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DIVERSITY OF TEN *RHIZOBIUM LEGUMINOSARUM* ISOLATED FROM VARIOUS EGYPTIAN SOIL ON PHENOTYPIC BASIS

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ABSTRACT: *We used ten Rhizobium leguminosarum isolates that obtained from different Egyptian soils with different pH . The isolates were characterized based on phenotypic traits , Temp, pH and their response to different antibiotics resistance. All isolates grew well at temperatures of 28,30 and 35 °C not able to grow at 45°C . Four isolates showed growth inhibition at 40°C . The isolates showed variation in growth at various pH levels in their medium at 4, 5, 9,10 and 11. Isolate number 9 showed the ability to grow at all used pH levels. Isolates number 4 and 8 showed inability to grow at all tested pH levels. We investigated the effects of various antibiotics including S10, MEM10, Cxm30, Va30, Ox1, T30, N30, B, Am10 and k30 on growth of Rhizobium isolates . We found all isolates resistant to antibiotic B and all isolates were sensitive to antibiotic k30.*

Key words: *Rhizobium leguminosarum, temperature, pH and antibiotic biodiversity.*

INTRODUCTION

Rhizobia are gram-negative bacteria. They are able to establish nitrogen-fixing symbiosis with leguminous plants. In the Rhizobium–legume symbiosis, the process of nitrogen fixation is strongly related to various environmental conditions. Several environmental conditions could be the limiting factors to the growth and activity of nitrogen-fixation. Typical environmental stresses faced by the legume nodules and the symbiotic partners may include antibiotics, soil pH and temperature , (Kucuk and Kivanc, 2008). Soil environmental conditions are critical factors to the persistence and survival of rhizobia. The changes in the rhizospheric environment can affect both growth and saprophytic competence, which will influence competitiveness and persistence (Dowling and Broughton, 1986). In arid and semiarid regions of the tropics, the soil temperatures near the surface can be very high. In Egyptian sandy soils, the temperature near the soil surface was 59 °C at the air temperature 39°C. However, the soil temperature decreased rapidly with depth, being moderate 35 °C, at 15 cm . It appears, that rhizobia are more resistant to high temperatures in soil than in laboratory medium (AbdelGadir and Alexander 1997). Every bacterium has its own optimum conditions, under which it grows at its best. For most rhizobia, the optimum growth temperature is 28 – 31 °C (Zahran 1999). Not only do the bacteria themselves have an optimum temperature range, but the processes within them do as well. Survival of *Rhizobium leguminosarum*, the microsymbiont of faba bean, may be affected mainly by extremes temperature. Differences in adaptation to high temperatures have been demonstrated from different climatic zones. Eaglesham and Ayanaba (1984) reported that more than 90 % of cowpea rhizobia isolated from hot dry Sahelian Savanna in Niger were able to grow at 40 °C while the rhizobia isolated form cooler humid regions of West Africa did not grow at this temperature (Munevar and Wollum 1981). Some rhizobial strains isolated from nodules in arid environments are able to grow at 40 °C or even higher (Eaglesham *et al.* 1981). Kluson *et al.* (1986) reported differences in the tolerance of *Bradyrhizobia* cultivated in liquid medium and in soil to temperatures within the range of 20 to 35 °C. Baldani and Weaver (1992) attributed heat tolerance of *Rhizobium leguminosarum biovar trifolii* strains to cryptic plasmids. These plasmids induce the synthesis of heat shock proteins upon exposure of bacteria above normal growth temperatures (Sen *et al.* 1990). Temperature is often pointed out as the

major factors in determining the bacterial community diversity (Fierer and Jackson 2006). Although the optimum temperature for rhizobial growth is 25-30 °C (Zhang and Smith 1995), however in both saprophytic and symbiotic life rhizobia are often subject to temperatures out of this range .

Agricultural soils are either alkaline or acidic that affect rhizobial growth and survival. Global warming and agricultural practices cause increase in the amount of soil effects by acidity. Each Rhizobium has its own optimum pH, under which it grows at its best. Although neutral conditions are generally optimum for bacteria, different species of Rhizobium display varying degrees of pH tolerance as measured by their ability to grow (not just survive) (Glenn and Dilworth 1994). Rhizobia is more sensitive to acidic condition. Indeed, it is in many cases the inability of the rhizobia to persist and survive under acidic conditions reduces the effectiveness of the symbiosis. Therefore, the selection of rhizobial strains tolerant to acidic conditions may improve the acid tolerance of the legume through an efficient symbiotic nitrogen fixation under acidity conditions. However, the relationship between low pH of soil and rhizobia competitiveness, and ability to persist under acidic stress is not always straight forward . Foster (2000) recorded that *S. meliloti* was viable only below pH for growth 5.5. Some rhizobia have wide range of pH such as *S. fredii* can grow well between pH 4 – 9.5 but *B. japonicum* cannot grow at the extremes of that range (Fujihara and Yoneyama 1993). These values are the extremes and hindered the growth and survival of bacteria between 1 and 2 pH units (Richardson and Simpson 1989). *Rhizobium* isolates have different wide range for antibiotics tolerance (Alexander *et al.,* 2006). The aim of this work is to detect the biodiversity of ten *Rhizobium leguminosarum* isolates that obtained from different geographical locations in Egypt on the basis of phenotypic characteristics such as pH , antibiotics and temperature.

MATERIAL AND METHODS.

1. Collection of Samples:

Ten samples were collected from contaminated soil in different locations and from different Egyptian governorates in (Table 1) . The sickle was used to dig into the soil around the plant. A plant was uprooted along with soil around them in such a way that entire root system was kept intact. The plant along with its rhizospheric soil was taken in plastic bags and brought to laboratory for *Rhizobium* isolation purpose (Bergersen, 1980).

Table (1): Isolates numbers and their origins in different Egyptian governorates.

Isolate No.	Source
RL.1	El-Shohada, Minufiya Governorate.
RL.2	Quesna , Minufiya Governorate
RL.3	El-Giza, Giza Governorate
RL.4	Asute, Asute Governorate.
RL.5	Kafer El-Shekh, Kafer El-Shekh Governorate.
RL.6	Nubaria, Behera Governorate.
RL.7	Baher El-Bakar, Sharkia Governorate.
RL.8	El-Ghabal El-Asfar, Qalubia Governorate.
RL.9	Khafer El-Zayat,Gharbia Governorate.
RL.10	El-Wady El-Geded, El-Wady El-Geded Governorate.

2. Processing of plant specimen:

The plant roots were held below running tap water to remove soil around roots followed by immersing root system in tap water with gently shaking to clean the root system. Further it was used for *Rhizobium* isolation according to Subba Rao (2006).

3. Isolation of Rhizobium:

Healthy, pink nodules from legume roots were selected . Rhizobium was isolated according to Vincent (1970) . It could be summarized as follows, after washing the root system of *Vicia faba* plants, nodules were taken from the roots with care not to damage the surface. Nodules were washed thoroughly in distilled water and sterilized in 2.5 % sodium hypochlorite for 3-5 min following a rinse in 95% ethanol and washed thoroughly in five changes of sterile water. The exudates were aseptically streaked onto surface on yeast extract mannitol agar (YEMA) plates by three phase streaking pattern and incubated at 28°C for 3 days . A well isolated colourless colony was restreaked onto same medium for several times and incubated at 28° C for 3 days for purification. Single colony isolates were picked and kept on YEM slants furthermore, The samples were kept in glycerol 30% and were stored at -20°C . YEM plates were prepared according to Vincent (1970) .

4. Phenotypic characteristics :

4.1. Tolerance to acidity, alkalinity and high temperature:

Tolerance to various pH levels were tested . This was done by adding HCL or NaoH to adjust media pH to 4,5,9,10 and 11 according to Wei et al. (2008). Furthermore tolerance to high temperature 30,35,37,40 and 45 were performed for all tested isolate on solid YEM plates. Comparisons were done with the optimum growth degree of 28°C . All tests were done in triplicate.

4.2. Determination of antibiotic resistance:

Antibiotic discs were placed on plates that seeded with different bacterial isolates. WE used ten antibiotics presented in Table (2). Plates were set in triplicates, and incubated at 28°C for three days . Data were scored as presence of inhibition of growth zone surrounding antibiotic discs (sensitive) or absence of it (resistant) according to Ahmed (2000) .

4.3. Data analysis:

The NTSYS-pc version 2.11 W (Numerical Taxonomy System) program was used to perform cluster analysis based on Jaccard's average (UPGAMA) clustering method. similarity coefficient. Dendrogram was constructed according to the Unweighted Pair-Group Method with Arithmetical.

Table (2). list of used antibiotics .

Abbreviation	Antibiotics
S10	Streptomycin
MEM 10	Meropenem
Cxm30	Cefuroxime Sodium
Va30	Vancomycin
Ox1	Oxacillin
T30	Oxytetracyline
N30	Neomycin
B	Bacitracin
Am10	Ampicillin

Results:

Data presented in Table (3) clearly show the ability of ten *Rhizobium* isolates to grow at various temperature . All isolates grew well at various degrees of temperatures 28 ,30 and 35 °C. Six isolates showed ability to grow at elevated temperature 40°C . At 45°C all isolates showed growth inhibition of all tested rhizobium isolates. In fact this may be due to presence of cryptic plasmid that induce the synthesis of heat shock protein according to what Sen *et al.*(1990) has been published .

This dendrogram presented in Fig (1), grouped rhizobia in two clusters . The first one include six similar isolates (Rlv1, Rlv3, Rlv5, Rlv9, Rlv7, Rlv8), while another one include four similar isolates (Rlv2, Rlv4, Rlv10, Rlv6).

Table (3). Response of various tested *Rhizobium* isolates to different degrees of temperature.

Isolates	Temperature °C				
	28 C	30	35	40	45
***Rlv* 1**	+	+	+	+	_
***Rlv* 2**	+	+	+	_	_
***Rlv* 3**	+	+	+	+	_
***Rlv* 4**	+	+	+	_	_
***Rlv* 5**	+	+	+	+	_
***Rlv* 6**	+	+	+	_	_
***Rlv* 7**	+	+	+	+	_
***Rlv* 8**	+	+	+	+	_
***Rlv* 9**	+	+	+	+	_
***Rlv* 10**	+	+	+	_	_

Whereas, (+) means resistance and (-) means sensitive.
- 28°C was used as a control.

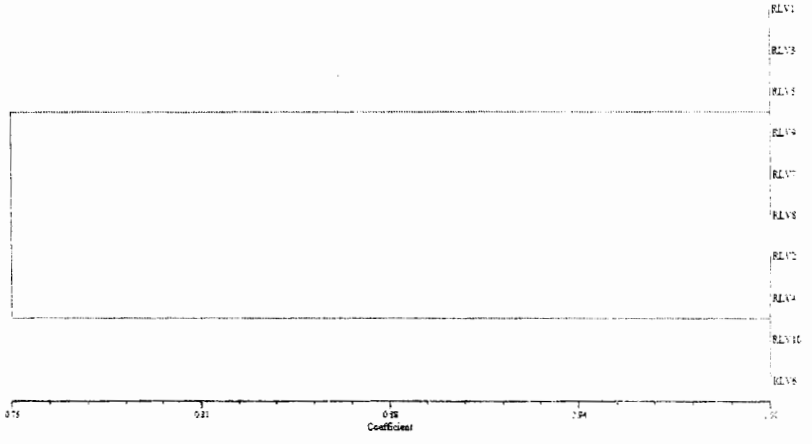

Figure (1). Dendrogram showing the similarities among ten tested isolates *Rhizobium leguminsarum* for their response to wide range of temperature.

Data in Table (4) clearly showed that all isolates are able to grow at pH 6.8 (control pH). There are four isolates 1,3,6 and 9 are able to grow at pH4. Isolate number 9 showed the ability to grow at all used pH levels ranging from 4 to 11. This is due to the pH level of the soil where this isolate originally obtained. Isolate number 4 showed inability to grow at all tested pH levels. The pH values of the soil that *Rhizobium* isolates were originated. It is clearly shown in Table (5) that pH level reflected the pH those isolates were able to tolerate of the soil where this isolate originally obtained. Isolates 5,7,9 and 10 were able to tolerate high pH levels ranging from 9 to 11.

Dendrogram presented in Fig. (2) showed two main clusters. First cluster contains seven isolates (Rlv1,Rlv9,Rlv6,Rlv3,Rlv5,Rlv7,Rlv10) this cluster contains another secondary clusters. Second cluster containing three isolates(Rlv2, Rlv4, Rlv8). It is clear that isolates(7 and 10) were similar and isolates (4 and 8) also similar. Isolates similarities here clearly reflect the original pH in soils where these isolates were originally obtained. These results were documented previously by Fujhara and Yoneyama (1993).

Results in Table (6) indicated differences between tested *Rhizobium leguminsarum* isolates toward ten different antibiotics. All isolates were able to grow in presence of antibiotic B and all isolates did not show any growth in presence of k_{30} . On the other hand there were some differences among isolates in resistance to the other antibiotics. All isolates showed sensitivity to antibiotic S_{10} except isolate No. 9 .All isolates were resistance to CXM $_{30}$ except isolate No.1. In the case of OX_1 all isolates showed resistance to it except isolates No. 1 and 4. Isolates No. 7 and 9 were resistant to antibiotic N_{30} . Isolates No. 1,3,5,6,8 and 10 showed sensitivity to antibiotic MEM_{10} . Isolates No. 1,2,4,6 and 10 showed sensitivity to VA_{30} . All isolates showed sensitivity to antibiotic T_{30} except isolates No. 4,6 and 9 . Five isolates (1,4,8,9 and 10) are resistant to antibiotic AM_{10} , whereas isolates (2,3,5,6 and 7) were sensitive to the same antibiotic.

Table (4). Response of various tested rhizobium isolates to different pH levels.

pH levels						
pH	6.8	4	5	9	10	11
Isolates	c					
***Rlv* 1**	+	+	+	+	+	–
***Rlv* 2**	+	–	+	–	–	–
***Rlv* 3**	+	+	+	+	–	–
***Rlv* 4**	+	–	–	–	–	–
***Rlv* 5**	+	–	+	+	+	+
***Rlv* 6**	+	+	+	–	+	+
***Rlv* 7**	+	–	–	+	+	+
***Rlv* 8**	+	–	–	–	–	–
***Rlv* 9**	+	+	+	+	+	+
***Rlv* 10**	+	–	–	+	+	+

Whereas, (+) means resistance and (-) means sensitive.
- pH 6.8 was used as a control.

Table (5). pH values of soils where the tested Rhizobium isolates were originally obtained.

Soil samples	**Rlv1**	**Rlv2**	**Rlv3**	**Rlv4**	**Rlv5**	**Rlv6**	**Rlv7**	**Rlv8**	**Rlv9**	**Rlv10**
pH values	7.88	7.92	8.05	7.30	7.89	7.87	7.81	7.94	8.46	8.24

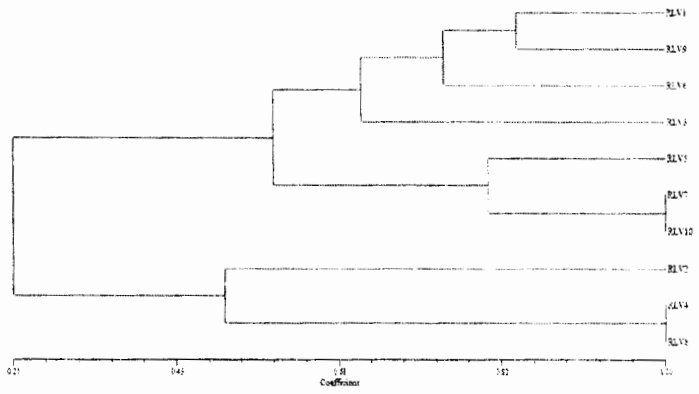

Figure (2). Dendrogram showing the similarities among ten tested isolates *Rhizobium leguminsarum* for their response to wide range of pH.

Table (6). Response of tested bacterial isolates to ten different antibiotics.

STRAINS	Antibiotics									
	S 10	MEM 10	Cxm 30	Va 30	Ox 1	T 30	N 30	B	Am 10	K 30
***Rlv* 1**	_	_	_	_	_	_	_	+	+	_
***Rlv* 2**	_	+	_	+	_	_	_	+	_	_
***Rlv* 3**	_	_	+	+	+	_	_	+	_	_
***Rlv* 4**	_	+	_	+	_	+	_	+	+	_
***Rlv* 5**	_	_	+	+	+	_	_	+	_	_
***Rlv* 6**	_	_	_	+	+	+	_	+	_	_
***Rlv* 7**	_	+	+	+	+	_	+	+	_	_
***Rlv* 8**	_	_	+	+	+	_	_	+	+	_
***Rlv* 9**	+	+	+	+	+	+	+	+	+	_
***Rlv* 10**	_	_	_	+	+	_	_	+	+	_

Whereas, (+) means resistance and (_) means sensitive.

Dendrogram presented in Fig. (3) showed a first cluster containing only one isolate(Rlv1). Another cluster containing secondary clusters included six isolates (Rlv2, Rlv4, Rlv3, Rlv5, Rlv8 and Rlv10) . The last cluster contained three isolates(Rlv6, Rlv7, Rlv9). It is clear that isolates 3 and 5 were similar.

Discussion:

Ten local Rhizobial isolates were collected from root nodules of field-grown faba bean in nine governorates in Egypt Table (1). Local Rhizobial isolates were phenotypically characterized in order to assess their tolerance ability under temperature, pH and antibiotic stress. Rhizobium are predominantly mesophilic and have optimum temperature for growth in culture at the range of 28-31 °C (Graham 1992). Maximum temperature degrees (T_{max}) for free-living rhizobia ranged between 35-45 °C (Zahran *et al.* 1994). Rhizobial strains obtained in the present study showed a relatively high (T_{max}), a large number of isolates showed growth at 40 and 45 °C.

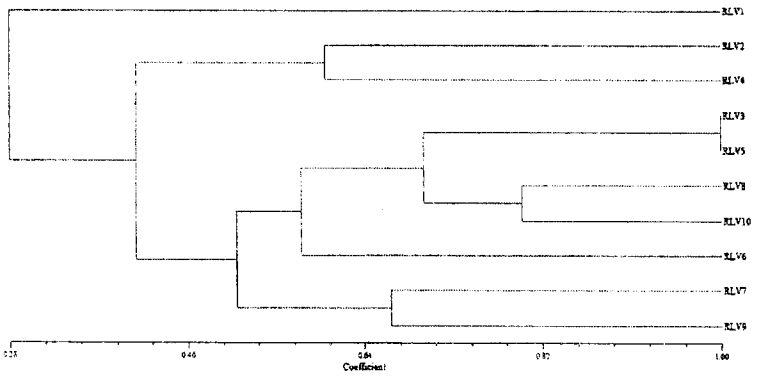

Figure (3). Dendrogram showing the similarities among ten tested isolates *Rhizobium leguminsarum* for their response to wide range of antibiotics.

This finding agreed with the results of previous studies on *R. leguminosarum* strains isolated from Nile Valley of Egypt, which showed tolerance to temperatures between 35-40 °C (Moawad and Beck 1991) and *C. arietinum rhizobial* isolates, which grew at 45 °C (Maatallah *et al.* 2002). The general responses to temperature stress in rhizobia were recently reviewed (Alexander and Oliveira 2012). Abundance of microorganisms (e.g. rhizobia) in soil can be limited with soil acidity and alkalinity (Brockwell et al. 1991). The majority of the isolates in this study grew well at pH ranges from 6 to 8. About 70 % and 28 % of the isolates in the present study tolerated alkaline pHs (9 and 10), respectively. The growth of our isolates at pH 6 and the alkaline pH 9, agreed with the results of earlier studies on rhizobial strains (Yadav and Vyas 1973), which showed that even pH 9,10 and 11 were not inhibitory to rhizobial strains and these strains were also not inhibitory to acidic conditions (pH_4) .

In addition, Hemphil and Jackson (1982) found that pHs of 5.6-6.4 were optimal for bean rhizobia. The isolates in this study showed variation in their resistance to the tested antibiotics. Most of our isolates were sensitive to antibiotic K30, but all isolates were resistant to antibiotic B.

In conclusion, we could performed that the isolated rhizobial strains were of good traits, e.g tolerant to high temperature levels, resistant to acidic and alkaline pH . The rhizobia are diverse, as shown from phenotypic characterization and numerical analysis.

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التنوع الحيوى لعشرة عزلات من بكتريا الريزوبيوم المعزولة من محافظات مصرية مختلفة

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الملخص العربي

استخدم في هذا البحث عشرة عزلات من بكتريا الريزوبيوم المعزولة من محافظات مصرية مختلفة لتحديد مدى التشابه والاختلاف بينهم . تم توصيف هذه العزلات على أساس مظهري بدراسة تأثير درجات الحموضة ، درجات الحرارة المختلفة وتأثرهم بوجود عشرة مضادات حيوية مختلفة وهى S_{10}, MEM $_{10}$, Cxm_{30} Va_{30}, Ox_1, T_{30}, N_{30}, B, Am_{10}, K_{30}. اتضح أن جميع العزلات قادرة على النمو في درجات الحرارة ٣٠،٢٨،٣٥ ° ، أظهرت أربعة عزلات عدم القدرة على النمو عند ٤٠ °. ظهرت اختلافات واضحة بين العزلات في النمو عند درجات حموضة مختلفة . دراسة استجابة العزلات المدروسة على النمو في وجود عشرة مضادات حيوية مختلفة أوضحت تباينا واضحا فى المقاومة والقدرة على النمو في وجود هذه المضادات الحيوية . يجدر الإشارة أنه بالرغم من أن العزلات مختلفة من أماكن متباعدة إلا أنه ظهرت بينهم تشابها بالنسبة للصفات السابقة .