

Molecular and Biochemical Characterization of Some Egyptian Genotypes Rhizobium (*Vicia Faba*) Isolates

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Abstract

Thirteen Rhizobial isolates were recovered from the root nodule of Faba bean (*Vicia faba* L.) grown in different geographic locations and soil properties in Egypt. The tested isolates were identified as *R. leguminosarum* sv. *Viciae* on the bases of morphological, biochemical characteristics and sequences of the gene encoding 16s rRNA. Rhizobium isolates were tested for their ability to utilize different carbon sources. Mannitol and Glucose were the best source of carbon. All tested isolates from *Vicia faba* differ in IAA production. The maximum amount of IAA production was in the range of (2.04 µg/ml) for Al Arish isolate to (7.5 µg/ml) for Ismailia isolate among the studied isolates. A great ability to degrade Roundup herbicide among the tested isolates was observed. Sues City isolate was the best active degrading Roundup herbicide on plates. All of the tested isolates showed resistances to (25 and 50 mg/ml) expect isolate RL7 was sensitive at high Roundup herbicide concentration, while isolates RL5, RL6, RL8, RL10 and RL13 were the most tolerance at 50 mg/ml herbicide. South Sinai, Zefta, Rafah, El-Menia, Cairo and Ismailia isolates shared a common band with mol. wt. of 70 KDa. New protein types were detected due to the differential response of the five isolates to the effects of the environment stress.

Keywords: Faba bean; Carbon sources; IAA; Herbicide tolerance; Protein pattern; 16s rRNA

Introduction

Bacteria belonging to the genera Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium [1] are generally called Rhizobia. They are best known for their ability to establish symbiotic relationships with leguminous plants of agricultural and environmental importance, a process that results in biological nitrogen fixation [2]. Nitrogen-fixing leguminous increase soil fertility and quality by enhancing soil N content, organic matter and soil aggregates [3]. Fundamentally, nitrogen (N) is one of the most vital nutrients required for the synthesis of the vast array of metabolites such as proteins, enzymes, chlorophyll, and nucleic acids and consequently, determines critically the health of all living organisms including microbes and plants [4,5]. Despite the presence of this element in the atmosphere in huge amounts (78%), the growing plants cannot utilize it in the present gaseous form (N₂). For this, the nitrogen fixing microorganisms residing in the specific organs of root systems of leguminous plants have devised a novel strategy to convert the plant-unavailable atmospheric N₂ into plant-utilizable forms (ammonia) through biological N₂ fixation (BNF) using enzymatic machinery known as nitrogenase [6]. BNF represents an economically beneficial and environmentally sound alternative to chemical fertilizers [7]. More than 50% of the applied of nitrogen fertilizers are somehow lost through different processes which not only represent a cash loss to the farmers but also consequently polluted the environment. Scientists all over the world are facing this alarming situation and they are trying to overcome this condition by exploring alternative sources which are cost effective and save the environment. Biofertilizer, an alternative source of N, especially rhizobia in legume symbiosis, is a promising technology. The United Nations Food and Agriculture Organization (FAO) estimates that the total demands for agricultural products will be 60% higher in 2030 than present time and more than 85% of this additional demand will come from developing countries. For over half a century, the world has relied on increasing crop yields to supply an ever increasing demand for food. Pesticides accumulation into soils at an abnormal level causes spectacular changes in composition, diversity

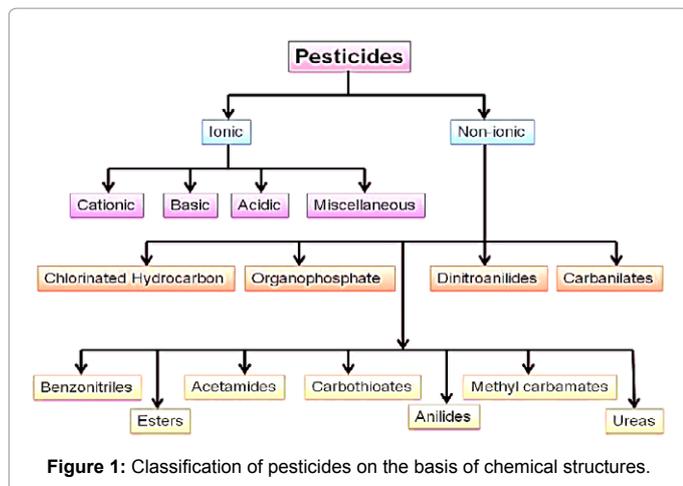
and functional activities of important soil microflora [8]. Round up causes decreased nodule numbers and their dry weight in cowpea roots with no nodule development on side [9]. Generally Pesticides also interact with rhizosphere microorganisms including rhizobia and restrict the root growth, in that way lead to the reduction in the number of the root sites available for the rhizobial infection [10]. Also decreases the total microbial biomass not only adversely affect the growth of bacteria, fungi and actinomycetes [11] by inhibiting protein synthesis, the metabolic enzymes including dehydrogenase and phsphatase. In addition to damaging structural proteins through biochemical alterations in membrane composition and geno-toxicity [12]. Pesticides are divided into categories according to the target organisms they are designed to control e.g., herbicides are used to kill unwanted weeds; insecticides control insects and fungicides eradicate phyto-pathogenic fungi. Pesticides are also classified according to their chemical structures or on the basis of their modes of action (Figure 1). Pesticides inhibit symbiotic signaling between host plants and rhizobia by interfering in the interaction between plant-secreted flavonoids and the rhizobial NodD proteins; thus they effectively block molecular communication and disrupt the initiation of symbiosis [13]. In addition, pesticides not only inhibit the biochemical signaling between the hosts and cognate rhizobia but also block the initial attachment of complementary rhizobia to lectins present on root hairs as the recognition sites by protecting them [14]. Herbicides may have negative effects on growth of rhizobia [15]. Protein profiles [16]

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Isolate No.	locations
Rh1	Quesna City, Al Menoufeya Governorate.
Rh2	Banha City, Al Qalyoubeya Governorate.
Rh3	Sadat City, Al Menoufeya Governorate.
Rh4	Zefta City, Al Gharbeya Governorate.
Rh5	Beni Suef City, Beni Suef Governorate.
Rh6	Sues City, Al Sues Governorate.
Rh7	South Sinai City, South Sinai Governorate.
Rh8	Al Arish City, North Sinai Governorate.
Rh9	Rafah City, North Sinai Governorate.
Rh10	New Al Menia City, AL Menia Governorate.
Rh11	Moshtohor City, Al Qalyoubeya Governorate.
Rh12	Cairo City, Cairo Governorate.
Rh13	Ismailia City, Al Ismailia Governorate.

Table 1: locations of Rhizobium leguminosarium sv. Viciae isolates.

have been studied in rhizobia. Proteins induced in response to stress may suggest that they have an important role in homeostasis and maintenance of vital cellular functions [17]. The SDS-PAGE analysis of whole cell proteins not only helps in identifying of the Rhizobial strains [18,19] but also useful in the differentiation among the isolates within the same group [20]. Also molecular tools for the identification of bacteria are now available and are used routinely in laboratories. The electrophoretic screening of large plasmids, although limiting the analysis to the extra chromosomal elements, has a particular importance in the screening of Rhizobium. These bacteria have large plasmids (> 50 kb in size) that account for a substantial portion of their genome and contain several genes coding for nodulation and nitrogen fixation [21]. These plasmids are often present in variable numbers and sizes; traits that provide the basis for an accurate strain characterization when the extracted plasmids are separated by gel electrophoresis [22]. The principle objectives of this study are isolation and evaluation the genetic diversity of some root-nodule bacteria isolates from Faba bean plants grown in different geographic locations in Egypt based on morphological, biochemical and molecular characteristics to determine the biodiversity among tested isolates. In addition, plasmid profile and herbicide tolerance.

Material and Methods

Collection and isolation of Rhizobium isolates

Thirteen representative sites were chosen from ten Egyptian

governorates as presented in Table 1 and Figure 2. Rhizobial isolates collected from the root nodules of Faba bean (*Vicia faba* L.) plants. Plant roots containing several nodules from each plant and transported in plastic bags (each plant in a separate bag). Bacteria were generally isolated in the same day as plant harvesting.

Isolation of rhizobium isolates

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 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 leguminosarium* were isolated according to [23] methods and restreaked 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.

Purification and storage of rhizobium isolates

Agar plates were incubated at 28 ± 1°C for 3 days. Individual colonies appearing over this period were re-streaked onto YEMA plates, and stored at 4°C until the time of processing.

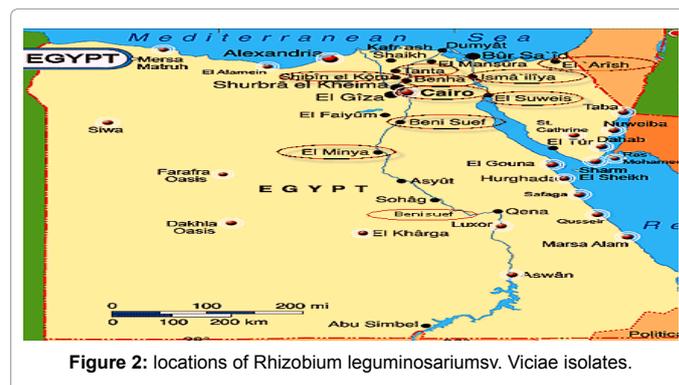
Phenotypic characteristics

Antibiotic resistance pattern: All isolates were evaluated for their responses as resistant or sensitive against 24 different antibiotic disks [24]. Growth rate at different carbon source: All of the Rhizobia isolates were tested for utilization of different carbon sources by replacing mannitol in YEM broth medium with equal amounts of different carbon sources including glucose, fructose, mannose, and maltose to study the effect of different carbon sources on growth of isolates. The growth was measured after 24h and 48h of incubation at 28 ± 1°C.

Roundup herbicide resistance test by agar plate and broth medium

Laboratory experiment: The sensitivity or resistance of herbicide on growth of Rhizobium was determined by two methods.

YEMA: The effect of Roundup on *R. leguminosarium* isolates under laboratory conditions were studied on Petri dishes containing YEM agar. In the first method bacterial sensitivity streak plate method, the



calculated amount of herbicide was mixed with YEMA medium and poured into sterile Petri plates. Plates without herbicide were prepared to use as control. Plates were streaked with broth suspension 10^7 cells ml^{-1} of test organism. The plates for Rhizobium were incubated at 28°C for 4 days, after which growth was examined. Growth was visually grouped into 4 categories: +++ (good), ++ (moderate), + (poor) and – (no growth) at different concentration of 0, 25, 50, 100 mg/ml respectively. Three replicates of Petri dishes were used to each isolate as well as three Petri dishes were used without Roundup as control. The Petri dishes were incubated at 28°C . After 4 days, were inspected to estimate the efficacy of Roundup against Rhizobium isolates.

YEM broth: The second method was bacterial sensitivity broth culture method in 150 ml flasks containing 50 ml YEM, pH of medium was adjusted to 7, and the control cultures were grown at 28°C . The flasks were incubated on rotary shaker for 4 days at 180 rpm. The growth of rate in Rhizobial isolates was measured by recording optical density (OD). The experiment was conducted in three replicates. The turbidity was measured using Jenway UV-VIS. spectrophotometer model UV-6305 at 600 nm against the blank (sterilized uninoculated YEM broth) and the mean generation time was calculated for each isolates.

Biochemical identification of rhizobia

IAA production: All the isolates were tested to IAA production in Yeast Extract Mannitol (YEM) broth [23] supplemented with $100 \mu\text{g/ml}$ L-tryptophan and added to YEM medium without L-tryptophan. The test tubes were covered with brown paper and incubated at 28°C for 5 days on a rotary shaker. The broth was centrifuged at 3,000 rpm for 30 minutes. 2 ml of supernatant was collected and 2-3 drops of o-phosphoric acid were added. The aliquots were shaken and 4 ml of Salkowski reagent (1 ml of 0.5 M FeCl_3 in 50 ml of 35% perchloric acid (HClO_4) was added then vortexed thoroughly. The samples were incubated at room temperature for 25 minutes. Development of a pink color indicates IAA production. IAA concentrations were quantified by Spectrophotometer, the developed pink color from previous reagent was read at 530 nm using spectrophotometer, and concentration of produced IAA was determined from a prepared standard curve of pure IAA ($10\text{-}100 \mu\text{gml}^{-1}$) [25]. Uses different IAA concentrations are prepared as aqueous solution of IAA ranging from 10 microgram/ml to 100 micrograms/ml, each 1ml of the standard, 2 ml of 2% 0.5 M FeCl_3 in 35% perchloric acid (Salkowski) reagent is added and readings are taken after 25 minutes at 530 nm by UV-Visible spectrophotometer.

Catalase test: The catalase enzyme decomposes hydrogen peroxide (H_2O_2) to water and O_2 . Hydrogenperoxide is very toxic for bacteria. Bacteria are smeared in a drop of hydrogen peroxide and checked for formation of oxygen gas bubbles.

Protein profile: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Glass slabs and spacers were washed with water and wiped with tissue paper. Rhizobia were grown on yeast extract mannitol broth [23] at 28°C for three days. According to [16] protein was extracted, Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as described previously [26].

DNA extraction and amplification of 16S rRNA Gene of rhizobia

DNA was extracted from bacterial cultures using SDS/CTAB Lysis and phenol/chloroform extraction method [27]. Sequence analysis of 16S rDNA and subsequent BlastN analyses indicated that the majority of isolated strains were Rlv [28].

Plasmid isolation from Rhizobium: Plasmid DNA was isolated by the method of [29].

Restriction fragment analysis: The extracted plasmids were digested with EcoRI and MSPI enzymes according to (Thermo scientific fermentase) as following: Combine the following reaction components at room temperature in order indicated: Water, nuclease-free $15 \mu\text{l}$, 10x FastDigest or 10x FastDigest Green Buffer $2 \mu\text{l}$, DNA $2 \mu\text{l}$, FastDigest enzyme $1 \mu\text{l}$, Total volume $20 \mu\text{l}$, Mix gently and spin down, incubate at 37°C in a heat block or water thermostat for (5-15) min and load an aliquot of the reaction mixture in 2% agarose with TBE (Tris -Boric-EDTA) buffer. Electrophoreses were carried out at 100 V for 1 h. Gels were stained with ethidium bromid"EtBr" ($2.5 \text{mg}/10 \text{ml}$) and photographed under UV illumination.

Statistical analysis

The data were subjected to one-way ANOVA using SPSS (2002) analysis program (version 11.5). The significant differences among individual means were analyzed by Duncan's multiple range tests and statistical significance was determined at 5% level.

Results and Discussion

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 isolates were tested by Congo red technique according to [30]. To ensure that all isolates were rhizobia and did not contaminated. 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. Antibiotic resistance pattern: All isolates were evaluated for their responses as resistant or sensitive against 24 different antibiotic disks [24].

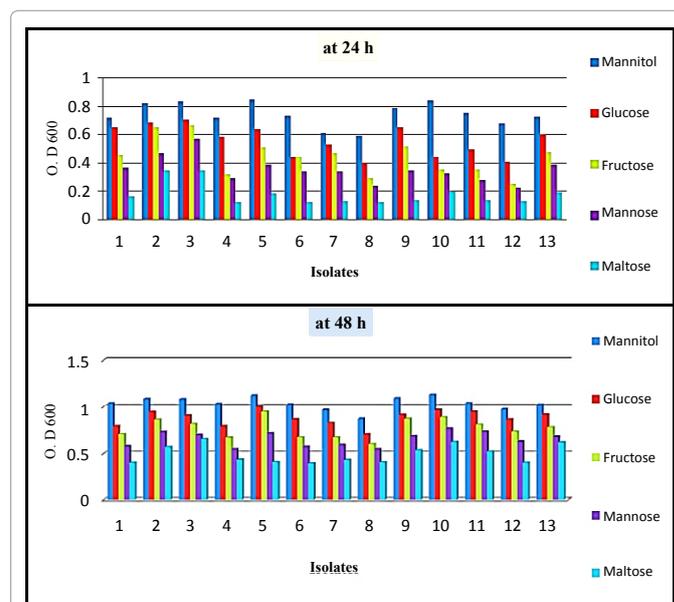


Figure 3: Effect of different carbon sources on growth of Rhizobium leguminosarum s.v. Vicia on YEM broth medium after 24 and 48 h from inoculation.

Time (h)	Carbon Sources	Rhizobium leguminosarum sv. Vicia isolates												
		1	2	3	4	5	6	7	8	9	10	11	12	13
24	Mannitol	0.720a	0.820a	0.836a	0.720a	0.843a	0.730a	0.611a	0.589a	0.786a	0.838a	0.751a	0.681a	0.726a
	Glucose	0.648a	0.686a	0.708ab	0.584b	0.634b	0.436b	0.528ab	0.395b	0.65ab	0.440b	0.492b	0.407b	0.598ab
	Fructose	0.453b	0.649a	0.658ab	0.313c	0.505bc	0.442b	0.465b	0.292d	0.512b	0.358b	0.354c	0.254c	0.477bc
	Mannose	0.366b	0.466b	0.571b	0.290c	0.387c	0.342b	0.341c	0.236d	0.350c	0.323bc	0.276c	0.223cd	0.387c
	Maltose	0.162c	0.349b	0.349c	0.121d	0.183d	0.121c	0.128d	0.121c	0.133d	0.194c	0.134d	0.126d	0.184d
48	Mannitol	1.039a	1.085a	1.081a	1.032a	1.120a	1.025a	0.966a	0.875a	1.092a	1.128a	1.040a	0.973a	1.022a
	Glucose	0.789b	0.945ab	0.910ab	0.789b	1.00a	0.871ab	0.827ab	0.707ab	0.914ab	0.966ab	0.950ab	0.867ab	0.918ab
	Fructose	0.708bc	0.866ab	0.810bc	0.673bc	0.947ab	0.675bc	0.675bc	0.597 b	0.875ab	0.890ab	0.805ab	0.732bc	0.779bc
	Mannose	0.576cd	0.730bc	0.700bc	0.544cd	0.717b	0.570cd	0.59cd	0.544bc	0.688bc	0.765bc	0.735bc	0.623c	0.682c
	Maltose	0.397d	0.568c	0.658c	0.428d	0.405c	0.391d	0.426d	0.402c	0.533c	0.618c	0.521c	0.398d	0.614c

a, b, c=Means of each trait within each column don't have the same letter are significantly different significantly at 0.05 level

Table 2: Estimation Growth of Rhizobium leguminosarum sv. Vicia isolates using different carbon sources after 24 and 48 (h) from inoculation.

Growth rate at different carbon source: The results of carbohydrate utilization by Rhizobium are summarized in Table 2 and Figure 3. All the thirteen Rhizobium isolates were tested to utilize a wide range of carbohydrates as a sole carbon source. The Rhizobium isolates in this present study also preferred monosaccharides as well as mannitol for proper growth [31]. In the present study, the Rhizobium isolates from *V. faba* showed better growth with mannitol than glucose, fructose, mannose and maltose when mannitol was replaced with four other carbohydrates in equal quantities. Our results showed that Rhizobia prefer mannitol and glucose as best carbon sources for growth [32]. Furthermore all the tested isolates were able to utilize almost all tested carbon sources in this investigate. All of the isolates obtained from *Vicia faba* nodules were able to utilize mannitol, glucose, Fructose, Mannose and Maltose as carbon sources. These carbon sources are generally utilized by bacteria of the genus Rhizobium [33]. The ability of the isolates to utilize a broad range of carbon substrates is also related to the survival of the isolates under acidic environments. Generally, Mannitol and Glucose were the best source of carbon. Mannitol measurements were ranged from (O.D 1.128) with RL10 isolate followed by (O.D 1.120) with RL5 isolate to (O.D 0.875) with RL8, which showed the lowest growth among the studied isolates. Also Glucose measurements were ranged from (O.D 1.00) with RL5 isolate followed by (O.D 0.966) with RL10 isolate to (O.D 0.707) with RL8 (Figure 4). On other hand Maltose measurements were ranged from (O.D 0.658) with RL3 isolate followed by (O.D 0.618) with RL10 isolate to (O.D 0.391) with RL6.

Roundup herbicide resistance test by agar plate and broth medium

It is clear that the higher dose of Roundup herbicide inhibited the growth of Rhizobium and we observed varied growth in vitro under different herbicide treatments between the tested isolates. In broth culture method, untreated rhizobia (control) had the highest OD (Figure 5). The OD (240) recorded with lower concentration of Roundup with isolate No.11 compared with control. However, The OD (364) recorded with lower concentration of Roundup with isolates No.2 and No.3. Further to the OD (0.048) recorded with highest concentration of Roundup with isolate No.7. However, The OD (0.117) recorded with highest concentration of Roundup with isolate No.13. Compared with control (without Roundup Herbicide), whereas growth was visually grouped into 4 categories: +++ (good), ++ (moderate), + (poor) and - (no growth) at different concentration of 0, 25, 50, 100 mg/ml respectively in solid media (Tables 3 and 4). Growth inhibition was shown at highest concentration. Generally, the higher concentration of herbicide has higher toxicity to the bacteria. Therefore, the number of colonies tolerant to Roundup decreased when concentration increased. About 38% of isolates were tolerant to the higher concentration.

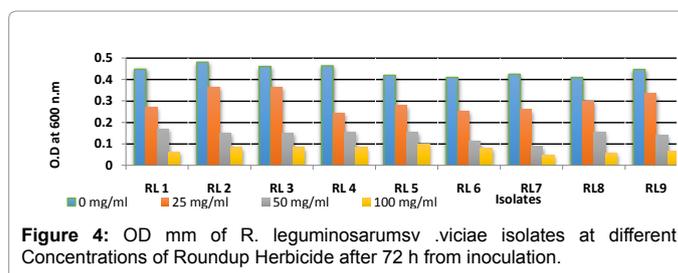


Figure 4: OD mm of *R. leguminosarumsv. viciae* isolates at different Concentrations of Roundup Herbicide after 72 h from inoculation.

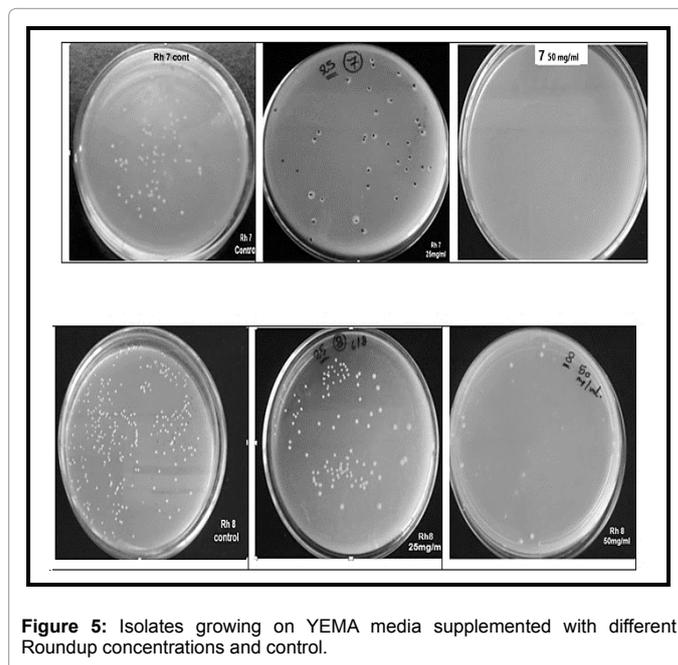


Figure 5: Isolates growing on YEMA media supplemented with different Roundup concentrations and control.

This experiment resulted in selected Rhizobium strains tolerant to environment stress i.e., high concentration of Roundup herbicide. Isolates from different locations in this study showed a great ability to degrade Roundup herbicide. They differed in their ability to Roundup degradation. It is clear that Ismailia City isolate was the most active degrading Roundup in this investigates as shown in Table 3. All isolates are tolerant to 25 mg/ml concentration of herbicide. All used isolates are very sensitive to 100 mg/ml concentration of herbicide and intermediate for 50 mg/ml concentration of herbicide. All Isolates showed inhibition of growth by increasing concentration of herbicide. All isolates grew on YEMA media [23]. The results of different

Isolate No.	Control	Roundup herbicide		
		25 mg/ml	50 mg/ml	100 mg/ml
1	+++	++	+	-
2	+++	++	+	-
3	+++	++	+	-
4	+++	++	+	-
5	+++	++	+	-
6	+++	++	+	-
7	+++	++	-	-
8	+++	++	+	-
9	+++	++	+	-
10	+++	++	+	-
11	+++	++	+	-
12	+++	++	+	-
13	+++	++	+	-

Whereas growth +++ (good), ++ (moderate), + (poor) and - (no growth)

Table 3: Response of Rhizobium isolates to different concentrations of Roundup herbicide.

Isolates	Herbicide concentrations mg/ ml			
	0	25	50	100
RL 1	0.448a	0.271bcd	0.171abc	0.063efg
RL 2	0.481a	0.364a	0.152bcd	0.084 bcd
RL 3	0.461a	0.364a	0.152bcd	0.084bcd
RL 4	0.464a	0.243d	0.157abc	0.084bcd
RL 5	0.420a	0.280bcd	0.154bcd	0.100ab
RL 6	0.410a	0.254d	0.115de	0.080bcde
RL 7	0.425a	0.260cd	0.088e	0.048g
RL 8	0.410a	0.300abcd	0.155abc	0.057fg
RL 9	0.447a	0.334abc	0.140cd	0.067defg
RL 10	0.475a	0.340ab	0.178abc	0.090bc
RL 11	0.431a	0.240d	0.154bcd	0.080bcde
RL 12	0.480a	0.251d	0.194a	0.070cdef
RL 13	0.500a	0.341ab	0.184ab	0.117a

a,b,c=Means of each trait within each column don't have the same letter are significantly different at 0.05 level (Same Colum)

Table 4: Effect of herbicides on growth after 72 hr. (measured as optical density) of Rhizobium isolates.

concentrations of herbicide effects on Rhizobial growth showed that 25 mg/ml concentration was the best concentration for all isolates growth; there was no growth at all at 100mg/ml for all isolates. As consequently different concentrations of herbicide decrease in the growth of rhizobia untreated rhizobia had the highest O.D. and each successive increase in the concentration of herbicide decreased the O.D. These results are in agreement with those obtained by [9] who studied the effect of different herbicides on the nodulation and growth of Rhizobial isolates. Also some studies have evaluated the effect of different herbicides on Rhizobium growth and nitrogen fixation activity. The effect depends on the herbicide, its concentration, and different weather conditions. Applied research methodology also may depend on the Rhizobium or Bradyrhizobium species and even the strain used [34].

Biochemical identification of rhizobia

IAA production: All isolated Rhizobia showed variations in red colour reaction with salkowaski reagent indicating their variations

ability to produce IAA. Comparison between the isolated Rhizobia showed variation for their ability to produce IAA. Rhizobium isolates from *Vicia faba* differ in IAA production. In majority of isolates; the amount of IAA produced was more than that in the control. The maximum amount of IAA production was in the range of 2.04 for isolate No.8 from Al Arish City to 7.5 µg/ml for isolate No.13 from Ismailia City in all the isolates studied. Comparison of means shows that there is a difference between isolates. Indole -3-acetic acid (IAA) is the common natural auxin, to produce auxin. This substance can be converted to IAA by soil beneficial bacterial activities. Tryptophan (L-trp) is physiologically a precursor of auxin biosynthesis in higher plants and microorganism [35]. Various reports showed Tryptophan derived microbial auxins present in the rhizosphere area play a very important role in growth and development of plant rooting system and crop yield [36]. Many of rhizobial species enable to produce IAA. Some studies shows that auxins play a key role in creating nodule in leguminous plants and generally, in establishing a symbiotic association with rhizobia. Also has been proved that flavonoids, promoting nodulation gene, increase the production of IAA by rhizobial bacteria [37]. Rhizobia are known to produce significant levels of IAA both in free living conditions and also symbiotically in nodules [38], (Table 5 and Figure 6).

Catalase test: Results present in Table 6 and Figure 7 revealed that the biochemical characteristics of Rhizobium isolates showed positive production of Catalase enzyme, by all of the tested isolates with exception of isolate RL 3 and RL 12. The genetic diversity among thirteen isolates representing the indigenous population of rhizobia was investigated using SDS-PAGE pattern. SDS-PAGE profile of protein extracted from thirteen *R. leguminosarum* isolates (Table 7, Figures 8 and 9). A dendrogram developed from similarity between the

Bacterial isolates	IAA Production (µg/ml)	O.D at 530 nm
1	3.18	0.057
2	3.47	0.063
3	4.56	0.086
4	4.13	0.077
5	3.50	0.065
6	3.30	0.056
7	3.20	0.058
8	2.04	0.033
9	3.30	0.060
10	2.30	0.039
11	4.90	0.094
12	6.57	0.128
13	7.50	0.148
control	1.28	0.017

Table 5: Mean comparison of IAA production Bacterial isolates using O.D.

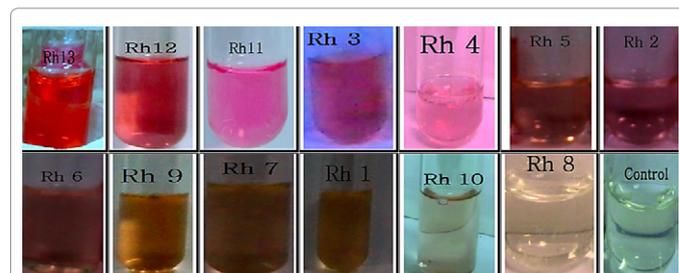


Figure 6: IAA production assay of the bacterial isolates in vitro by Salkowski assay.

Rhizobial isolates												
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
+	+	-	+	+	+	+	+	+	-	+	-	+

Whereas, (+) means resistance and (-) means sensitive

Table 6: Effect of different isolates on Catalase production.

Rh1	Rh2	Rh3	Rh4	Rh5	Rh6	Rh7	Rh8	Rh9	Rh10	Rh11	Rh12	Rh13
1.00												
1.00	1.00											
1.00	1.00	1.00										
0.90	0.90	0.90	1.00									
1.00	1.00	1.00	0.90	1.00								
1.00	1.00	0.90	1.00	1.00	1.00							
1.00	1.00	1.00	0.90	1.00	0.81	1.00						
0.81	0.81	0.81	0.72	0.81	0.90	0.81	1.00					
0.90	0.90	0.90	0.81	0.90	1.00	0.90	0.90	1.00				
1.00	1.00	1.00	0.90	1.00	1.00	1.00	0.81	0.90	1.00			
1.00	1.00	1.00	0.90	1.00	1.00	1.00	0.81	0.90	1.00	1.00		
1.00	1.00	1.00	0.90	1.00	1.00	1.00	0.81	0.90	1.00	1.00	1.00	
1.00	1.00	1.00	0.90	1.00	1.00	1.00	0.81	0.90	1.00	1.00	1.00	1.00

Table 7: Similarity between different tested isolates based on Protein banding pattern analyses.

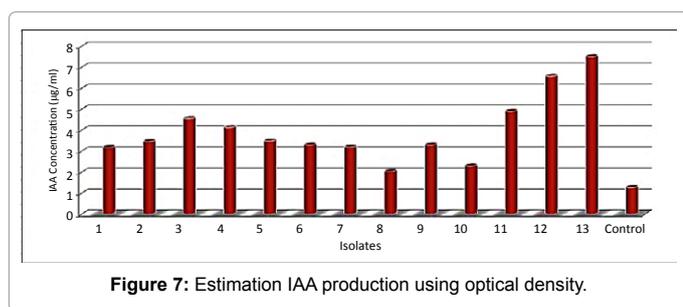


Figure 7: Estimation IAA production using optical density.

Isolate No.	Control	Roundup herbicide		
		25 mg/ml	50 mg/ml	100 mg/ml
1	120	50	30	0
2	140	40	30	0
3	100	40	30	0
4	130	50	30	0
5	120	70	40	0
6	120	80	60	0
7	70	36	0	0
8	120	100	40	0
9	140	30	30	0
10	150	80	50	0
11	100	50	30	0
12	120	50	30	0
13	150	90	50	0

Table 8: Effect of different concentrations of Roundup herbicide on number of colonies at Rhizobium Leguminosarum sv.vicia (107cells ml⁻¹).

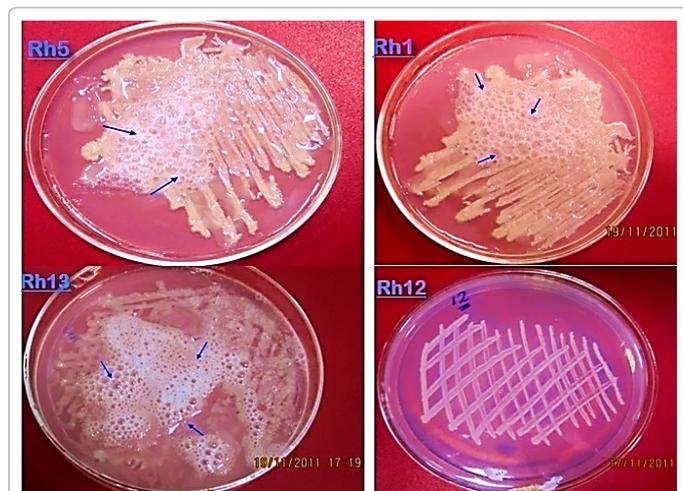


Figure 8: Estimation catalase production using H₂O₂. Protein banding pattern of thirteen Rhizobial isolates.

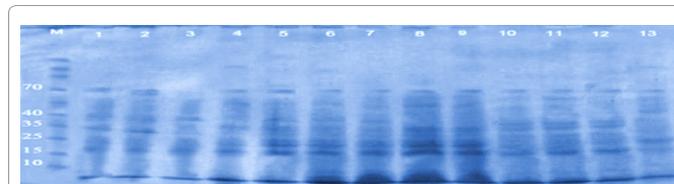


Figure 9: Stained SDS-PAGE pattern of total soluble protein of thirteen Rhizobial isolates from different locations. M, molecular weight standard (PageRulerTM protein ladder, Fermentas).

different isolates based on Protein banding pattern formed two groups of proteins were identified in Rhizobium leguminosarum sv vicia isolates (Table 8). The first group contained eleven isolates, whereas the second group contained remains isolates RL8 and RL9 located in South and North Sinai respectively. It showed a total of 78 bands with different molecular weight values ranged from 130 to 15 KDa. The protein bands of the tested isolates varied in numbers of bands.

The electrophoresis banding pattern of thirteen R. leguminosarum isolates showed either absence or presence of different polypeptides in this study. One band was for only one isolate RL4 (130 KDa), some of these bands appeared in each of the studied isolates such as 70, 40, 35, 25, 20 and 15 KDa which were considered monomorphic bands. Whereas each isolate contained at protein banding range 5-7 bands. Our results were agreement with [39] who mentioned that protein patterns, peroxides isozymes and DNA fingerprints were used for analysis of somaclonal variations among three R. leguminosarum

isolates to determine Rhizobium genetic diversity under environment stress. Preparation of total protein from all rhizobial isolates showed seventy eight bands ranged from (15 to 130 KD), these bands were not necessarily present in all isolates. Among tested Rhizobium isolates there were some variations in protein banding pattern. As presented in Figure 10. There were sixty five bands common in all Rhizobial isolates and thirteen bands showed variability among them. This may be due to the differences of their genetic background according to the different locations; similar conclusion was also obtained by [40] who stated that Rhizobial isolates could be distinguished by protein banding pattern. Our results indicate that under salt stress, SDS-PAGE revealed a powerful characterization of method Rhizobium leguminosarium genotypes. On the other hand, phenotypic and genotypic diversity among tested strains play important role for their tolerance or sensitive stress. The results finding is in agreement with [41] that used protein pattern to analyze the genetic variations among three Rhizobium leguminosarium and observed variation in protein banding pattern (10, 12 and 11 polypeptide bands). This study was conducted to employ SDS-PAGE as simple tool to determine the genetic variability among R. leguminosarium isolates (Figure 11).

Plasmid isolation from Rhizobium

Plasmid profile of Rhizobial isolates were presented in Figure 12. Results showed variation in number of bands based on variation in size

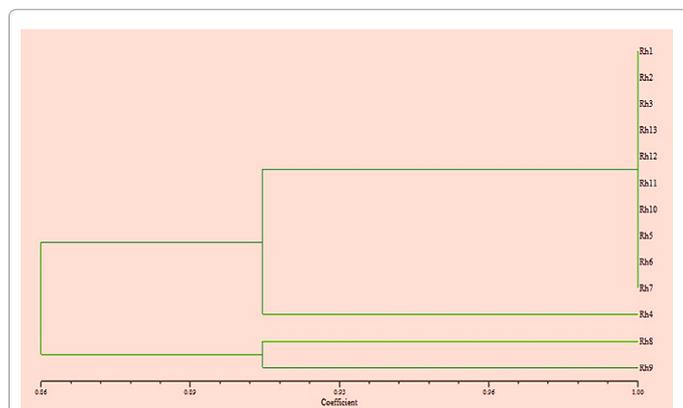


Figure 10: Phylogenetic relationship between 13 Rhizobial isolates using UPGMA method based on Protein banding pattern analyses.

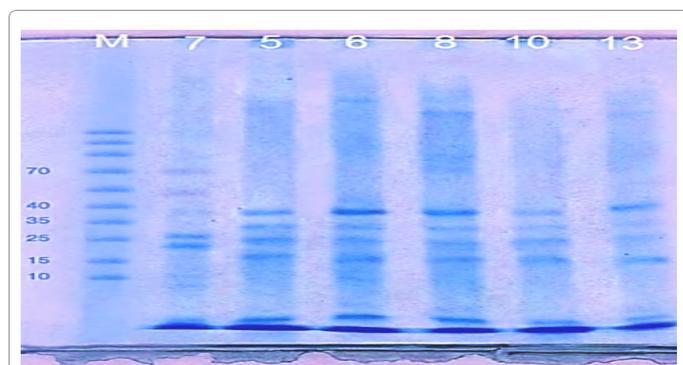


Figure 11: Stained SDS-PAGE pattern of total soluble protein in RL7, 5, 6, 8, 10 and 13 grown in YEM medium and incubated with 50mg/ml Roundup herbicide concentration, sensitive isolate (lane 7) and tolerant isolates (lane 5, 6, 8, 10 and 13), M, molecular weight standard (PageRulerTM protein ladder, Fermentas).

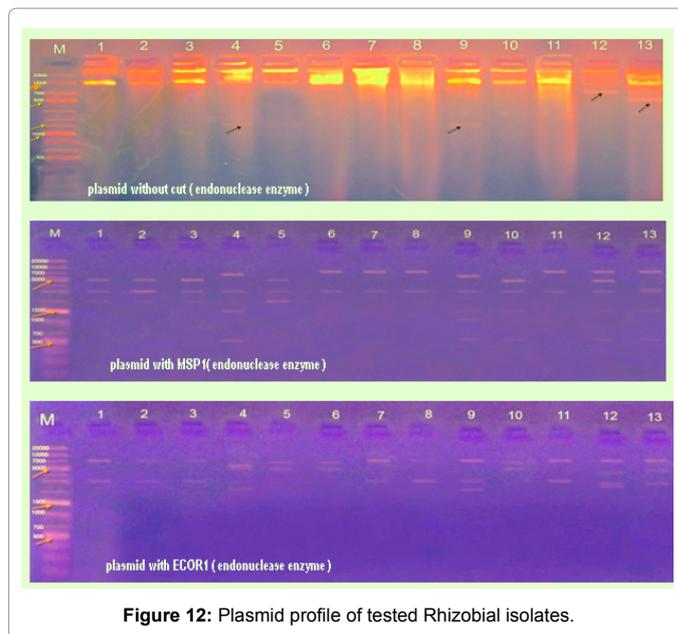


Figure 12: Plasmid profile of tested Rhizobial isolates.

of plasmids. Plasmid genotypes of rhizobia nodulating Faba bean from various Egyptian locations were extracted and characterized. Plasmid profile analysis revealed different plasmid types having differ of size ranging from 10 to 0.5 Kb pairs. A variation in number of symbiotic (Sym) plasmid was observed. The size of these plasmids showed marked diversity among the isolates and varied in size from 10 to 0.5 Kbp. Isolates were characterized according to their plasmid profiles. All studied isolates contained a 10 Kbp plasmid and four isolates RL4, RL9, RL12 and RL13 contained additional small plasmid with molecular weight about 2 Kbp, 2 Kbp, 7 Kbp and 5 Kbp respectively as shown in Figure 12. These data are parallel to that achieved by [42-44] who found that R. leguminosarium sv. *Viciae* strain T83K3 which had six plasmids of 480, 440, 300, 255, 210 and 155 kb.

Conclusion

This research determines the phenotypic diversity of the rhizobia that nodulates *Vicia faba* through their growth characteristics, e.g. carbon source utilization. This could lead to an improvement in the selection of strains that efficiently nodulate and fix nitrogen and may be used as inoculants for their host plant.

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