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Diversity of Rhizobia Nodulating Faba Bean (*Vicia faba*) Growing in Egypt

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Abstract

The aim of this work was to characterize and to describe the diversity and phylogeny of rhizobial bacteria associated to nodules of faba bean in four different geographical regions of north Egypt. Eight rhizobial isolates from healthy faba bean roots were isolated. They were identified as Rhizobium leguminosarum morphologically. They showed sensitivity to Kanamycin, Neomycin and Sulphemethooxazole antibiotics. Mannitol was the best source of carbon source for their growth. However, two isolates Rl. 2 and Rl. 10 indicated better tolerance to high NaCl concentrations than the other isolates and their plasmid profiles contained additional large plasmid with molecular weight about 23 kb. A relationship between salt tolerance and extra plasmid was suggested. Analysis of similarity among rhizobial isolates by using the RAPD-PCR technique showed a high level of genetic polymorphism, grouping the rhizobial isolates into two different clusters. These clusters reflexed the similarity among genotype of isolates independent of their geographic locations. 16S rDNA sequences of three representing isolates were determined and were aligned and compared with the 16S rDNA sequences of other members of the family Rhizobiaceae available in the Gene Bank database. The obtained dendogram indicated that the isolates belonging to Rhizobium leguminosarum biovar viciaen.

Keywords: Rhizobium leguminosarum biovar viciae; Salt tolerance; Plasmid profile; RAPD diversity; 16S rDNA sequencing

Introduction

Bacteria belonging to the genera Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium are generally called rhizobia [1]. Rhizobia are best known for forming nitrogen-fixing nodules in legumes, they are soil bacteria, endophytes and also successful rhizosphere colonizers of legumes and non-legumes [2]. The ancestral lifestyle of rhizobia was probably rhizospheric, and the degradative capabilities of rhizospheric bacteria have been highlighted [3]. It is well known that crop plants belonging to the genera Vicia, Lathyrus, Pisum and Lens are effectively nodulated by isolates of Rhizobium leguminosarum biovar viciae [4]. The symbiotic interaction between rhizobia and legumes is initiated by an exchange of complex molecular signals that confer host-specificity. Legume roots and seeds exude a number of organic compounds (e.g. flavonoids), which differ between species [5]. Rhizobia respond to these by one or more of the constitutive genes called Nod Factors [6]. Salinity seriously conisolatescrop yield in irrigated agriculture throughout the world. Also, salinity is a serious threat to agriculture in arid and semi-arid regions. Nearly 10% of the world's land surface can be classified as endangered by salinity. Most of these areas are in the tropics and Mediterranean regions [7]. Increasing salt concentration may have detrimental effects on soil microbes because of direct toxicity as well as through osmotic stress [8]. Chloride and sulfate salts are predominant in saline soils. Plant growth, nutrient uptake and metabolism, and protein synthesis are all thought to be adversely affected by salt stress conditions [9]. All these factors lead to unsuccessful legume nodulation [10]. However, the development and increased availability of molecular biology techniques have made it possible to obtain information regarding the genomic organization and diversity of rhizobia populations in different soils [11]. Most information on the role of plasmids in the ecology of rhizobia in soil has been obtained using rhizobial derivatives lacking a single plasmid or more [12]. Genomic DNA fingerprinting using Random Amplification of Polymorphic DNA (RAPD) has been found to be useful in differentiating between very closely related bacteria. The RAPD technique is a Polymerase Chain Reaction (PCR) based assay that was developed to detect polymorphisms in genomic DNA. Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identifying Rhizobium isolates [13]. This study have mainly focused on the assessment of rhizobial diversity and characterization of native rhizobial isolates from different areas in north Egypt, compared them for their potential use in salinity soil by a polyphasic approach including the evaluation of biochemical properties as well as molecular characteristics.

Materials and Methods

Collection of nodules

Naturally occurring root nodules from healthy faba bean (Vicia faba) plants were collected at several locations within Egypt as follows; Rl. 1, Rl. 8 and Rl. 10 from Khafer El-Shekh., Rl. 2 from El-Dakahlya., Rl. 3 from El-Sharkya., Rl. 4, Rl. 5 and Rl. 6 from Dameitta. The collected nodules were excised from roots, washed with water and stored till isolation.

Bacterial isolation

Root nodules were surface sterilized with 70% ethanol for five min and exhaustively washed in sterile distilled water. Afterwards, they were crushed and bacteria were isolated on Yeast Extract-Mannitol (YEM) agar plates containing 250 µg cycloheximide/ml [14]. Differentiating of Rhizobia from closely related soil microorganisms such as Agrobacterium was done according to Hahn (1966) [19].

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Isolate Code	Site
Rl 1	Khafer El-Shekh
Rl 2	El-Dakahlya
Rl 3	El-Sharkya
Rl 4	Dameitta
Rl 5	Dameitta
Rl 6	Damietta
Rl 8	Khafer El-Shekh
Rl 10	Khafer El-Shekh

Table 1 Sites from where faba bean root nodulating rhizobia were isolated.

Isolates were grown at 28°C, individual colonies were selected and the purity of the colonies was checked by repeated streaking of single colonies on YEM medium and by microscopy examination. Eight bacterial isolates representing the different geographical area showing characteristics corresponding to rhizobia were selected for subsequent analysis (Table 1).

Morphological and growth characteristics of bacteria

The nodule bacteria were examined for Gram reaction, structure of colonies, their color, consistency, and presence or absence of gummy substances [16]. The active movement of bacteria was assessed on 0.3 % YEM agar medium by their capacity to migrate away from the spot of inoculation.

Intrinsic antibiotic resistance

The intrinsic antibiotic resistance was evaluated according to Eaglesham's technique on YEM agar medium supplemented with one of the following antibiotics at the indicated concentrations (g ml-1): Aztreonam 30, Gentamicin 120, Ceftriaxone 30, Cefoxitin 30, Imipenem 10, Mafenide 10, Meropenem 10, Nopeloxacin 10, Kanamycin 5, Sulphemethooxazole 25, Vancomycin 5, Bagitracin 10, Neomycin 30, Amikacin 30 and Oxacillin 5 [17].

Utilization of carbon sources

Assimilation of different substrates as a sole carbon sources was tested on BS agar medium where mannitol was replaced by other carbon compound at the final concentration of 1 % (w/v). The following carbon substrates were tested: pectin, manitol, sorbitol, sucrose, galactose and lactose [14].

Growth in saline liquid media

The effect of different concentrations of NaCl on the growth of Rhizobial isolates was achieved by measurement of Optical Density (OD)420 after 48 hours of isolates growth in liquid culture [14]. The concentrations of different NaCl were 50 mM, 100 mM, 200 mM and 300 mM.

Plasmid extraction

Plasmid extraction from isolates was performed according to manufacturers' directions of Gene JET ™ plasmid mini-prep kit (Fermentas). The extracted plasmids were digested with BamHI enzyme, separated in 2% Agarose gel, stained with Ethidium bromide and photographed.

RAPD analysis

Total genomic DNA was extracted from Rhizobial isolates and RAPD analysis was performed according to using six primers; OP-A1, OP-A2, OP-A11, OP-B6, OP-B9, and OP-H10 [18]. The PCR products were analyzed by electrophoresis in 2% agarose gel and the RAPD patterns were visualized and photographed. The size of each produced band was estimated by computer program using a standard curve that was generated using DNA ladder and Microsoft excel 2010 (Microsoft corporation, USA). The presence and absence of alleles for each RAPD marker were recorded for all isolates and converted into a genetic matrix. Genetic similarities between two isolates were computed based on Gower. A cluster diagram was constructed based on these similarities by the UPGMA method to develop a dendogram. The similarity matrix and dendogram analysis were computed using Numerical Taxonomy and Multivariate Analysis system, using computer program NTSYS-pc Version 2.1 [19].

16S rDNA gene amplification

The 16S rDNA gene from the genomic DNA of each strain was amplified with the primers rD1 (3'-AAGGAGGTGATCCAGCC-5') and fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'). These primers were able to amplify almost the full-length of 16S rDNA sequence in many bacterial genera [20]. 2X PCR Master Mix (Fermentas®, Lithuania) was used for PCR reactions. Each reaction contains all necessary reagents (dNTPs 200 nm of each and 0.6 unit of Taq DNA polymerase) except primers and DNA template for performing 25 µl reaction. 50 ng of genomic DNA and 10 pmol of each primer were added and conditions used for 16S rDNA gene amplification were according to [20]. The presence of PCR products was ascertained by agarose (1% w/v) gel electrophoresis at 100 V for 1 h.

16S rDNA sequencing

A single DNA fragment of about 1044 bp representing the 16S rDNA gene was amplified in all isolates. This fragment was purified from three salt tolerant isolates (Rl1, Rl2 and Rl10) using QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA) and sequenced using an Applied Biosystems model 373A DNA sequencer (Sigma Co., Hamburg City, Germany). The sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, WI). BLAST searches were conducted using the NCBI server of http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The 16S rDNA sequences for the Egyptian faba bean-nodulating Rhizobium isolates Rl1, Rl2 and Rl10 were deposited in Gene Bank under the accession numbers JQ303080, JQ303081and JQ303082 respectively..

Sequence analysis of 16 S gene

The nucleotide sequences of 16 S genes of isolates Rl1, Rl2 and Rl10 were aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.10. The forward and reverse sequences were checked and edited manually when needed. Then, a consensus sequence was generated from each alignment made. The sequences were then compared with the sequences deposited in GenBank database using Basic Local Alignment Search Tool (BLAST), where a nucleotide blast program was chosen. The phylogeny tree of these sequences was draw using (MEGA) programmed version 5.10.

Results

The eight bacterial isolates representing the different geographical area; Khafer El-Shekh, El-Dakahlya, El-Sharkya and Dameitta (Table 1) were characterized and also, they were molecularly analyzed.

Characterization of rhizobial isolates

The eight isolates of Rhizobia were isolated from healthy Vicia faba nodules collected from four different geographic areas from

north Egypt. These eight isolates were tested by Congo red technique to ensure that all isolates were Rhizobia and did not contaminate with Agrobacterium. The growth characteristics and cell morphology of the isolates of faba bean rhizobia were examined microscopically to insure their purity and indicate probable differences among them. The tested isolates were found to be microscopically similar and showed normal cell morphology and were identified as Rhizobium leguminosarium according to the following examinations. Firstly, gram stained rhizobial cells developed after 72 hours on Yeast Extract Mannitol agar (YEM) showed negative reaction. Secondly, isolates were found to be motile in hanging drop preparations from three days old YEM broth cultures. Thirdly, on YEM agar slant, the growth was generally moist, whitish, smooth and gummy.

Results presented in (Table 2) indicated different antibiotic resistance patterns among the eight rhizobial strains. All isolates (100%) were resistant to Cefoxitin, 75% to Meropenem, 62.5% to Ceftriaxone, 50% to Aztreonam and Bagitracin, 37.5% to Impipenem and Mafenide, 25% to Vancomycin and Oxacillin, 12.5% to Centamicin, Sulphemethooxazole and Nopeloxacin. Finally, all isolates were sensitive to Neomycin and Amikacin.

At the same time, isolates abilities to utilize different carbon sources as a sole carbon source were investigated. Six sole carbon sources were tested (Table 3). Generally, results indicated positive spectrophotometer measurements toward all substances tested (lactose, Mannitol, Sucrose, Sorbitol, Galactose and Pectin) as hard carbon source. This means that the isolates were able to solubilize the given compounds. Mannitol showed the highest measurements where the values ranged from 1.76 to1.95. It was the best source of carbon was assimilated by all isolates. However, Pectin as hard carbon (non-sugar source) showed very low measurements range (0.08 to 1.14). Finally, within the five sugar sources used, sucrose was the least metabolized, its measurements ranged from 0.93 to 1.53.

Finally, the effect of NaCl on the growth of rhizobial isolates was examined. The obtained data was presented in (Table 4). The effects of NaCl concentrations were significant in the all cases of 48 hours

Isolates	Antibiotics resistance														
	Atm	Gm	Cro	Fox	Ipm	Ma	Mem	Nor	Km	Smx	Va	Bac	N	Ak	Ox
RI 1	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
RI 2	+	+	-	+	+	-	+	-	-	-	-	+	-	-	+
RI 3	+	-	+	+	-	+	+	-	-	+	+	+	-	-	-
RI 4	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-
RI 5	-	-	+	+	-	+	+	-	-	-	+	-	-	-	+
RI 6	+	-	-	+	+	-	+	-	-	-	-	+	-	-	-
RI 8	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
RI 10	+	-	+	+	-	+	+	-	-	-	-	+	-	-	-

Atm = aztreonam, Gm = gentamicin, Cro = ceftriaxone, Fox = cefoxitin, Ipm.= imipenem, Ma = mafenide, Mem = meropenem, Nor = nopeloxacin, Km = kanamycin, Smx = sulphemethoxazole, Va = vancomycin, Bac = bacitracin, N = Neomycin, Ak = amikacin, Ox = oxacillin, (+) = resistant and (-) = sensitive

Table 2 Intrinsic antibiotic sensitivity/resistance of rhizobial isolates.

Isolate	Carbon Source					
	Pec	Man	Sor	Suc	Gal	Lac
RI 1	1.02^{b}	1.92^{a}	1.21^{de}	1.08^{e}	1.09^{f}	1.04^{d}
RI 2	1.11^{a}	1.95^{a}	1.33^{d}	0.93^{d}	1.29^{d}	0.78^{f}
RI 3	0.9^{b}	1.95^{a}	1.27^{d}	1.02^{e}	1.17^{e}	0.91^{e}
RI 4	0.08^{e}	1.92^{a}	1.53^{c}	1.38^{b}	1.42^{c}	1.71^{a}
RI 5	0.67^{c}	1.93^{a}	1.93^{a}	1.34^{bc}	1.61^{b}	1.64^{b}
RI 6	0.32^{d}	1.83^{b}	1.56^{c}	1.53^{a}	1.88^{a}	1.45^{c}
RI 8	1.03^{b}	1.77^{c}	1.78^{b}	1.33^{c}	1.86^{a}	1.74^{a}
RI 10	1.14^{a}	1.76^{c}	1.16^{e}	0.86^{f}	1.09^{f}	1.04^{e}

Pec. = pectin, Man. = mannitol , Sor. = sorbitol, Suc. = sucrose, Gal. = galactose, and Lac. = lactose.

Means within a column followed by the same letter is not significantly different at the p = 0.05 level according to the least significant difference test (Snedecor and Cochran, 1967).

Table 3 Growth of rhizobial isolates measured as $(OD)_{420}$ at different carbon source.

Isolate	NaCl concentrations				
	0 mM	50 mM	100 mM	200 mM	300 mM
RI 1	1.71^{c}	1.63^{c}	1.61^{c}	1.55^{b}	0.82^{b}
RI 2	1.99^{a}	1.73^{b}	1.71^{b}	1.62^{a}	0.94^{a}
RI 3	1.75^{bc}	1.73^{b}	1.63^{c}	1.53^{bc}	0.60^{c}
RI 4	1.76^{b}	1.78^{a}	1.61^{c}	1.66^{a}	0.56^{c}
RI 5	1.78^{b}	1.65^{c}	1.54^{d}	1.52^{c}	0.47^{d}
RI 6	1.67^{c}	1.72^{b}	$1.58c^{d}$	1.50^{c}	0.32^{e}
RI 8	1.76^{b}	1.52^{d}	1.58^{cd}	1.58^{b}	0.56^{c}
RI 10	1.74^{bc}	1.78^{a}	1.76^{a}	1.64^{a}	0.96^{a}

Means within a column followed by the same letter is not significantly different at the p = 0.05 level according to the least significant difference test (Snedecor and Cochran, 1967).

Table 4 Growth of rhizobial isolates measured as $(OD)_{420}$ at different NaCl concentrations.

incubation period. Generally, results showed moderate effects of NaCl at all concentrations where (OD)420 values ranged from 1.99 with Rl.2 at 0 mM NaCl to 0.32 with Rl. 8 at 300 mM NaCl. However, Rl 2 and Rl 10 showed highest growth values with all salt concentrations used. Therefore, they were considered as the best isolate that tolerate high NaCl concentrations with high turbidity values comparing with other isolates.

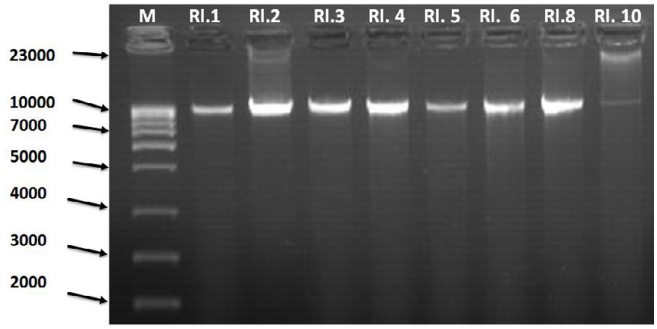

Figure 1: Plasmid pattern of eight rhizobial isolates separating in 2 % Agarose gel.

Molecular characterization of rhizobial isolates

Rhizobia usually contain large plasmids which can comprise 25 % or more of its total DNA and its molecular weight usually, ranged between 100 to 300 kilo base pairs. In order to study the relationship between salt tolerance of rhizobial isolates and plasmid content, plasmids were isolated and digested with BamHI enzyme. Plasmid profile of rhizobial isolates were presented in (Figure 1). Results showed variation in molecular weight of plasmids. All isolates contained a 10 kb plasmid and two isolates Rl. 2 and Rl. 10 contained additional large plasmid with molecular weight about 23 kb. Therefore, a probable relationship between salt tolerance and the indicated 23 kb plasmid was suggested.

Genetic diversity of tested rhizoibal isolates

The genetic diversity among eight isolates representing the indigenous population of rhizobia was investigated using RAPD-PCR technique. Genetic polymorphism among the isolates was determined using genomic DNA from the eight strains. A total of 83 fragments were generated (Table 5). Some primers were very successful in generating more polymorphism than others. The average number of fragments per primer was 13.83, the average polymorphic fragments were 10.5 and the average polymorphic fragment percentages were 75.12%. (Figure 2) showed DNA profiles produced by the six random primers and the fragment sizes ranged from 50 – 1520 bp. Accordingly, the scored

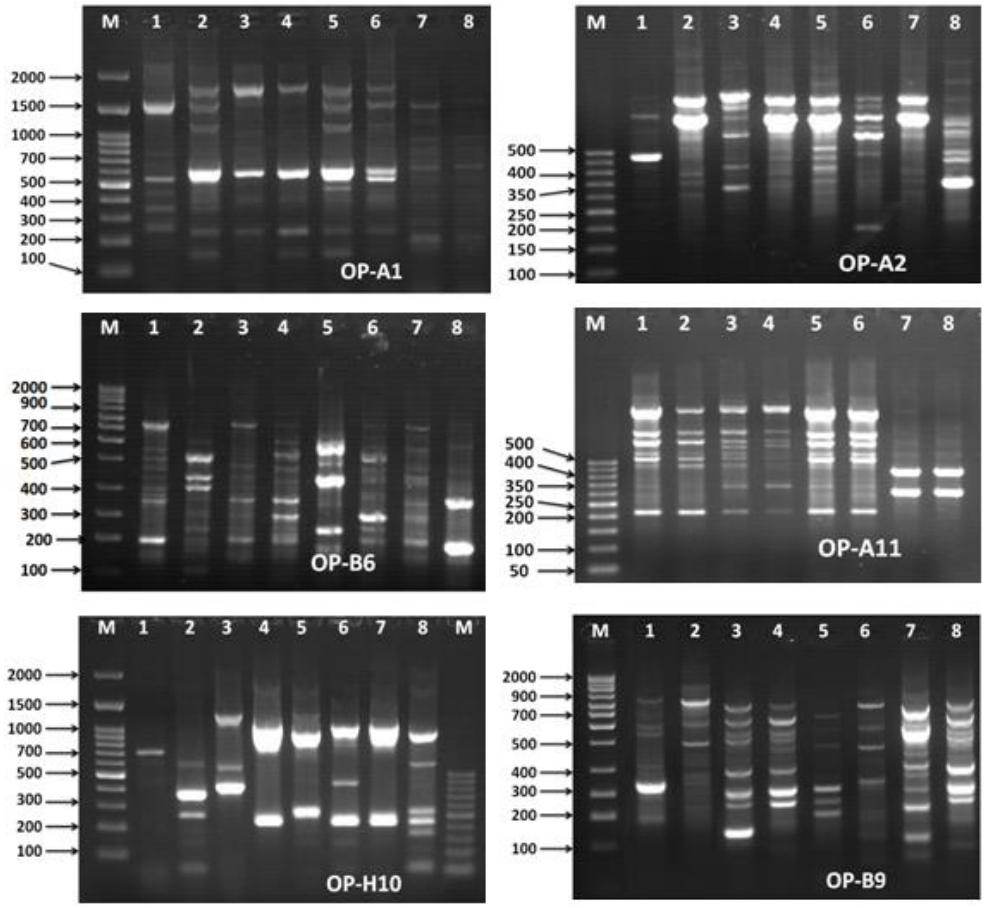

Figure 2: RAPD profile of rhizobial isolates generated with different primers; M = Marker (100 bp DNA ladder); 1 = Rl. 1, 2 = Rl. 2, 3 = Rl. 3, 4 = Rl. 4, 5 = Rl. 5, 6 = Rl. 6, 7 = Rl. 8 and 8 = Rl. 10.

Primer	Total No of fragments	No. of Monomorphic fragments	No. of Polymorphic fragments	% Monomorphic fragments	% Polymorphic fragments
OPA-01	14	4	10	28.6	71.4
OPA-02	15	5	10	33.3	66.7
OPA-11	12	6	6	50.0	50.0
OPB-06	13	3	10	23.1	76.9
OPB-09	14	2	12	14.3	85.7
OPH- 10	15	0	15	00.0	100
Average	13.83	3.33	10.5	24.88	75.12

Table 5 Primers used in RAPD analysis with eight strains of Rhizobium leguminosarum biovar viciae under-study, total number of fragments detected by each primer and number of polymorphic fragments.

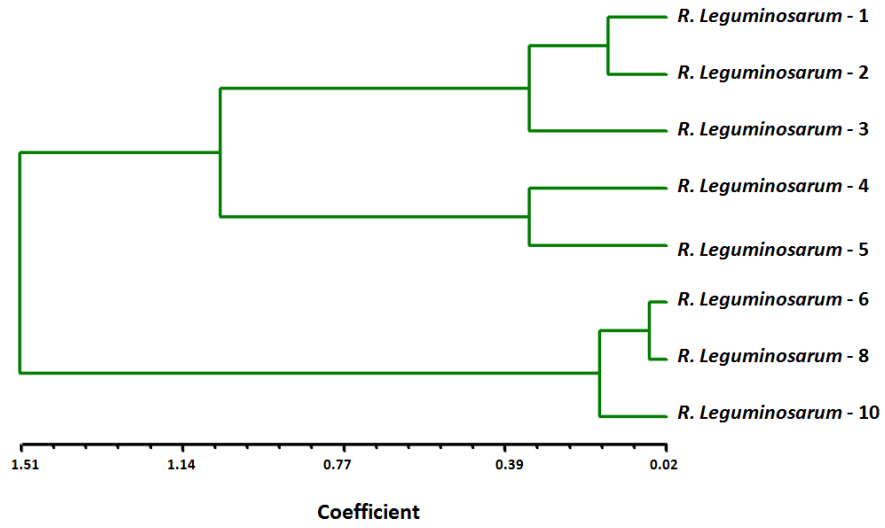

Figure 3: Dendogram based on RAPD markers developed for eight isolates of Rhizobium leguminosarum biovar viciae using Gower coefficient (Gower, 1971).

polymorphic fragments were analyzed by NTSYS-PC Statistical Package (version 2.1) software program, using Gower coefficient (Gower, 1971). Dendogram developed by the RAPD markers was presented in (Figure 3). It separated the eight isolates into two clusters Rl 1, Rl 2, Rl 3, Rl 4 and Rl 5 belong to the same cluster while Rl 6, Rl 8 and Rl 10 belong to the other cluster. These clusters were based on similarity level which was a reflex of the similarity in genotype independent of their geographic locations. For example, within the first cluster Rl 4 and Rl 5 isolates were isolated from almost the same location and they were only related by a similarity value of 77 % in the dendogram. On the other hand, Rl 6 and Rl 8 isolates were very similar in the dendogram (about 85% similarity) and they were isolated from different locations. While isolates Rl 2 and Rl 10 were quite different with a low similarity of about 47%. Therefore, RAPD analysis seems to be one of the powerful tools for detecting polymorphism and could discriminate among all tested isolates on the genetic level.

16S rDNA sequence analysis

The 16S rDNAs of three isolates (Rl 1, Rl 2 and Rl 10), which included the two most salt tolerant isolates, were amplified. The stringency conditions of PCR allowed the amplification of a single fragment of about 1044 bp for all the isolates tested. The 16S rDNA sequences of the following isolates representing the three identified Rhizobium genotypes were determined: Rl. 1, Rl. 2 and Rl. 10. These sequences were aligned and compared with the 16S rDNA sequences of other members of the family Rhizobiaceae available in the Gene Bank database. (Figure 4) showed a dendogram which demonstrated the phylogenetic relationships of these unclassified rhizobia and the previously named species of Rhizobiaceae, and these isolates are deposited in Gene Bank under the accession numbers JQ303080, JQ303081and JQ303082. Phylogenetic tree based on 16S rDNA sequences showed the position of the novel species. The bacteria isolated showed a high diversity at the genus, species, and strain level regardless of the geographical origin of the host plant. Nucleotide sequences of 16S rRNA genes obtained in this research and from the Gen Bank database were aligned, sequence similarities were calculated and a phylogenetic tree generated, bootstrapped with 1000 subsamples and visualized as described. Abbreviations, an extended tree including a wider range of reference sequences is available as supplementary material in NBCI data base.

Discussion

Rhizobia are special bacteria that can live in the soil or in nodules formed on the roots of legumes. In root nodules, they form a symbiotic association with the legume, obtaining nutrients from the plant and producing nitrogen in a process called biological nitrogen fixation [5]. Several diagnostic tests were performed to compare the eight rhizobial isolates. Our results were broadly classified the native isolates as fast growing based on their growth on YEM media. The rhizobial isolates were shaped like short rods, as seen under the microscope. They measure 0.5 to 0.9 µm wide and 1.2 to 3.0 µm long (data not shown). Like most living things, they need a supply of air to live.

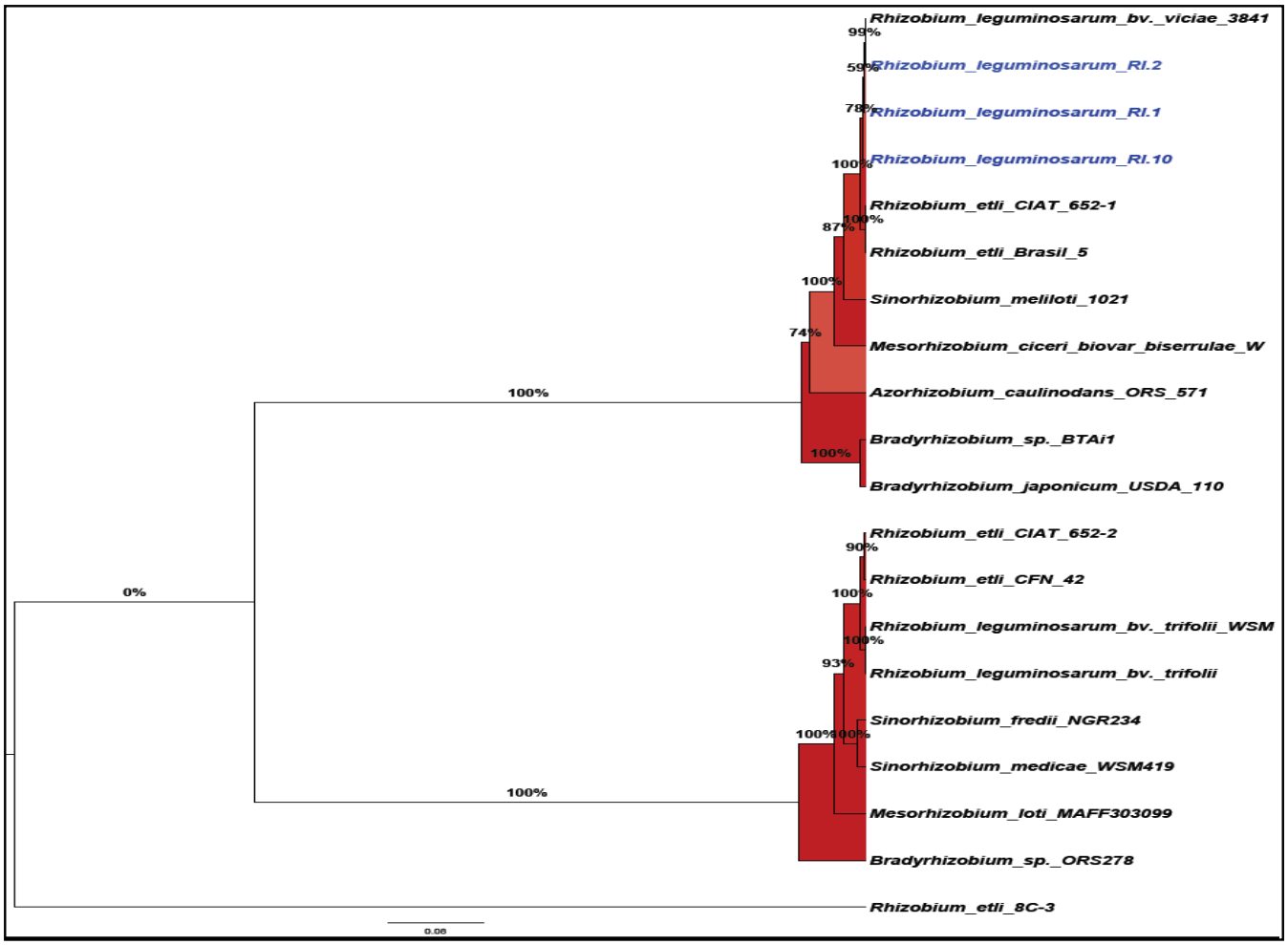

Figure 4: Phylogenetic tree of rhizobia based on partial sequence analysis of the 16 S rRNA gene, constructed using the neighbor joining method in MEGA programmed version 5.10.

The Rhizobium isolates were sensitive to kanamycin, Neomycin and Sulphemethooxazole antibiotics. There are three known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic and the Rhizobium that showed a high level of resistance did not take up the antibiotics [21].

In order to visualize the ability of the isolates to solubilize different carbon sources, six different compounds were tested as a sole source of carbon. Five sugar sources tested namely; mannitol, sorbitol, sucrose, galactose and lactose and also, a non-sugar source pectin was examined. All isolates were able to assimilate all carbon sources used and they showed growth responses to these sole carbon sources. However, mannitol was the best source of carbon source because it assimilated by all strains. On the other hand, pectin as hard carbon source (non-sugar source) was the hardest source to metabolism with isolates. At the same time, sucrose was the least carbon source (sugar source) utilized by isolates. Similar results have been reported before by [22].

It is well known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Researchers have proposed that salt stress may decrease the efficiency of the Rhizobium legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function [23]. To date, some rhizobial isolates have been shown to grow under high salt conditions [24]. In general, results of this research indicated that isolates were able to grow on 200 mM of NaCl but they grew slowly on higher concentrations 300 mM. These results indicated that these isolates were relatively sensitive to the salt stress except two isolates Rl.2 and Rl.10. Similar observations were reported [23,24].

Despite that several genes have been identified in rhizobia response to salinity, the tolerance mechanisms of rhizobia to overcome salt stress remains unknown, mainly due to the fact that response and adaptation to salinity stress is a complex phenomenon involving many physiological and biochemical processes that likely reflect changes in gene expression. Rhizobium leguminosarum bv. viciae generally contains 1 to 10 plasmids which vary in size from 30 kb to more than 800 kb [25]. Most of the genes required for Nodule Formation (nod) and nitrogen fixation (nif and fix) are carried on plasmids [26]. Thus, loss of plasmids affected the relationship between Rhizobium and legume. Isolated correlated plasmids from R. trifolii and they showed that Nod-

mutants resulting from a prolonged treatment at high temperature were due to either loss of plasmid DNA or internal deletions in the plasmid [11]. Zurkowski reported that at high temperature stress may cause the loss of the plasmid during cell division [11]. Also, he added that the transfer of the plasmid into the Nod- isolates converted them to a Nod+ phenotype. Result of this research demonstrated extra plasmid with molecular weight about 23 Kb only in the two most salt tolerant isolates understudy. Therefore, it was concluded that salinity treatment may cause the presence of an extra plasmid in most native isolates. This result was in agreement with [27].

The biodiversity of the indigenous isolates assessed by DNA fingerprinting method-RAPD-PCR further demonstrated a high level of genetic diversity among Faba bean rhizobial isolates. The eight primers were used to obtain RAPD fingerprint patterns because it has been shown to distinguish genomic variation within members of the same species of Rhizobium [28]. From the analysis of eight faba bean isolates different RAPD fingerprint patterns were revealed. These fingerprint patterns varied in distribution according to the field site of isolation. Therefore, the population of bean-nodulating rhizobia in soils of Egypt was shown to be diverse, which allowed the selection of representative isolates for further analysis [29].

Thus, this result is further evidence that PCR-RAPD is a useful tool to conduct persistence and competitiveness studies in rhizobia [30-32]. Also, it is in agreement with who tested 28 of indigenous rhizobia nodulating chickpea in India using RFLP to classify their isolates and who found that all RAPD primers detected one or more polymorphic DNA fragments among the studied rhizobia species and that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of Rhizobium. Also, Valverde, et al. (2011) found that RAPD fingerprint patterns were able to distinguish genomic variation within members of the same species of Rhizobium [33-35].

Finally, from a phylogenetic perspective, legume root bacterial symbioses do not form a single monophyletic group, and different lineages within the α-proteobacteria are today recognized according to the systematic of Garrity et al. [36]. The phylogenetic analyses performed in this research confirmed the wide diversity of bacterial isolates associated to O. ridentata. The phylogeny of the 16S rRNA gene yielded two main clades. The first one grouped members of the Rhizobiaceae (Rhizobium and Sinorhizobium) and Phyllobacteriaceae (Mesorhizobium), with 100% bootstrap support, and the second clade brought together members of the Bradyrhizobiaceae, such as Bradyrhizobium, with full bootstrap support. The genus Rhizobium was grouped in a single cluster in which most of the sequences of R. leguminosarum isolates were placed. These results are similar with those obtained with Rincon et al. [37]. The classification of most isolates obtained after 16S rRNA sequence analysis was in a good agreement with classification obtained using RAPD data, however, 16S rRNA sequence analyses allowed better identification at species level [34].

Conclusion

The main goal of this research was to characterize and to describe the diversity of eight rhizobial isolates nodulating faba bean from different geographical regions of Egypt. They showed sensitivity to Kanamycin, Neomycin and Sulphemethooxazole antibiotics and mannitol was the best source of carbon source for their growth. Two isolates indicated tolerance to high NaCl concentrations and their plasmid profiles contained additional large plasmid. RAPD-PCR technique showed a high level of genetic polymorphism, grouping the rhizobial isolates into two different clusters. The comparative 16S sequencing identified that the isolates belonging to Rhizobium leguminosarum biovar viciae.

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