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Phenotypic and Genotypic Diversity of Rhizobia Nodulating Faba Bean from Various Egyptian Locations

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Abstract

Thirteen Rhizobial isolates from root nodules of Faba beans (*Vicia faba* L.) were obtained from eleven governorates, representing different agro-ecological, agro-climatic and soil conditions in Egypt. A polyphasic approach, including phenotypic and genotypic techniques were used to study their diversity. Rhizobium isolates were examined for their ability to tolerate salt stress and antibiotic resistance. We used four NaCl concentrations ranging from 0.1%, 1%, 3% and 5% in liquid media and plates, moreover we used 24 different antibiotic disks to determine resistance or sensitive among the tested isolates on plates. The obtained results after salt tolerance and antibiotic response of the R. *leguminosarium strains* showed that the isolate No. RL9 was the superior strain for salt tolerance . The following strains for salt tolerance were the isolates (RL4) and (RL12), although the isolate No. RL11 was the superior strain for antibiotic resistant then isolates No. RL 13 and Rh3. Growth of all isolates were inhibited above 3% NaCl except five isolates RL4, RL9, RL10, RL12 and RL 13. Also the antibiotic resistance of the isolated strains showed a high level of resistance against Cefoxitin (FOX_{30}) and Cefuroxime (Cxm_{30}). The genetic diversity was studied using RAPD-PCR technique and we used specific primer (*nodC*) amplification. In this study we found that OPE_{15} and OPG_{04} primers showed the highest polymorphism level among the tested isolates, however OPJ_{20} and OPC_{06} primers showed the lowest polymorphism level.

Keywords: Faba bean; Antibiotic resistance; Salt tolerance; RAPD-PCR; nodC

Introduction

Faba bean (*Vicia faba* L.) is a major leguminous crop grown worldwide, it is most intensively cultivated in the Middle East and in North East Africa [1]. Rhizobium leguminosarium symbiovar. Vicia forms a nitrogen-fixing root nodule symbiosis with faba bean [2,3]. Since Faba bean has been grown for centuries in Egypt, it was of interest to determine the diversity of rhizobia forming nitrogen-fixing nodules with this important legume crop. The soils used in our studies distributed in geographically different locations in Egypt. Rhizobia are soil bacteria which are capable of forming nitrogen-fixing nodules with different leguminous plants and have a significant role in nutrient cycling due to biological nitrogen fixation and enhancing crop productivity [4]. The symbiotic relationships between rhizobia and leguminous plants provide soil with nitrogen. Many studies have addressed the diversity level of *V. faba rhizobia*; mainly focusing on rhizobial populations from the same location [5] or for comparison with Rhizobium leguminosarum isolates from other legume species [6]. The diversity of rhizobia provides valuable bio resource for the search of bacterial isolates in attempt to find isolates that maximize nitrogen fixation, and hence increase legume crop productivity [7]. Salinity stress is one of the most serious factors limiting the productivity of agriculture. High salt can directly impair rhizobia–legume early interactions during nodule formation [8]. In general, Egypt suffering from increasing population in both drought and arid climate. The symbiotic interaction between rhizobia and legumes is initiated by an exchange of complex molecular signals that confer host-specificity. Rhizobia respond to these by one or more of the constitutive nod D genes encoding for a soluble cytoplasmatic protein activating the other nod genes when they interact with the appropriate plant signal compounds. This leads to the biosynthetic enzymes of lipo-chitin oligosaccharides (Nod Factors) encoded by nod ABC genes. Laguerre et al., [9] used the nod C gene, a common nod gene essential for nodulation in all rhizobial species, to characterize a collection of 83 rhizobial strains which represented 23 recognized species distributed in the genera Rhizobium, Sinorhizobium, Mesorhizobium and Bradyrhizobium. Many techniques were developed and widely used to detect polymorphisms in many organisms including bacteria. Among these techniques, RAPD technique is a polymerase chain reaction (PCR) to detect the polymorphisms in genomic DNA [10]. Genomic DNA Fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between Rhizobial strains. [11] proposed this method for identification and phylogenetic grouping of Rhizobium isolates. RAPD PCR technique provides reliable information on the diversity of Rhizobium populations in soils [12].

The objectives of this study are to isolate salt tolerant Rhizobium and investigate the effects of antibiotics and NaCl on growth of isolates. Furthermore detect the diversity of these isolates by using RAPD.

Material and Methods

Isolation of Rhizobia from nodules

Thirteen Rhizobial isolates isolated from the root nodules of Faba bean (*Vicia faba* L.) isolates were obtained from eleven governorates as presented in (Table 1). All nodules were cut off in a laminar flow cabinet with small pieces of root and washed thoroughly with 2.5% NaOCl and sterile water. Nodules were surface sterilized with 70% ethanol

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Isolates	Nitrogenase Activity	Nodules No.	Shoot Dry (g)	Shoot Fresh (g)
1	1.60	23.0	2.10	13.70
2	0.80	14.7	1.30	11.30
3	1.35	17.3	3.00	12.50
4	1.75	23.0	4.60	19.90
5	1.00	18.7	2.00	12.70
6	0.75	24.3	26.01	14.60
7	2.15	24.3	1.00	11.10
8	0.50	15.3	2.60	16.50
9	3.30	32.3	8.80	29.00
10	2.00	22.7	5.00	24.60
11	2.50	28.3	4.10	24.70
12	2.70	25.3	6.90	26.10
13	3.10	30.7	7.50	27.60

Table 1: Locations of isolates measured for each isolate.

alcohol for 5 min and exhaustively washed in sterile distilled water. After washing the root nodules were taken from the roots with care not to damage the surface, washed thoroughly in distilled water. Nodules were then transferred to 0.2% (W/V) solution of mercuric chloride for 3-5 minutes. Each nodule was crushed under aseptic conditions and streaked onto a Yeast-Mannitol Agar plate (YMA) using a sterile loop and incubated at 28°C. Single colonies were picked up from the original streaked plates. Pure cultures of *Rhizobium leguminosarum* were isolated according to Vincent methods and retreated on YMA containing Congo red to ensure purity before storage in 20% glycerol at -20°C. Stock cultures of the Rhizobium isolates were maintained on slants of YEM at 4°C and refreshed periodically.

Phenotypic characterization of isolates

Antibiotic resistance pattern: All isolates were evaluated for their responses as resistant or sensitive against 24 different antibiotic disks as shown in Table 2.

Rhizobium isolates were tested on YMA agar supplemented with following antibiotic disks that presented in Table 2 according to Eaglesham's technique [13]. This was done as the following each isolate plated on YEM plates and the antibiotic disks were distributed on the surface of the plate. The plates were incubated for 3 days at 28°C. After this period the presence or absence of an inhibition zone around antibiotic disks was recorded, indicating sensitivity or resistance, respectively.

Effect of Salinity on tested Rhizobium isolates:

- YEMA: The ability of the Rhizobial isolates to grow in various concentration of NaCl was tested by plating them on YEM agar Petri dishes. YEMA was prepared with different concentrations of NaCl ranging from (0.1%, 1%, 3% and 5% wt/vol) NaCl. All the plates were incubated at 28°C for three days in triplicate in addition to control.
- YEM broth: Testing of salt tolerance was measured as the ability of the bacterial cells to grow and divide under the stress of used NaCl concentration. Test tubes (10 ml) each containing 5 ml YEM medium, were inoculated with a bacterial isolates and incubated at 28°C for 72 hours. 100 µl of suspension (1×10^{-7} cfu/ml) of the grown isolate was used for the inoculation of 5 ml YEM medium containing various NaCl concentration (0.1 %, 1%, 3% and 5% wt/vol) and the test tubes were incubated at 28°C for different time ranging from zero time, 12, 24, 36, 48, 60 and 72 h of incubation and the growth rate in Rhizobial isolates tested using optical density (OD) at three replicates. The turbidity was measured using Jenway UV-VIS. spectrophotometer model UV-6305 at 600 nm against the blank (sterilized uninoculated YEM broth) (Table 4).

DNA isolation

Total genomic DNA of each isolate was extracted from bacterial cultures grown in yeast extract mannitol media (YEM) according to Ausubel et al., [14] method and kept at -20°C. The cells were lysed with sodium dodecyl sulphate (SDS), protein–lipopolysaccharides complexes were removed by using cetyl tri methyl ammonium bromide (CTAB), and DNA was precipitated from solution using isopropanol.

RAPD–PCR reaction and amplification

Oligonucleotide sequences of the random primers:

OPE20---5′AACGGTGACC 3′---OPA04---5′AATCGGGCTG 3′

OPJ20---5′AAGCGGCCTC 3′---OPE 15---5′AACGGTGACC 3′

OPG 04---5′AGCGTGTCTG 3′---OPC 06---5′GAACGGACTC 3′

Total genomic DNA was extracted from exponentially grown cultures and PCR reactions were carried out using an arbitrary primer RAPD-PCR were used in this study RAPD-PCR was performed in the total volume of 25 µl of reaction mixture containing 1 µl of DNA template, 1 µl dNTPs mix,4 µl (10 pmol) of an arbitary primer and 1 µl of Taq polymerase with 2.5 µl Taq buffer and 15.5 µl sterilized water.

PCR conditions were as follows template DNA was denatured at 94°C for 4 min then the PCR was carried out for 35 cycles (1 min at 94°C, 1 min at 34°C, 2 min at 72°C, for each cycle). Finally, a 7 min extension period at 72°C was performed. Amplified products were resolved on a 1% (w/v) Agarose-TBE gel electrophoresis and separated by running 5 µl of PCR reaction mixture at 100 V for 1 h and stained

No.	Disks	Name
1	AK30	Amikacin
2	AM 10	Ampicilin
3	ATM 10	Aztreonam
4	B 0.04	Bacitracin
5	C 30	Chloramphenicol
6	CIP5	Ciprofloxacin
7	CLR 15	Cefuroxime sodium
8	CN 10	Gentamicin
9	CRO 30	Ceftriaxone
10	Cxm30	Cefuroxime sodium
11	E 15	Erythromycin
12	FOX 30	Cefoxitin
13	IPM 10	Imipenem
14	K 30	Kanamycin
15	MA 30	Cefamandole
16	Mem 10	Meropenem
17	N 30	Neomycin
18	NOR10	Norfloxacin
19	OX 1	Dloxaciolin
20	S 10	Streptomycin
21	Sam 20	Sulbactampicillin
22	SXT25	Sulphamethoxazole
23	T 30	Tetracycline
24	VA 30	Vancomycin

Table 2: Antibiotic disks used in this study.

Antibiotic Isolates	AK 30	AM 10	ATM 10	B 0.04	C 30	CIP 5	CLR 15	CN 10	CRO 30	CXM 30	E 15	FOX 30	IPM 10	K30	MA 30	Mem 10	N 30	Nor 10	OX 1	S 10	Sam 20	SXT 25	T 30	VA 30
t	-	-	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-
Rh 2	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	-	+	-	-	-
Rh 3	-	+	+	+	-	-	-	-	+	+	-	+	-	-	+	+	-	-	-	-	-	+	-	+
Rh 4	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	+	-	+	+	-	+	-	-	-
Rh 5	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	+	-	-	+	-	-	-
Rh 6	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	-	-	+	-	+	-	-	+
Rh 7	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-	+	+	-	+	-	-	-
Rh 8	-	-	+	+	-	-	-	-	-	+	-	+	+	-	-	+	-	-	-	-	+	-	-	-
Rh 9	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-
Rh 10	-	-	+	+	-	-	-	-	+	+	-	+	-	-	+	+	-	-	-	-	+	-	-	-
Rh 11	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+	+	-	+	+	+	+	-	-	-
Rh 12	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-	+	-	+	-	-	-
Rh 13	-	+	+	+	-	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-	+	-	+	-

Table 3: Response of Rhizobium isolates to 24 different types of antibiotic disks.

Isolates	RhI	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	RhS	Rh9	RhI 0	Rh11	Rh12	Rh13
RhI	1												
Rh2	0.3	1											
Rh3	0.33	0.3	1										
Rh4	0.27	0.87	0.28	1									
Rh5	0.33	0.62	0.23	0.75	1								
Rh6	0.4	0.5	0.5	0.45	0.4	1							
Rh7	0.44	0.4	0.31	0.5	0.44	0.5	1						
Rh$	0.44	0.75	0.47	0.67	0.62	0.36	0.4	1					
Rh9	0.67	0.37	0.27	0.33	0.43	0.5	0.37	0.37	1				
Rh10	0.55	0.5	0.64	0.45	0.4	0.6	0.5	0.67	0.5	1			
Rh11	0.38	0.46	0.47	0.54	0.38	0.43	0.58	0.46	0.23	0.58	1		
Rh12	0.33	0.62	0.23	0.55	0.5	0.55	0.44	0.44	0.43	0.4	0.5	1	
Rh13	0.45	0.42	0.54	0.5	0.45	0.38	0.42	0.54	0.4	0.64	0.57	0.33	1

Table 4: Similaritybetween different tested isolatesbased on antibiotics resistant analyses.

with ethidium bromide (0.5 µl/ml). The sizes of the amplified fragments were determined by comparison with 100 bp.

Plus DNA Ladder (Gene ruler) as molecular weight marker. Presence of amplified bands were observed using UV-transilluminator and the gel was photographed. Variations between amplified bands were recorded as presence or absence of DNA fragments. The dendrogram was constructed based on UPGMA cluster analysis according to dice square co-efficiant method. The dendrogram tree among the studied Rhizobium isolates based on RAPD markers were constructed using NTSYSpc version 2.1 programs depending on the similarity matrix present in Table 5.

Amplification of the nodC gene

The nodC gene from the Rhizobium isolates was amplified by PCR by using forward primer nodCf (5-GCTGCCTATGCAGACGATG-3) and reverse primer nodCr (5-GGTTACTGGCTTTCATTTGGC-3), PCRs were carried out in 50 ml reaction mixtures [14] using the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 3 min at 72°C. A final extension was done at 72°C for 7 min. PCR products were separated on 2% agarose gels at 120 V for 1.5 h in TBA buffer, stained with ethidium bromide, and visualized under UV light.

Statistical analysis: Data obtained were statistically analyzed using SPSS analysis program (version 11.5). The significant differences among individual means were analyzed by Duncan's multiple range tests.

Isolate No.	Control (0.1%)	NaCl at different concentration		
		1%	3%	5%
1	130	110	50	0
2	150	130	70	0
3	110	100	80	0
4	140	120	90	50
5	120	90	40	0
6	120	110	40	0
7	90	80	50	0
8	100	80	60	0
9	150	120	90	50
10	160	120	50	40
11	100	70	40	0
12	140	110	90	60
13	170	140	100	80

Table 5: Response of tested isolates to different concentrations of NaCl on YEMA Plates ($1*10^7$cfu/ml).

Results and Discussion

Faba bean has been cultivated for over 6000 years, and is grown in 57 countries worldwide. Since faba bean has been grown for centuries in Egypt, it was of interest to determine the diversity of rhizobia

forming nitrogen-fixing symbioses with this important legume crop. The soils used in our studies differed in texture and class, and were distributed in geographically different sites in Egypt.

Phenotypic characteristics

On the basis of morphological parameters, we have confirmed differences between the isolates. Table 1 represent thirteen isolates of *Rhizobium leguminosarum* symbiovar *Vicia* isolated from nodules of Egyptian Faba beans (*Vicia faba* L.) collected from different geographic areas. This ten isolates were tested by Congo red technique [15], to ensure that all isolates were rhizobia and did not contaminated. These results are in agreement with those obtained by [16] they sampled two hundred and eighty seven isolates of Rhizobium in France from geographically distant field populations and characterized by their colony morphology. Rhizobial isolates were found to be microscopically similar, isolates identified as Rhizobium *leguminosarium symbiovar. Vicia*. According to the negative reaction to gram stain. Strains were found to be motile. On YEM agar, the growth is generally moist, whitish, smooth and gummy. The culture on YEM broth showed uniform turbidity, white sediment and need 8-10 days or longer time to attain maximal growth. These results are in agreement with those obtained by [17]. Colonies on agar after 2-3 days were large, gummy, translucent, spreading about 2–4 mm in diameter, similar results were reported by [18].

Antibiotic resistance pattern

Resistance to different disks of antibiotic is one of the simple and rapid methods for rhizobial isolates characterization and identification. Resistance patterns of the isolates to various antibiotics were studied to provide phenotypic data for differentiating the rhizobial isolates from each other and to determine the diversity among the isolates. Apparently, the antibiotic resistance of the tested strains showed a high level of resistance against Cefoxitin (FOX30r) and Cefuroxime (Cxm30 r). More than 70% of the isolates showed resistance to Meropenem (Mem10r) except isolate No.1,7 and 9. Some isolates were resistant to Sulbactampicillin (Sam20r). Moreover all the isolates showed generalized sensitivity to Amikacin (AK30s), Chloramphenicol (C30s), Ciprofloxacin (CIP5s), Cefuroxime sodium (CLR15s), Gentamicin (CN10s) and Erythromycin (E15s). Furthermore all the isolates showed sensitivity to Tetracycline (T30s) except isolate No.13, The generalized sensitivity to Tetracycline in this study agrees with the results reported previously by Jordan (1984) for the genus Rhizobium and by Hagedorn [19] for *R.leguminosarum sv. trifolli*. Finally all the isolates showed Sensitivety to Streptomycin (S 10 s) except isolate No.1

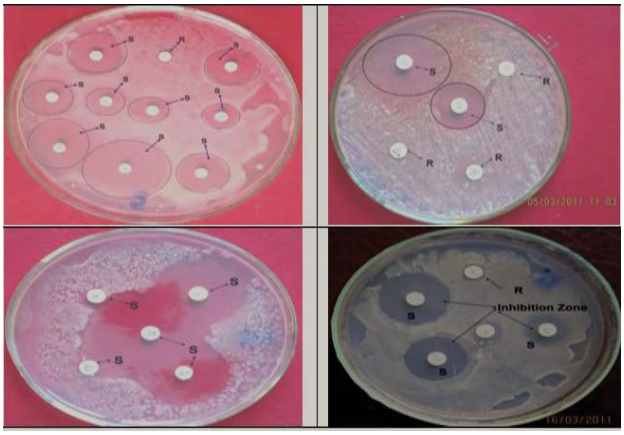

Figure 1: Response of different isolates to kinds of different antibiotic discs.

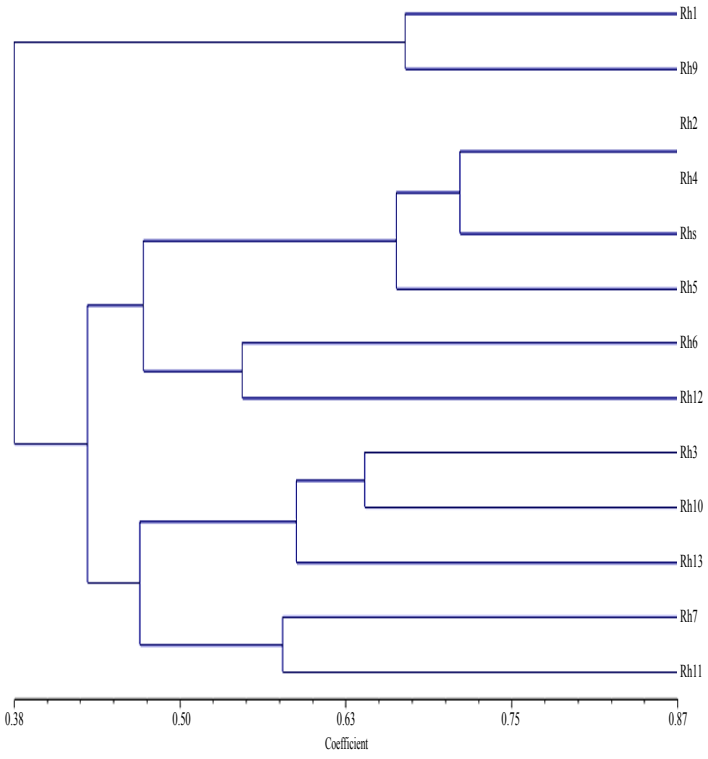

Figure 2: Phylogenetic relationship between 13 isolates using UPGMA method based on antibiotics resistant analyses.

and isolate No.11. Whereas isolates No. Rh11, Rh13 and Rh3 showed 50% resistance to the tested antibiotics as shown in Table 3, The same Table 3 and Figure 1 showed the wide range of variations among the isolates for its resistant to these discs. These results were similar to those reported by [20].

Salt tolerance: These strains were first screened to examine their resistance to environmental stresses e.g. salt tolerance. The majority of the tested strains were sensitive to salt stress . All 13 rhizobial isolates tolerated 3% NaCl. Whereas only five isolates are able to grow at 5% NaCl. only 5 isolates still grew, but 8 isolates failed to grow at the same concentration (Rh1, Rh2, Rh3, Rh5, Rh6, Rh7, Rh8 and Rh11) and showed great inhibition . In fact,Ismailia isolate showed the most salt tolerancy (Figures 2 and 3).

Results in Table 5 showed that all of the 13 isolates were able to survive in salt concentrations ranging between 0.1% till 3.0% NaCl

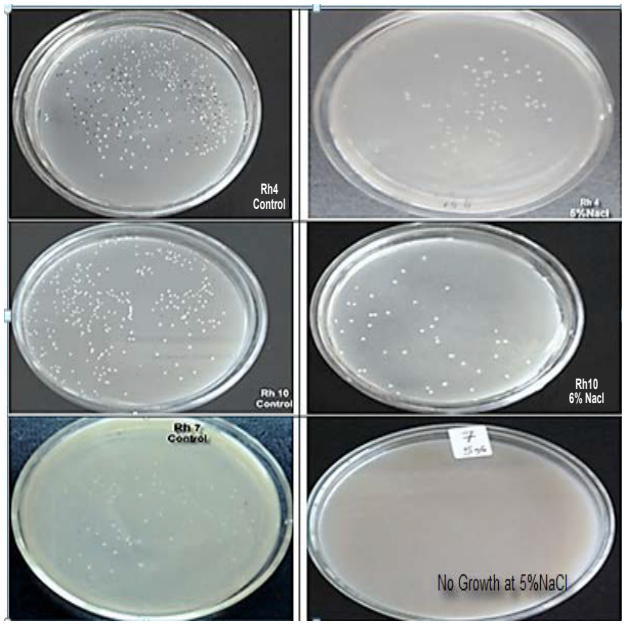

Figure 3: Some isolates growing on YEMA media supplemented with NaCl as a control and 5% NaCl concentration.

and a few of them tolerated and survived at 5% NaCl. These tolerated isolates were isolate No.4 from Zefta City, Ghrbia Governorate ,isolate No.9 from Rafah City, North Sinai Governorate, isolate No.10 from El-Menia Governorate , isolate No.12 from Cairo City, Cairo Governorate and finally isolate No.13 from Ismailia City, Ismailia Governorate. These results were similar to those reported by [21] who found that the Faba bean-nodulating strains EFBRI 35, 41, 92 and 93 that we isolated were salt tolerant, and grew well in 1% NaCl, and a large number of strains that we later identified as S. *meliloti* and isolated from Faba bean nodules were resistant to 3% NaCl. Similar results were reported by who noted that strains belonging to this species are salt tolerant.

The O.D. of all isolates was affected significantly by salt concentration (Figure 4 and Table 6). O.D. decreased with increasing salinity above 1% concentration. Most isolates had maximum O.D. At 1% NaCl; all isolates continued to grow strongly at 3% NaCl, whereas growth of all isolates was inhibited 5% NaCl except for isolate No.4, 9, 10, 12 and 13. As a result, the salt tolerance of rhizobia is critical for symbiosis in that it facilitates the survival and growth of rhizobial strains in saline conditions and, in particular, is one of the key mechanisms to enhance the symbiosis under saline conditions [22]. The Ismailia strain was able to tolerate the maximum concentration tested at 5% NaCl, while without NaCl we noticed heavy growth of Rh.7 (control), whereas plate No.7 at 5% NaCl showed no bacterial growth at all. Therefore this isolate is very sensitive to NaCl at used concentration.

Data in Table 6 showed that the isolate No. RL9 was the superior strain for salt tolerance The following strains for salt tolerance were the isolates RL12, RL4, RL10 and RL13 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.

Genetic diversity of Faba bean (*Rlv*) rhizobia using different molecular approaches

RAPD PCR: Several investigators have studied the genetic diversity of Rlv isolated from several countries around the world [23-26].

RAPD profiles were used to show the genetic polymorphism among isolates of *Rhizobium leguminosarum bv. Viciae* and to discriminate their genetic differences [26]. The genetic diversity of Rhizobium isolated from several countries around the world was studied by [21]. The genetic diversity of thirteen studied strains were

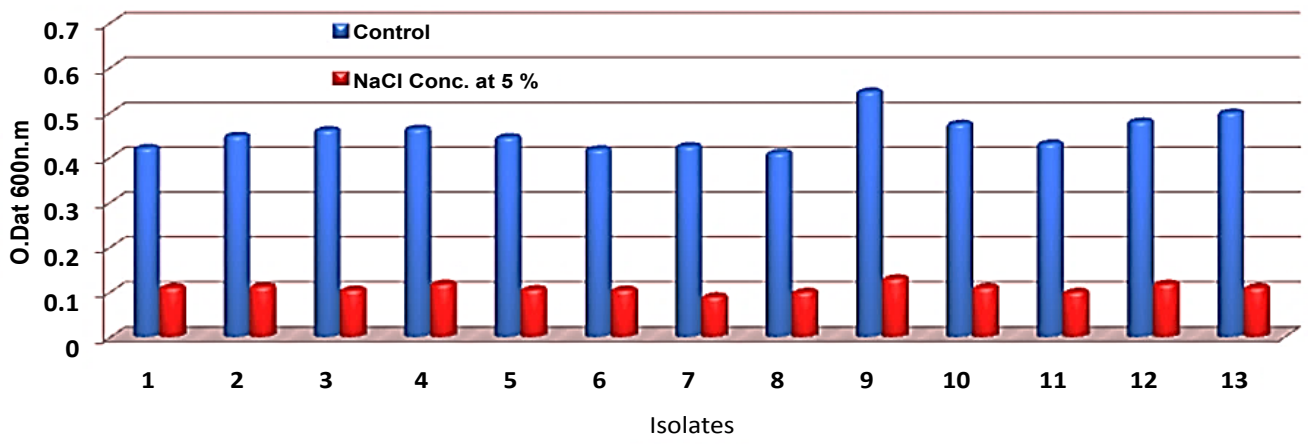

Figure 4: OD of all isolates at High Concentrations of NaCl (5%) after 72(hr) from inoculation.

	Isolates												
Conc. (%)	**RL 1**	**RL 2**	**RL 3**	**RL 4**	**RL 5**	**RL 6**	**RL 7**	**RL 8**	**RL 9**	**RL 10**	**RL 11**	**RL 12**	**RL 13**
0.1	0.421[a]	0.448[a]	0.461[a]	0.464[a]	0.445[a]	0.418[a]	0.425[a]	0.410[a]	0.547[a]	0.475[a]	0.431[a]	0.480[a]	0.500[a]
1	0.256[b]	0.361[b]	0.256[b]	0.253[b]	0.321[b]	0.258[b]	0.321[b]	0.321[b]	0.265[b]	0.248[b]	0.361[b]	0.321[b]	0.266[b]
3	0.150[c]	0.160[c]	0.140[c]	0.155[c]	0.172[c]	0.150[c]	0.172[c]	0.172[c]	0.18[bc]	0.145[b]	0.160[b]	0.150[c]	0.152[c]
5	0.110[c]	0.112[c]	0.105[c]	0.118 [c]	0.106[c]	0.105[c]	0.090[c]	0.100[c]	0.130[c]	0.110[c]	0.100[b]	0.118[c]	0.108[c]

a,b,c=values of each trait within each column don't have the same letter are significantly different

Table 6: Effect of different concentrations of NaCl on Growthafter 72 hr. (measuredas optical density) of isolates.

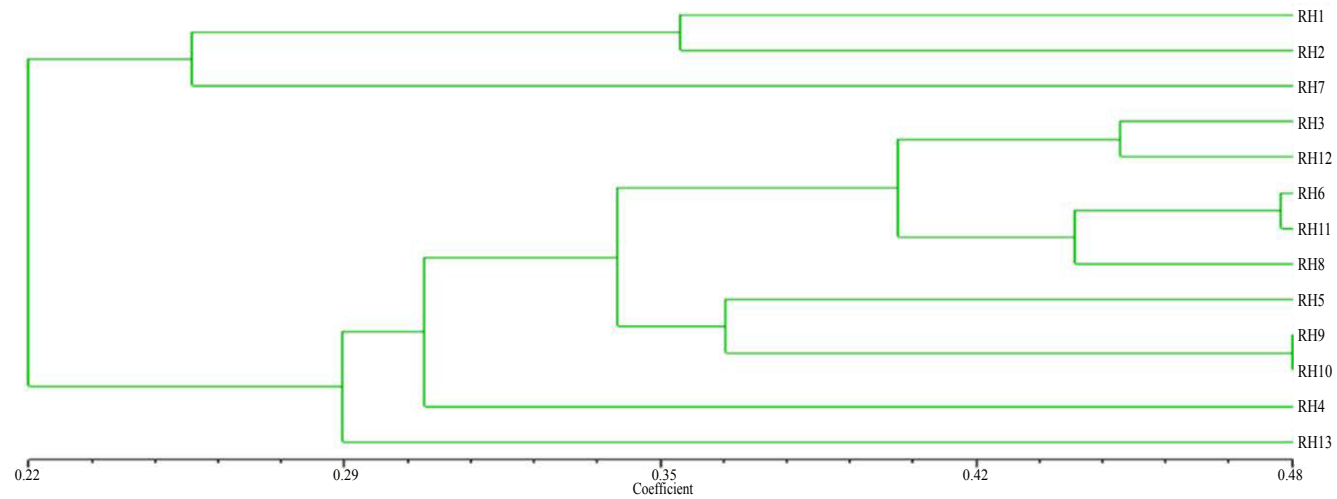

Figure 5: Phylogenetic relationship between 13 Rhizobial isolates using UPGMA method based on RAPD analyses.

examined: Rh1,Rh2, Rh3 , Rh4 , Rh5 , Rh6 , Rh7 , Rh8 , Rh9 , Rh10 , Rh11 , Rh12 and Rh13 using six random primers (OPE 15, OPA 04, OPC 06, OPE 20, OPJ 20 and OPG 04), and were used to screen for the polymorphism between the tested isolates. The results of RAPD analysis showed that all used primers were polymorphic. OPE 15 and OPG 04 primers showed the highest polymorphism level among the tested isolates, while OPJ 20 and OPC 06 primers showed the lowest polymorphism level Figure 6.

The similarity matrix values were converted into Dendrogram using UPGMA (Unweight Pair Group Method with the Arithmetic average) clustering method. Cluster analysis divided into two main clusters (Figure 5). The first cluster included isolates from Quesna, Benha, and South Sinai; whereas the second cluster included isolates from Sadat, Cairo, Sues, South Sinai , Beni Suef , Al- Dakahlia , North Sinai , El-Menia , Zefta, and Ismailia. Remarkably, the first cluster was divided into three groups for the isolates were highly related to each other. The second cluster presented ten isolates, the isolates within each group were overlapped, principal coordinates analysis showed similar pattern as cluster analysis. The analysis was based on the number of markers that were similar between any given pair of isolates Table 7.

All Rhizobia isolates genotypically characterized by RAPD_PCR. Total genomic DNA amplified with six oligonucleotide primers, amplification patterns revealed a high level of polymorphism. All primers produced multiple DNA products ranging in size from 0.2 to 1.5 Kb. Primer OPJ20 Produced multiple DNA products ranging in number from one in isolates Rh 11 and Rh 12 to three bands in Rh 1, Rh 3, Rh 4, Rh 5, Rh 6, Rh 8, Rh 10 and Rh 11. Whereas only Rh 2, Rh 7 and Rh 13 produced two bands. Primer OPE15 Produced multiple DNA products ranging in number from TWO bands in isolate Rh 5 to five bands in isolates Rh 3 and Rh 13. Whereas isolates Rh 4, Rh 8, Rh 10, Rh 11 and Rh 12 produced four bands . Also isolates Rh 1, Rh 2, Rh 6, Rh7 and Rh 9 produced three bands. OPC 06 Produced multiple DNA products ranging in number from two bands in isolates Rh 4, Rh 10, Rh 12 and Rh 13 to four bands in isolates Rh 1, Rh 3 and Rh 6 . On the other hand isolates Rh 2, Rh 5, Rh 7, Rh 8, Rh9 and Rh 11. OPG 04 Produced multiple DNA products ranging in number from three bands in isolates Rh 2, Rh 3, Rh 5, Rh 6 and Rh 8 to five bands in isolates Rh 7, Rh 11 and Rh12. Whereas isolates Rh 1 , Rh 4 ,Rh 9 , Rh10 and Rh 13 showed four bands. OPA 04 Produced multiple DNA products ranging in number from three bands in isolates Rh 2, Rh 3, Rh 7 and Rh 11 to six in isolates Rh9. Also five bands in isolates Rh 4 and Rh10 were produced, further to isolates Rh 1, Rh 5, Rh 6, Rh8, Rh 12 and Rh 13 showed four bands. Finally primer OPE 20 produced multiple DNA products ranging in number from two bands in only isolate Rh 2 to five bands in several isolates Rh 10, Rh 12 and Rh 13. Also the same primer produced four bands in isolates Rh 1, Rh 3, Rh 7 and Rh 9. Whereas isolates Rh 4, Rh 5, Rh 6, Rh 8 and Rh 11 showed only three bands. All six primers were successfully generated reproducible polymorphic bands to evaluate the degree of genetic diversity and calculating the genetic distances of Rhizobia based on the DNA nucleotide sequence using RAPD and used to identify the genetic variability and genetic relationship among the thirteen genotypes. Also we used Sequence analysis of 16S rDNA and subsequent BlastN analyses indicated that the majority of isolated strains were *Rlv.* [27].

To further confirm the taxonomic status of the Faba bean

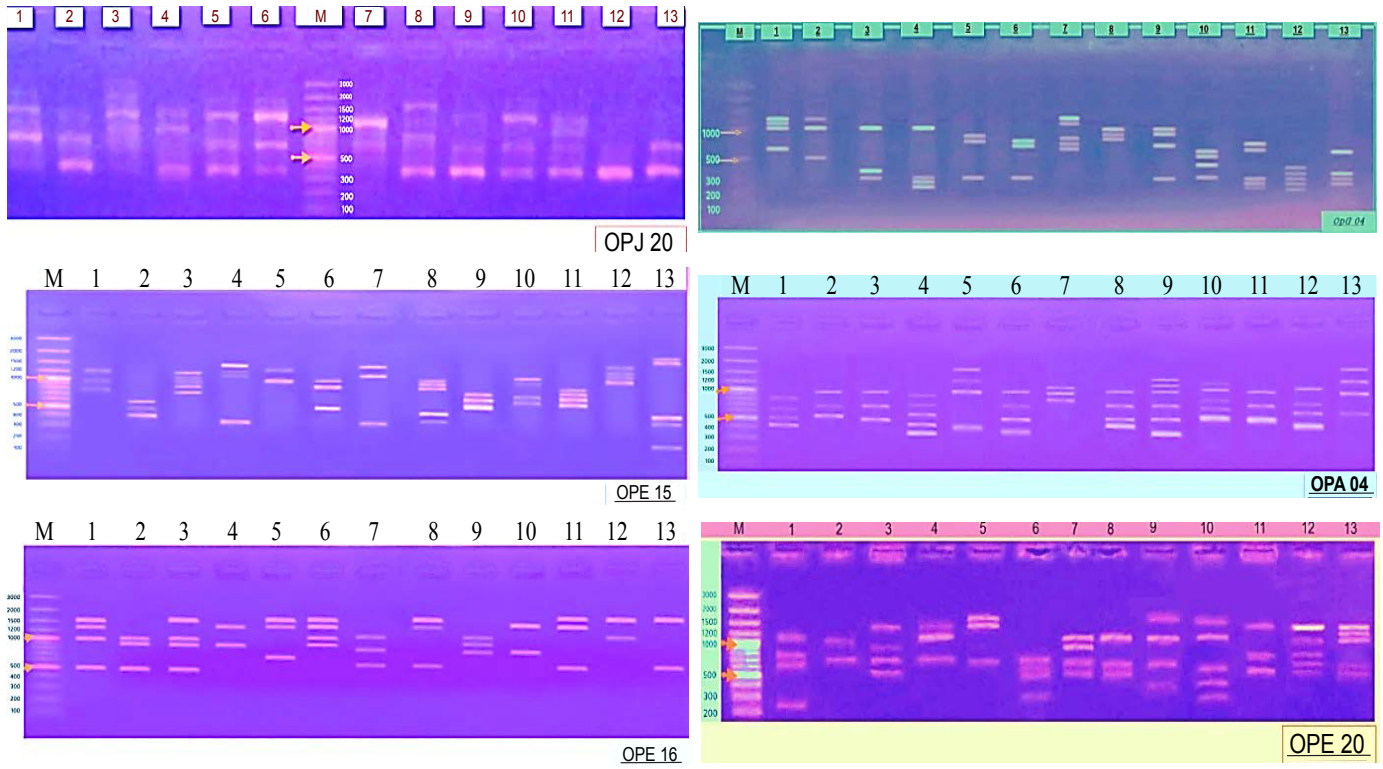

Figure 6: 1% Agarose gel electrophoresis of RAPD PCR products. (M) DNA size marker (Gene Ruler™ 100bp Plus DNA Ladder, Fermentas); 1=Rh. Quesna; 2=Rh. Banha; 3=Rh. Sadat ; 4=Rh. Zefta ; 5=Rh. Baneswaif; 6=Rh. Sues; 7=Rh. South Sina; 8=Rh. Al Arish; 9=Rh. Rafah; 10=Rh. New El-Menia; 11=Rh. Moshtohor; 12=Rh. Cairo and 13=Rh. Ismailia.

Isolates	Rh 1	Rh 2	Rh 3	Rh 4	Rh 5	Rh 6	Rh 7	Rh 8	Rh 9	Rh 10	Rh 11	Rh 12	Rh 13
Rh 1	1												
Rh 2	0.35	1											
Rh 3	0.34	0.27	1										
Rh 4	0.31	0.16	0.32	1									
Rh 5	0.17	0.06	0.25	0.26	1								
Rh 6	0.23	0.16	0.36	0.29	0.35	1							
Rh 7	0.31	0.2	0.28	0.25	0.22	0.21	1						
Rh 8	0.34	0.19	0.35	0.36	0.39	0.41	0.32	1					
Rh 9	0.15	0.3	0.29	0.3	0.36	0.38	0.22	0.25	1				
Rh 10	0.18	0.21	0.33	0.22	0.36	0.38	0.13	0.29	0.48	1			
Rh1 1	0.2	0.2	0.36	0.29	0.35	0.48	0.25	0.46	0.38	0.48	1		
Rh 12	0.22	0.15	0.44	0.36	0.34	0.41	0.28	0.44	0.25	0.37	0.46	1	
Rh 13	0.15	0.18	0.26	0.27	0.29	0.2	0.23	0.3	0.21	0.25	0.35	0.43	1

Table 7: Similarity between different tested isolates.

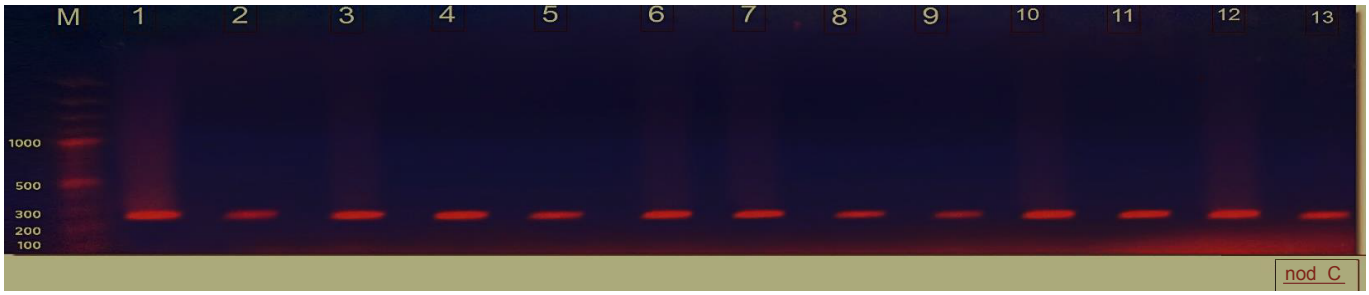

Figure 7: Identification of Rhizobial isolates by amplifying a specific region of nodC gene (220 bp).

nodulating Rlv strains, and to examine variation in a plasmid-borne, symbiotic specific gene, primers nodCf and nodCR were designed to amplify a region of the nodC specific for *Rlv*. as well as the nodC gene amplification results (Figure 7). PCR analyses done in this study showed that all of the strains that were identified as Rlv produced about a 220 bp DNA fragment using a nodC-specific primer pair.

Conclusions

Salt-stress are the major constraints to plant productivity and isolation of effective rhizobia to inoculate the leguminous crop plants could be an important strategy to improve the efficiency of rhizobium-legume symbiosis and thereby productivity. In our study we isolated of Faba plants thirteen *R. leguminosarum sv. Viciae* isolates, identified by phenotypic and genotypic characteristics.

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