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ORIGINAL ARTICLES

Molecular and biochemical characterization of new *Rhizobium leguminosarum* bio *viciae* strains isolated from different located of Egypt

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ABSTRACT

Eight locally isolates of *Rhizobium* sp. obtained from different soils location were characterized and determined of their nodulation efficiency. The obtained results exhibited that all the isolated strains were *R. leguminosarium* bio *viciae* and the eight isolates produced the highest mean of nodules per plants which reached (104 nodules) for isolate RL8 . The obtained results after salt tolerance and antibiotic response of the *R. leguminosarium* strains showed that the isolate No. RL7 was the superior strain for salt tolerance. The following strain for salt tolerance was the isolate (RL2). The high growth rate of the *R. leguminosarium* strains may be considered as an indicator of salt tolerance. Moreover, the isolated strains (RL3, RL4 and RL7) showed the highest level of antibiotic resistance since they were resistant to five antibiotics. However, high level of polymorphism RAPD technique was observed among the eight *R. leguminosarium* genotypes. All three primers were successfully generated reproducible polymorphic bands. Regarding the phylogenetic tree and similarity index based on Jacard's coefficient the isolate RL2 was grouped with RL6 in one cluster with 40% similarity. However, isolate RL4 was highly diverged with lower similarity about 10 % with the other rhizobia strains. The growth in the presence of NaCl generally altered the whole-cell protein patterns in SDS-PAGE in *Rhizobium* strains. Some bands disappeared and new bands appeared in treatment by salt. The similarity matrix between eight *R. leguminosarium* strains, when use the SDS-PAGE technique, varied from 0.120-0.889. The highest similarity coefficient (0.889) was detected between isolates RL6 and RL7. However, the lowest similarity was computed between strains RL3 and RL5 which presented in two different clusters. SDS-PAGE revealed a powerful characterization of method *R. leguminosarium* genotypes under study. The variations appeared between strains could reflex their different abilities in nodulation, antibiotic resistance and salt tolerance.

Key words: *Rhizobium leguminosarum*, Isolation, Identification, Nodulation test, DNA-fingerprinting, Protein-fingerprinting.

Introduction

The symbiosis between the root nodule bacteria of the genus *Rhizobium* and legumes results in the fixation of atmospheric nitrogen in root nodules. This symbiotic relationship is of special significance to legume husbandry as seed inoculation with effective strains of *Rhizobium* can meet the nitrogen requirements of the legume to achieve increased yields (Somasegaran and Hoben 1985). Rhizobia are Gram-negative soil bacteria which are able specifically to induce nitrogen-fixing nodules on the roots of leguminous plants. Among fast-growing rhizobia, on the basis of physiological properties and nucleic acid hybridization studies, a cluster can be identified grouping strains of *R. leguminosarum, R. phaseoli* and *R. trifolii*, which is called the *R. legurninosarum* cluster, whereas strains of *R. meliloti* form a separate cluster (Moffet and Colwell 1968; Gibbins and Gregory 1972). Recent genetic evidence has confirmed this separation in two clusters (Kondorosi *et al*. 1980).

The variability in the effectiveness of native *Rhizobium* isolates even on a single cultivar of a legume crop, gives the impression that the nitrogen-fixing ability of *Rhizobium* could be improved either by strain selection or by genetic manipulation. Since the nitrogen-fixing ability is expressed only in symbiotic association, it is not possible to find out whether the restriction on the bacterial gene expression is due to the bacterial genome or the plant.

Increased salt-tolerance of crops has therefore been a major objective of symbiotic N_2-fixation programmes for regions in Egypt where soil salinity is high and water quality is poor. Higher concentrations of NaCl cause

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marked changes in growth patterns, dry matter allocation, ion transport, water status, physiological processes and biochemical reactions of both partners of symbiotic N_2 fixation (Rai 1992; Cardovilla *et al*. 1994). Salinity affects the infection process by inhibiting root hair growth and by decreasing the number of nodules per plant and the amount of N_2 fixed per unit weight of nodules. Thus, in saline soils the yield of leguminous crops is decreased due to the lack of successful symbiosis (Hafeez *et al*., 1988). Salt tolerance in plants is a complex phenomenon that involves morphological and developmental changes, as well as physiological and biochemical processes (Greenway and Munns 1980). Survival and growth in saline environments are the result of adaptive processes, such as ion transport and compartmentation, osmotic solute synthesis and accumulation, which lead to osmotic adjustment and protein turnover for cellular repair (Rai *et al*. 1985; Munns and Termaat 1986).

High salt can directly impair rhizobia–legume early interactions during nodule formation (Singleton and Bohlool, 1984) and nodule development (El-Hamdaoui *et al*., 2003a). Moreover, several studies attributed salt-mediated inhibition of nitrogen fixing activity to a reduction of nodule respiration (Delgado *et al*., 1994; Ikeda *et al*., 1992) and to a decrease of cytosolic proteins, including leghemoglobin production (Delgado *et al*., 1993, 1994). Reduction of photosynthetic activity by salt can also reduce N_2-fixation (Georgiev and Atkias, 1993) although it does not seem to be a major factor in nitrogenase inhibition (Soussi *et al*., 1998). Velagaleti, *et al* (1990).The Fast growing *Rhizobium* strains were more salt tolerant, then the strain *Bradyrhizobium* (slow growers); however, salt tolerance in both genera is dependent upon ionic species, pH value and temperature (El sheikh, 1998).

Rapid and unambiguous identification of marker strains amongfield isolates has greatly benefited from recent advances in DNA finger printing methods based on the polymerase chain reaction (PCR). Random amplified polymorphic DNA PCR (RAPD-PCR) (Berg *et al.,* 1994; Dooley and Harrison, 1993; Williams, *et al.,* 1990), the interspersed repetitive sequences PCR (rep-PCR) (Versalovic, *et al.,* 1991 and 1994) or the finger- printing of bacterial genomes using ribosomal genes or operons (Schmidt, 1994) are now routinely used to index prokaryotes.

The SDS-PAGE of whole-cell proteins of rhizobial strains from wild legumes, exhibited protein profiles with peptide bands ranging from 5–19 bands per profile was reported by Zahran *et al*. (2003). The SDS-PAGE analysis of whole cell proteins not only helps in identifying of the rhizobial strains (Roberts *et al*., 1980; Fabiano and Arias, 1990) but also useful in the differentiation among the isolates within the same serogroup (Broughton *et al*., 1987). By conducting the SDA-PAGE analysis of four *Azorhizobium* strains isolated from stem nodules of *Sesbania rostrata* Dreyfus *et al*. (1985) concluded that all the four strains have identical protein gel electropherograms and are closely related.

The aims of this study were to: (1) isolation and characterization of natural *Rhizobium* strains from nodules of faba bean plants which were collected from different Governorate in Egypt, (2) analysis of their phenotypic, biochemical characteristics, salt tolerance, antibiotic response and nodulation test, and (3) analysis their diversity and relationships based on RAPD and SDS-PAGE patterns, to clarify the wide genetic biodiversity of the isolated *Rhizobium*.

Materials and Methods

Isolation and collection of Faba bean and Purification of Rhizobium isolates:

Eight *Rhizobium* isolates were isolated from nodules of Faba bean plants which were collected from different Governorate in Egypt, as well as; El-Zarka district and Dimaitaa city, Dimaitaa Governorate; the Kafr El-Dwar district and Moderiate El-Tahrire, El-Behara Gov.; Ismailia Gov.; Zagazig Gov. and experimental farm of Fac. Agric. Al-Azhar Univ. Nasser city, Egypt. Methods described by Vincent (1970) were used for characterization, employing yeast extract mannitol medium(YEM) broth (g/L): (0.5 g KH_2PO_4, 0.2 g $MgSO_4$. $7H_2O$, 0.1 g NaCl, 0.5 g yeast extract, 10 g mannitol and Congo red) for 48 h at 30^{o}C. The isolates were inoculated on N-free Burk's medium to check for contamination by free N_2 fixers, and characterized on the basis of morphological, biochemical and plant infection tests (Vincent 1970).

Identification of the Rhizobium isolates:

All isolates were identified according to Allen (1958); Vincent (1970) and George (2010), to distinct Rhizobia from other closely related soil microorganisms such as Agrobacteria the following test were preformed. The growth behavior on YMA medium was observed on (medium 79) Allen (1961), the Rhizobia produced good growth on this medium within 3-5 days. The Congo red test was tested of Congo red dye (0.025gm/L) into YMA medium is helpful in differentiating Rhizobia from closely related soil microorganisms such as *Agrobacterium* according to Hahn (1966). The growth behavior on PGA medium was tested which

grow poorly in peptone glucose agar medium (PGA containing 0.5% Bromocreasol purple) at 38C (Vincent, 1970), this medium was differentiated between for *Rhizobium* isolates and *Bradyrhizobium* isolates.

Nodulation test:

Seeds of Faba bean cultivar Giza 1 were obtained from Legumes Research Dep. Agronomy Research Institute, Agricultural Research Center, Giza, Egypt. The different isolates were tested their ability to nodulate on specific host plant (*Faba bean*), grown in plastic pots washed sterilized fine sand. All isolates of *Rhizobium* were grown up to 6 days at 30 ^{o}C on the liquid yeast extract mannitol medium. Seeds of the Faba bean cultivar were slurry inoculated with *Rhizobium* isolates by soaking the seeds with 100 ml bacterial solution (381×10^{7} Cfu/ml) and sown in plots. Plants were carefully uprooted after 45 days from plantation and pink reddish nodules were counted.

Biochemical identification of the Rhizobium isolates according to assimilation of different carbon and nitrogen source:

The yeast extract mannitol medium was used as a control for estimation the effect of carbon sources on the growth of *Rhizobium* isolates. Different carbon sources were used Glucose; Sorbose; Maltose; L-Arabinose; Gluconate; Citrate; Inositol; Succinate and Glycerol. They were supplemented by the rate of 1% to *Rhizobium* medium instead of Mannitol. Nitrogen sources; KNO_3; NH_4Cl; Glycine and peptone were used Thiamin and Biotin as vitamin sources were supplemented to *Rhizobium* medium compared of yeast extract. The detection of growth was observed after incubated for 5 days at 30^{o}C and recorded results as positive or negative growth

Testing of salt tolerance:

Salt tolerance was measured as the ability of the bacterial cells to grow and divide under the stress of known NaCl concentration. Test tubes (12 cm) each containing 5ml YM medium, were inoculated with a bacterial isolate and incubated at 30 ^{o}C for 48 hours. 10 µl of the grown strain was used for the inoculation of 20 ml YM medium containing of known NaCl concentration (0, 50, 100 and 200 mM) and the conical flask were incubated at 30 ^{o}C for 2, 5 and 7 days. The Shimadzu UV-VIS spectrophotometer model UV-240 was used for the measurement of growth (in turbidity units) at 600 nm where none inoculated medium was used as a blank.

Testing of antibiotic response:

The antimicrobial resistance patterns of bacterial isolates were routinely tested by the single-disk diffusion method using Muller-Hinton agar against the following antibiotics: Streptomycin (Sm, 10 µg/ml), Tetracycline (TE, 30 µg/ml), Norfloxacine (Nor, 10 µg/ml), Ampicilln (Ap, 25 µg/ml), Gentamycin (Gm, 10 µg)**,** Rifampin (RA, 5 µg Vancomycin (VA, 30 µg), Tobramycin (TOB, 10 µg), Eryhromycin (E, 15 µg) and Nalidixic acid (NA, 30 µg). Zone sizes were interpreted by using standard recommendations.

Isolation of total DNA from isolated strains:

Total DNA was isolated according to i-genomic BYF DNA extraction Mini Kit, iNtRON Biotechnology, Canda. Genetic diversity was done using three primers as showed in Table (1). The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95^{o}C for five min. 35 cycles each consists of the following: Denaturation at 95^{o}C for one min; primer annealing for two min., according to GC ratio of each primer and incubation at 72^{o}C for two min. for DNA polymerization. Then, 72^{o}C for 5 min., at the end, hold the PCR at 4^{o}C till analysis. The amplified DNA products from RAPD analysis were electrophoresed on 1.5% agarose gel and 1 X TBE buffer at consistent 100 volt. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using Gel Documentation System with UV-Transeliminator.

RAPD-Data analysis:

RAPD amplification profiles for the used eight isolates were scored as a binary data, where (1) means presence and (0) means absence of band. The distance coefficients were calculated by the following statistical

equation:

$$F= 2Nxy / (Nx + Ny)$$

Where, F is the distance coefficient in which Nx and Ny are the numbers of fragments in genotypes x and y, respectively, and Nxy is the number of fragments differed by the two genotypes (Lynch, 1990). The electrophoretic patterns of the reproducible banding patterns of each primer which produced by RAPD were chosen for analysis. Pair wise comparisons between mutants were made to calculate the Jaccard coefficient using PAST program (PAleontological Statistics Version 1.94b) adapted by Hammer *et al*., (2001). Cluster analysis was performed to produce a denderogram using UPGMA method.

Table 1: Primers used and their respective base sequences.

Primer Name	Sequence (5′ → 3′)
P-2	CAT ACC CCC GCC GTT
P-3	GTG TTG TGG TCC ACT
P-5	TGA GTG GTC TAC GTG

SDS-protein electrophoresis:

For miniscale preparations, 1.5 ml of stationary cultures (48h) were pelleted and suspended in 40µl of Laemmli Sample Buffer, 5µl of 10%SDS and 5µl of β–mercaptoethanol then boiling the mixture for 5 min and centrifugation to obtain the supernatant which contains protein fractionations. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Samples prepared by adding Protein fractionation electrophoresis was performed on 12% acrylamide gel using the apparatus manufactured by LABOCONCO. Gels were analyzed using TotalLab TL100.

Results and Discussion

The present study was designed to isolate new *Rhizobium* isolates with high nodulation rate. To achieve this goal, different strains of *Rhizobium* isolates were firstly collected, identified and tested for their nodulation rate by cultivar of *Vicia faba*.

Morphological and biochemical characteristics of the Rhizobium isolates:

The morphological cells of *Rhizobium* isolates were examined under light microscope has short rods Gram negative; nonsporeforming; motile and aerobic. The isolates produce circular, low convex to convex; mucous; and opaque white to beige colored colonies, with an entire edge and a diameter of 2–4 mm after 5–6 days of incubation at 30 C. The isolates were observed to lack the ability to absorb congored from a yeast extract mannitol mineral salts medium containing this dye, this colonies were colorless white or very faintly pink colonies. Congo red is thought to form colored colloidal complex with ions on the cell surface, the colonies absorb little dye and remain colorless or became slightly pink after 2-3 days of incubation; marked absorption by *Agrobacterium* produces bright red colonies. By prolonged incubation some Rhizobia colonies became surrounded by blue halo and eventually became totally blue due to acid production by these isolates, but in *Rhizobium leguminosarium* the prolonged incubation, up to 10 days, its colonies are still colorless (Barbara, *et al.*, 1983). The growth behaviors of the *Rhizobium* isolates on PGA medium were which grow poorly in PGA medium and change the Bromocreasol purple to yellow for all *Rhizobium* isolates.

The nodulation test:

The purified isolates of *Rhizobium* were checked for their efficiencies to nodule the roots of faba bean plants, the number of nodules per plants which showed well recognized that isolates within a rhizobia population show great variation in their symbiotic effectiveness on hosts. Data presenti in Table (2) cleared that the inoculation with eight isolates of *R. leguminosarum* produced the highest mean of nodules per plants which reached (104 nodules) for isolate RL8, while reach (71 nodules) for RL4.

Rhizobium isolates were identified according to their morphological and physiological characteristics as proposed by (Vincent, 1970) and George (2010). Results present in Table (2) revealed that the biochemical characteristics of *Rhizobium* isolates showed positive assimilation of Glucose with production of acid;

assimilate and Mannitol; Sucrose; Maltose; Gluconate; Inositol; Succinate; L-Arabinose and Glycerol, respectively, while could not assimilate Sorbose; and Citrate. On the other hand, the ability of the Rhizobium isolates were showed positive assimilation of nitrogen sources NH_4Cl; KNO_3 and Glycine but different degree. The *Rhizobium* isolates were gave good assimilation yeast extract as source of nitrogen and glycine, while, slight assimilation peptone and ammonia and nitrate. However, some of the *Rhizobium* isolates under study requirements of growth factor; Biotin and Thiamine depended of different between these isolates, which is the specific carbon source for the growth of this isolates. The eight isolates of *Rhizobium* were identified to species *Rhizobium leguminosarum* bio *viciae*. The results finding is in agreement with (Scowcroft and Gibson, 1975), who reported that the various carbon sources were examined as alternatives for Arabinose in supporting nitrogenase activity after 5 days incubation, nitrogenase activity in the cultures containing Ribose or Xylose was similar to that in the Arabinose control. With other carbon sources the nitrogenase activity was less than that with arabinose, Inositol has no effect of on activity in the presence of Mannitol. The vitamin components of medium, Thiamine, Nicotinate, and Pyridoxal, were constitutents of the medium. Biotin, Thiamine and Pantothenate are most commonly required by fast growing rhizobia (Graham, 1963). Ammonium and nitrate ions are regarded as inhibitors of nitrogenase synthesis in free living diazotrophs (Brown *et al.*, 1974).

Table 2: The Main Morphological and Biochemical Characteristics of the *Rhizobium* isolates.

Rhizobium isolates / Characters	*isolate RL1*	*Isolate RL2*	*isolate RL3*	*Isolate RL4*	*isolate RL5*	*Isolate RL6*	*Isolate RL7*	*Isolate RL8*
Morphology	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods	Short rods
Gram's reaction	G-	G-	G-	G-	G-	G-	G-	G-
Motility	+	+	+	+	+	+	+	+
Acid production from glucose	++	+	+	++	+	++	+	+
Carbon source								
Mannitol	+++	+++	+++	+++	+++	+++	+++	+++
Glucose	+++	+++	+++	+++	+++	+++	+++	+++
Sucrose	+++	+++	+++	+++	+++	+++	+++	+++
Sorbose	-	-	-	-	-	-	-	-
Maltose	+++	+++	++	+++	+++	+++	++	++
L-Arabinose	+++	+++	++	++	++	+++	+++	++
Gluconate	+	++	++	++	+	++	+	+
Citrate	-	-	-	-	-	-	-	-
Inositol	++	+++	++	++	++	++	++	++
Succinate	++	++	+	+	++	+	++	++
Glycerol	++	+++	+++	++	++	+++	++	+++
Nitrogen source								
Yeast extract	+++	+++	+++	+++	+++	+++	+++	+++
Peptone	+	+	+/-	+	+	+	++	+
Glycine	++	++	++	++	+++	++	++	++
NH_4Cl	++	++	++	++	++	++	++	++
KNO_3	+	+	++	+	+	++	+	+
Growth factor required								
Biotin	+	+	+	+/-	+/-	+	+	+/-
Thiamine	+	+	+/-	+/-	+	+	+	+/-
Nodulation test								
Mean No. of nodules/plants	96	87	84	71	91	81	97	104

Salt tolerance of isolated bacterial strains:

The effect of different concentration of NaCl on the growth of *Rhizobium* isolates may be considered as an indicator of salt tolerance. So, in order to study the effect of different of NaCl on the growth of *Rhizobium* isolates, they were grown in YEM medium supplemented with different concentration of NaCl, e.g., 50, 100 and 200 mM. The growth was measured as turbidity units at 600 nm following the incubation at 30 C for 2, 5 and 7 days.

Data in table (3) showed that the isolate No. RL7 was the superior strain for salt tolerance. The following isolate for salt tolerance was the isolate No. RL2. The higher growth rate of the *Rhizobium* isolates may be considered as an indicator of salt tolerance and subsequently, could be considered as an estimate parameter for high nodulation under the saline soil.

Table 3: Effect of different concentration of NaCl on the growth (measured as optical density) of *Rhizobium* isolates.

NaCl Conc.	C				50 mM				100 mM				200 mM			
Time (days) / Isolate Code	0	2d	5d	7d	0	2d	5d	7d	0	2d	5d	7d	0	2d	5d	7d
RL1	0.01	0.53	0.76	0.76	0.01	0.22	0.53	0.57	0.01	0.22	0.53	o.53	0.01	0.05	0.07	0.12
RL2	0.02	1.08	1.44	1.52	0.0	0.02	1.27	1.27	0.02	0.89	1.25	1.34	0.02	0.30	0.50	0.55
RL3	0.03	0.56	0.66	0.68	0.03	0.48	0.55	0.63	0.03	0.34	0.42	0.39	0.03	0.17	0.15	0.15
RL4	0.01	0.82	2.25	2.24	0.01	0.75	2.21	2.18	0.01	0.08	0.11	0.13	0.01	0.09	0.12	0.12
RL5	0.02	0.51	0.72	0.82	0.02	0.39	0.53	0.61	0.02	0.16	0.15	0.14	0.02	0.09	0.08	0.07
RL6	0.02	0.89	1.49	1.53	0.02	0.71	1.42	1.44	0.02	0.51	1.01	1.02	0.02	0.06	0.10	0.18
RL7	0.02	0.92	2.29	2.33	0.02	0.90	1.57	1.56	0.02	0.66	2.14	2.18	0.02	0.46	1.06	1.23
RL8	0.02	0.75	2.23	2.23	0.02	0.68	2.22	2.22	0.02	0.07	0.14	0.13	0.02	0.01	0.10	0.11

In previous studies, the selection of *Rhizobium* sp. strains for saline soils was made on the basis of the ability of strains to grow separately in saline yeast extract mannitol agar media (Yadav and Vyas 1971; Singleton *et al*., 1982). However, the salinity tolerance of free-living rhizobia is generally much higher than that of the host plant, and also, the tolerance of free-living *Rhizobium* sp. strains on yeast extract mannitol agar media does not correlate well with their symbiotic ability under saline conditions (Subbarao, 1984).

Antibiotic response of isolated bacterial isolates:

Table (4) presents the *Rhizobium* isolates response to 10 different types of antibiotics. Results showed that, all the *Rhizobium* isolates were resistant to AP_{25}. The isolated strains (RL3, RL4 and RL7) showed the highest level of antibiotic resistance since they were resistant to five antibiotics. On the other hand, the isolated strains (RL1, RL5, RL6 and RL8) were resistant to two antibiotics. Meanwhile, they were sensitive to the other antibiotics.

Table 4: Effect of different antibiotics on the growth of *Rhizobium* isolates.

Strain No.	Sm_{10}	TE_{30}	NOR_{10}	AP_{25}	Gm_{10}	RA_5	VA_{30}	TOB_{10}	E_{15}	NA_{30}
RL1	S	S	R	R	S	S	S	S	S	S
RL2	S	S	S	R	S	S	S	R	S	S
RL3	S	R	S	R	S	R	R	S	R	S
RL4	R	S	S	R	S	S	S	R	R	S
RL5	S	S	S	R	S	S	S	S	S	R
RL6	S	S	S	R	S	S	S	R	S	S
RL7	R	S	S	R	R	S	S	S	R	S
RL8	S	S	S	R	S	S	S	S	S	R

S, Sensitive; R, Resistant; Sm_{10}, Streptomycin(10 µg/ml); TE_{30}, Tetracycline(30 µg/ml); NOR_{10}, Norfloxacine(10 µg/ml); AP_{25}, Ampicilln (25 µg/ml); Gm_{10}, Gentamycin(10 µg/ml); RA_5, Rifampin(5 µg/ml); VA_{30}, Vancomycin(30 µg/ml); TOB_{10}, Tobramycin(10 µg/ml); E_{15}, Eryhromycin(15 µg/ml) and NA_{30}, Nalidixic acid(30 µg/ml).

Polymorphism of Rhizobium by RAPD markers:

To evaluate the degree of genetic diversity and calculating the genetic distances of Rhizobia based on the DNA nucleotide sequence using RAPD, three random primers were used to identify the genetic variability and genetic relationship among the eight genotypes. High level of polymorphism was observed among the studied genotypes. All primers were successfully generated reproducible polymorphic bands. The polymorphic patterns of the scorable five RAPD primers among the studied genotypes were shown in Table (5) and Fig's. (1-3).

The fragment patterns of RAPD exhibited a total of 150 amplified fragments. All of the total recorded bands were polymorphic. The highest band No., were scored by primer P-2 (63 bands) followed by primer P-3 gave 47 bands. However, primer P-5 scored the lowest band No. (47 bands), among the three primers.

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Table 5: Numbers and types of the amplified DNA bands as well as the total polymorphism percentage generated by RAPD primers.

Primer	Total bands	Monomorphic bands	Polymorphic bands	Polymorphic percentage
P-2	63	0	63	100
P-3	47	0	47	100
P-5	45	0	45	100
Total	150	0	150	100

Regarding the phylogenetic tree and similarity index based on Jacard coefficient the isolate RL2 was grouped with RL6 in one cluster with 40% similarity. However, isolate RL4 was highly diverged with lower similarity about 10 % with the other rhizobia strains as shown in Table (6) and Fig. (4).

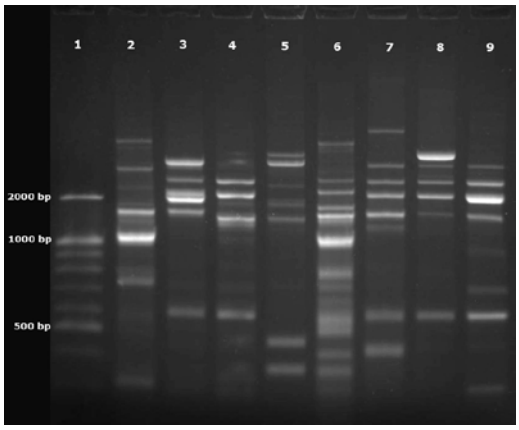

Fig. 1: Photograph of DNA amplified banding patterns based on RAPD for eight wild type strains of *Rhizobium leguminosarum* (lane 2 to 9) and 100 bp ladder DNA marker (lane 1) using primer No.2(P2). wild type strains sequence as follows: (lane 2 to 9), RL 1, RL 2, RL 3, RL 4, RL 5, RL 6, RL 7 and RL 8.

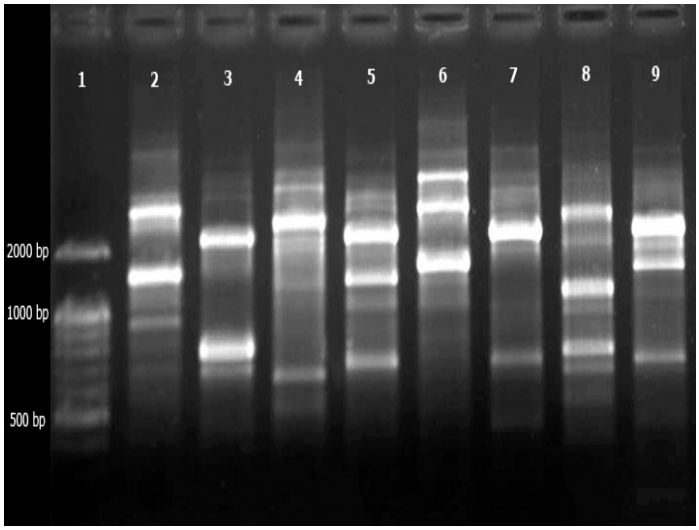

Fig. 2: Photograph of DNA amplified banding patterns based on RAPD for eight wild type strains of *Rhizobium leguminosarum* (lane 2 to 9) and 100 bp ladder DNA marker (lane 1) using primer No.3(P3). wild type strains sequence as follows: (lane 2 to 9), RL 1, RL 2, RL 3, RL 4, RL 5, RL 6, RL 7 and RL 8.

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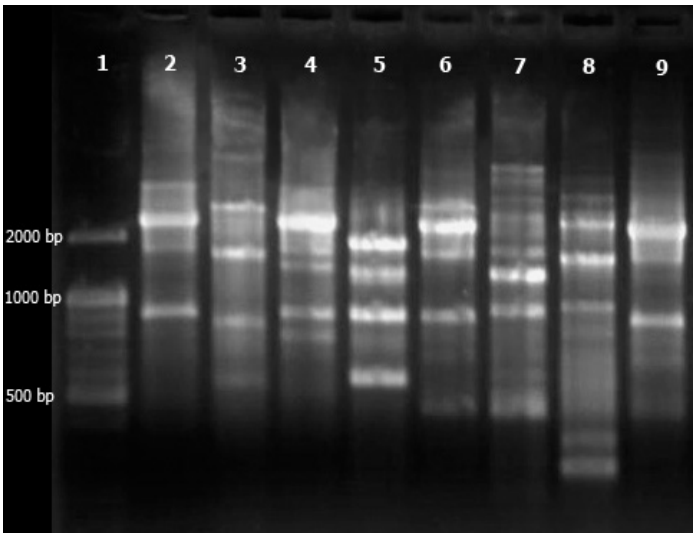
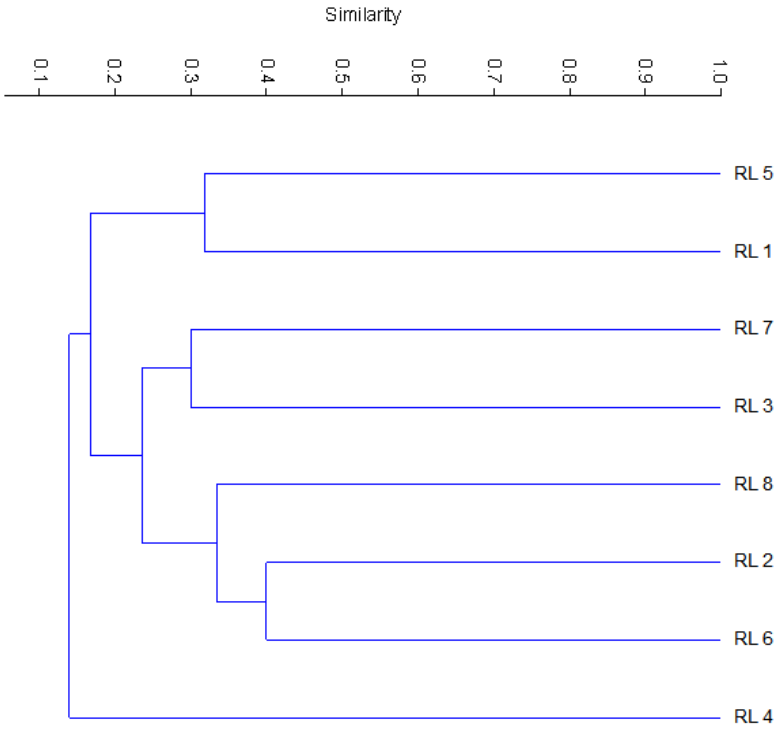


Fig. 3: Photograph of DNA amplified banding patterns based on RAPD for eight wild type strains of *Rhizobium leguminosarum* (lane 2 to 9) and 100 bp ladder DNA marker (lane 1) using primer No.5(P5). wild type strains sequence as follows: (lane 2 to 9), RL 1, RL 2, RL 3, RL 4, RL 5, RL 6, RL 7 and RL 8.



Fig, 4: RAPD based dendogram of the eight rhizobia genotypes constructed using UPGMA method based on Jacard coefficient.

It is worthy to mention that, diversity is important for genetic characterization with different nitrogen fixing capacities. Using diversity analysis novelties of new *Rhizobium* sp with high nitrogen fixing potential under the saline soil could be revealed which have not been identified ever before. Similar results were concluded in previous study concerning the characterization of *Sinorhizobium meliloti* indigenous strains (Bradic *et al.*, 2003), as well as *Bradyrhizobium japonicum* (Sikora *et al.*, 2002). This finding is in agreement with Blazinkov *et al.* (2007), who reported that PCR performed was less reliable for differentiating among different strains of *Rhizobium leguminosarum bv. viciae* isolated from different locations. These data are in full harmony with the previous studies in which PCR genomic fingerprinting is an adequate technique for differentiating *Rhizobium* strains (Zribi *et al.*, 2004 and Ogutcu *et al.*, 2009) and many other closely related sub-species or strains and/or for determining phylogenetic relationship (Pinto *et al.*, 2007).

Table 6: Similarity matrix among the eight rhizobia genotypes based on RAPD analysis based on Jacard coefficient.

Strains	RL 1	RL 2	RL 3	RL 4	RL 5	RL 6	RL 7	RL 8
RL 1	1							
RL 2	0.045	1,000						
RL 3	0.050	0.200	1,000					
RL 4	0.048	0.250	0.095	1,000				
RL 5	0.318	0.231	0.304	0.107	1,000			
RL 6	0.087	0.400	0.182	0.125	0.214	1,000		
RL 7	0.042	0.217	0.300	0.125	0.133	0.250	1,000	
RL 8	0.250	0.353	0.313	0.222	0.318	0.316	0.250	1

Characterization of Rhizobium isolates by SDS-PAGE:

SDS-PAGE protein analysis was carried out for eight *Rhizobium* isolates to identify and classify these strains. *Rhizobium* isolates from different Egyptian locations essentially grouped in two separate protein gel electrophoretic clusters (I and II). The results are presented as a similarity dendrogram in Fig. 5. Whereas, the cluster I divided to two subgroups (A and B). The subgroup A consisted of isolates RL5, RL8, RL6 and RL7. However the subgroup B contained only the strain RL1. On the other hand, the cluster II comprised three strains (RL2, RL4 and RL3). The similarity matrix between *Rhizobium* isolates varied from 0.120-0.889 (Table 7). The highest similarity coefficient (0.889) was detected between isolates RL6 and RL7. However, the lowest similarity was computed between isolates RL3 and RL5 which presented in to two different clusters. SDS-PAGE a revealed powerful characterization method and classification of *Rhizobium* isolates under study. That is agreement to Fabriano and Arias, 1990 and Irisarri *et al*., 1996 who reported that the whole cell soluble protein pattern (SDSPAGE) has been used not only to identify rhizobial strains, but also to differentiate among isolates within the same serogroup (Broughton *et al.*, 1987). Moreover, we can report that the high diversity appeared between the isolated *Rhizobium* strains by SDS-PAGE could be produced in genetic of these strains by environmental affects like pesticides and abiotic stresses. Our results demonstrated by others to often result from prevailing site-specific environmental variables imposing general genetic adaptations on soil rhizobia. Moreover the authers suggested that a relatively rapid rate of genetic change within the rhizobia populations and some evidence that soil texture may influence genetic diversity of the bacterium in the region (Farooq and Vessey, 2009). Consequently, as strains become adapted to a given environment, they also become distinct from strains in other environments resulting in increased diversity between sites (Mothapo, *et al*., 2013). It seems to be the horizontal gene transfer contributed in genetic modifications of *Rhizobium* bacteria. The field inoculation with compatible rhizobia can increase the genetic diversity of resident rhizobia (de Fatima *et al*., 2007) likely by presenting new strains of rhizobial bacteria into the environment with the potential of genetic material transferring between strains. Several studies have suggested horizontal gene transfer that may also potentially occur between existing strains in fields to be a major factor contributing to rhizobial diversity (Zhang *et al*., 2001, Barcellos *et al*., 2007 and Aoki *et al*., 2010).

Table 7: Similarity matrix among the eight rhizobia genotypes as estimated using protein banding pattern based on Jacard's coefficient.

strains	RL1	RL2	RL3	RL4	RL5	RL6	RL7	RL8
RL1	1							
RL2	0,185	1,000						
RL3	0,292	0,438	1,000					
RL4	0,259	0,444	0,389	1,000				
RL5	0,370	0,160	0,120	0,148	1,000			
RL6	0,286	0,400	0,421	0,429	0,375	1,000		
RL7	0,357	0,364	0,381	0,455	0,400	0,889	1,000	
RL8	0,414	0,269	0,185	0,207	0,652	0,423	0,500	1

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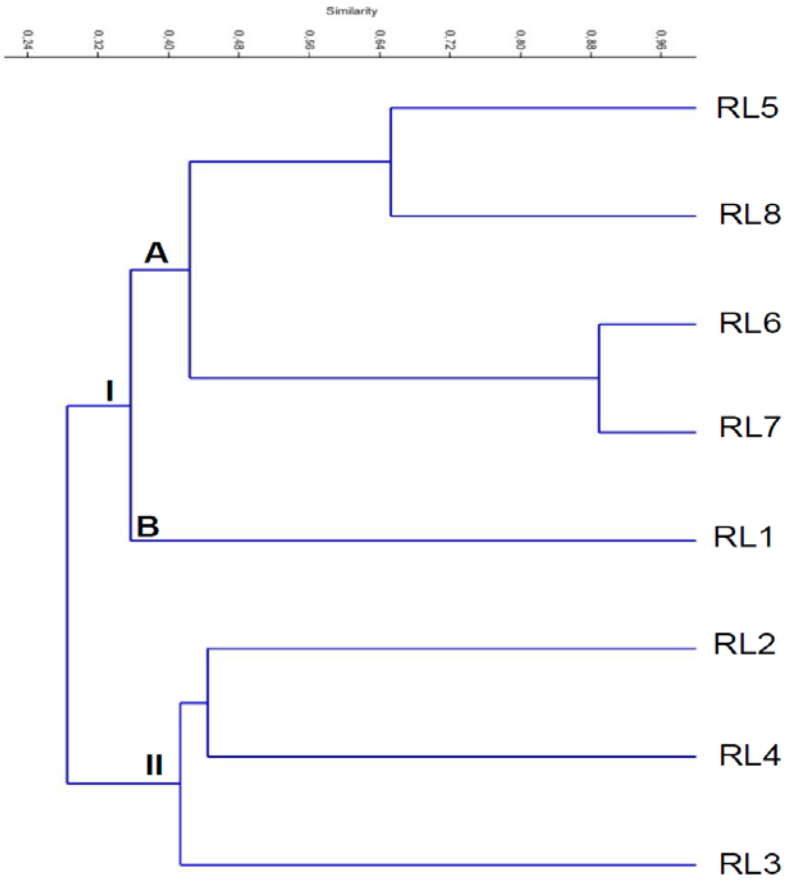

Fig. 5: Dendrogram showing the similarity among the electrophoretic protein patterns (SDS-PAGE) of eight *Rhizobium* strains based on Jaccard's similarity coefficient values which were grouped by the UPGMA method.

Salt-tolerant affects on R. leguminosarium protein banding pattern:

Improved isolates of *Rhizobium* for saline lands must have salt resistance mechanisms to enable them to grow and survive in these areas. To study the resistance of eight isolated *Rhizobium* isolates, we treated them with different concentrations of NaCl. The growth in the presence of NaCl generally altered the whole-cell protein patterns in SDS-PAGE in *Rhizobium* isolates. Some bands disappeared and new bands appeared in treatment by salt (Fig. 6). Under salt stress, seven bands 190, 125, 93, 63 and 16 KDa appeared in strains RL2 (200 mM), RL4 (50 mM, 100 mM and 200 mM), RL2 (100 mM), RL2 (200 mM) and RL4 (200 mM), respectively. Two pair bands with molecular weight 70 and 67 KDa appeared in isolates RL2 (200 mM) and RL4 (50 mM, 100 mM and 200 mM). Furthermore, the 90 and 16 KDa bands appeared in the control of isolates RL3 and RL1, respectively and they disappeared in the treatment with 50 mM of NaCl at the same isolates. The 65 KDa disappeared all salt stress treatments in isolates RL4. But the other strains RL5, RL6, RL7 and RL8 did not exhibit any response to salt stress. The genetic diversity of all rhizobia isolates under study can play a role for their resistance or sensitive to salt stress. It could be salt induces genes related salt-resistance and/or sensitive. Understanding *Rhizobium* ability to tolerate salt stress has been to identify stress-induced changes of individual proteins (Natarajan *et al*. 1996) under the assumption that stress adaptation results from alterations in gene expression. These findings agree with the protein profile showed major alterations at salinity levels (Soussi, *et al*., 2001 and Unni and Rao 2001). Pereira *et al*., 2006, concluded that there is a relationship between *Rhizobium*'s resistance and the alterations in protein pattern.

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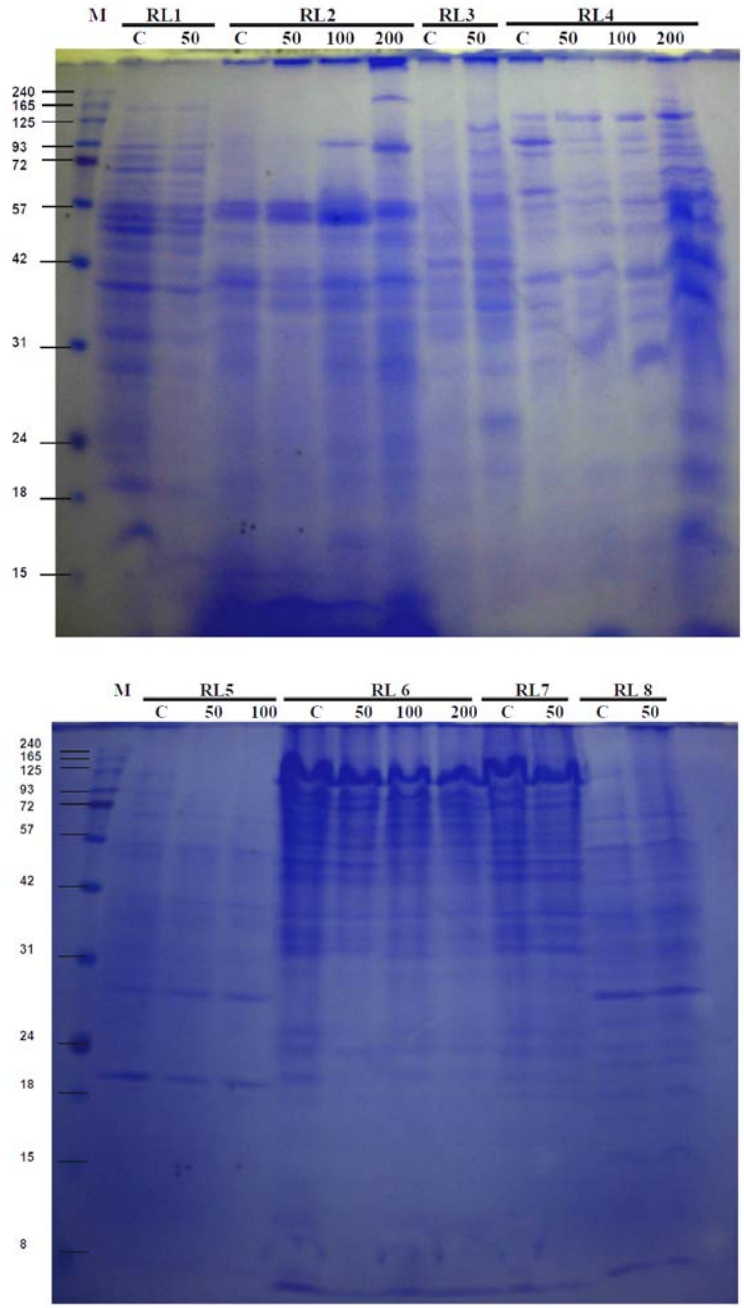

Fig. 6: Protein banding pattern for strains RL1, RL2, RL3, L4, RL5, RL6, RL7 and RL8 under control (C) and different concentrations of salt stress. M: Standard marker.

In conclusion, the differentiations appeared between the different Rhizobium isolates can refer to the plasticity of these organisms to the environmental changes which can influence in the Rhizobium genome and epigenetic stats that are essential for efficient nodulation.

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