resistant. The majority of the isolates including the reference strains were resistant to erythromycin and sensitive to tetracycline, rifampicin and streptomycin.

4. Discussion

Seventeen rhizobial isolates were recovered from wild leguminous plants seemed to be fast-growing depending on the time of colony development, acid production in growth medium , where the first colony was found to appear between 3 and 5 days [27].

The different morphological shapes of colonies, the development of yellowish, and red or rosy colonies by some isolates (RT1 and RT8) from *Trigonella* plant indicated the occurrence of genetically diverse rhizobia inside the nodules of this plant. The isolates that produced large gum and showed rapid growth are similar to *Sinorhizobium* species that nodulate *Medidago* plants [28] and generally have common characteristics of rhizobia from arid and semi-arid lands [29].

All isolates formed nodules when were re inoculated on their host plants. Thus, all isolates from *M. Polymorpha* were rhizobia and the resulst reported here were different from that reported by some investigators [10-11] who found occurrence of *Agrobactrium* and rhizobia (*Ensifer meliloti*) inside nodules of this plant. *Sinorhizobium medicae* was reported to fix nitrogen on *M. polymorpha* wheras *S. meliloti* formed ineffective symbiosis with this plant [28]. In this study, Isolates from *M. polymorpha* were unable to fix nitrogen on field cultivated *M. sativa*, therefore, they could be *S. medicae*. In contrast to *M.* polymorpha, isolates from *Trigonella satellata* formed effective nodules on *M. sativa*. In this case, they behaved similar to *S. meliloti*.

The physiological characteristics showed that most of isolate (group D and RM14) from both plants were highly tolerant to temperature and they grew at 44Ċ. In this case, they were distinguished from the reference strains and rhizobial strains from annual *Medicago* species including *M. polymorpha* [28]. Rhizobial isolates which grew at extreme temperatures were previously recovered from wild legumes growing in the arid regions of Libya. This result was in conformity with other observations which suggest that dry regions harbor selections of rhizobia tolerant to high temperature [12, 33].

Rhizobia nodulating annual *Medicago* were not usually tolerant to high acidity i.e. unable to grow below pH 5 [12, 28]. In this study, most of the tested isolates were shown to have wider range of pH profile for their growth. This was to some extent in agreement with other study [32, 33] Isolates with high adaptability to acidic and alkaline pH can be used as inoculant for legume production where acidity and alkalinity is a problem.

Rhizobia are different in their response to salinity. Those isolated from wild legumes appeared to be highly tolerant to salinity [1]. Isolates tested in this study, ranged from sensitive to highly tolerant, grew up to 4% NaCl. Salt tolerance is a characteristic of rhizobial isolates from *Medicago* species such *S. meliloti* and *S. medicae* [1, 12, 31-32]. Tolerance to salinity could help rhizobia survive and tolerate water stress in environments affected by salinity and drought such as in the arid and semi-arid regions of Libya.

Diluted salt solutions of heavy metals had a toxic effect on most bacteria including rhizobia. However, differences among rhizobia in their resistance to heavy metals were reported. In this study, most of the tested isolates behaved similar to *S. medicae* and S. *meliloti* from arid regions of Morocco [12] with respect to their Resistance to some heavy metals such as Zn, but were different in their response to . Pb and Hg. Resistance to heavy metals could give advantages to rhizobia to live and perform in soils irrigated by treated sewage waters that contain those heavy metals.

Rhizobia isolates were sensitive or had low resistance to

tetracycline, streptomycin, chloramphenicol and erythromycin. This result was support of that reported for rhizobial isolates from lentil plants [30], but was different from the result recorded for *Sinorhizobium* species from arid and salt affected regions [12]. It has been reported that fast-growing strains are more sensitive to antibiotics than slow-growing rhizobia (Maâtallah et al., 2002) [33]

5. Conclusion

The present study shows the physiological and symbiotic characteristics of Rhizobial isolates of wild legumes, *(Trigonella stellate* L. and *Medicago sativa* L.). All isolates from *T. stellata* and *M. polymorpha* form effective symbiosis with their respective host plant and with cultivated *Medicago sativa*. Some of the isolates showed tolerant to a wider range of salt, pH, and temperature. All isolates forming the four groups, including reference strains, were sensitive to $CuCl_2.2H_2O$, but they grew in the medium containing $HgCl_2$. The rhizobial isolates showed a great diversity in their ability to react with the antibiotic from sensitive to resistant. This study recommends characterizing the test isolates further using modern molecular techniques in order to elucidate the proper identity of the strains

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