Characterization and evaluation of rhizobium isolates from vicia faba for some plant growth promoting traits

Ismael, Rabab, R¹, Omar, M.N.A¹, Azzam, Clara, R², Ahmad, Enas, S³,4 Abdel-Fattah, M³ and Zahran, H.H³.

¹Department of Microbiology, Research Institute of Soils, Water and Environment, research institute, Agricultural Research Center, Giza, Egypt
²Department of Cell Research, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt
³Department of Botany and Microbiology, Faculty of Science, University of Beni-Suef, Beni-Suef, Egypt.
4Department of biology, Faculty of education, Majmaah University, Al-Majmaah, Saudi Arabia.

*Correspondence: Clara_azzam@yahoo.com Accepted: 18 Aug. 2018 Published online: 23 Nov. 2018

The aim of this work was to characterize and identify the root-nodule bacteria isolated from broad bean (Vicia faba) plants grow in the cultivated lands of Beni-Suef Governorate, Egypt. The cultural, biochemical, and molecular characteristics were determined. Two isolates were able to grow at 6% NaCl. Four isolates showed catalase, urease and gelatinase activity, two isolates have ability for ammonia production, and only one isolate that could produce Siderophore and HCN. Three isolates were capable of solubilizing phosphate. In the presence of tryptophan, the four isolate (RV1, RV2, RV3 and RV4) produced indole acetic acid (134, 90, 154 and 75 µg/ml), respectively, in the growth medium. All isolates were capable of inhibiting the growth of pathogenic fungal strains of Fusarium oxysporum and Rhizoctonia solani. The strains from V. faba had 99% identity with Rhizobium leguminosarum. Increasing of salt concentration decreased growth, nodulation and nitrogen fixation. The present study reveals that Rhizobium leguminosarum strains (RV1 and RV3) has a growth-promoting characters under salt stress and can be used as a biofertilizer inoculant beneficial for broad bean cultivation.

Keywords: Salt stress, broad bean (Vicia faba) Rhizobium, 16SrDNA gene, nodulation, N₂ fixation.

INTRODUCTION

High salinity is one of the major environmental stress factors that reduces growth and significantly limits crop productivity (Acosta-Motos et al., 2015). Increasing of salt concentration decreased growth, nodulation and nitrogen fixation in legumes. About 20% of the world's cultivated land is affected by salinity, which results in the loss of 50% of agricultural yield (Shrivastava and Kumar 2015). Salt stress causes ion toxicity due to increased concentration of Na and Cl ions. Salinity adversely affected plant growth and development, hindering seed germination, seedling growth, enzyme activity (Seckin et al., 2009), DNA, RNA, protein synthesis and mitosis (Javid et al., 2011). Salinity also affects photosynthesis mainly through a reduction in leaf area, chlorophyll content and stomatal conductance, and to a lesser extent, through a decrease in photosystem II efficiency (Netondo et al., 2004). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that colonize roots of plants actively and increase plant growth and development (Bhattacharyya and Jha 2012, Gupta et al., 2015). Different bacteria that have
been reported as PGPR belong to the genera *Pseudomonas*, *Bacillus*, *Azospirillum*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, *Enterobacter* and *Phyllobacterium*. The PGPR promote plant growth directly by phosphate solubilization, production of phytohormones (Indole-3-acetic acid, IAA), nitrogen fixation, production of deaminase and Siderophores or indirectly by their biocontrol properties such as antibiotic production, production of lytic enzymes, competition with phytopathogens for nutrients and colonizing sites, and induced systemic resistance (Omara et al., 2017).

Rhizobia are soil bacteria able to form nodules on roots or stems of leguminous plants. Rhizobia comprise diverse members of the class Proteobacteria, and have large number of species (up to 180 species (Velázquez et al., 2017). The rhizobia Belongs to eight recognized genera of alpha Proteobacteria, Including *Allorhizobium Azorhizobium*, *Brdyrrhizobium*, *Mesorhizobium*, *Neorhizobium*, *Pararhizobium Rhizobium* and *Sinorhizobium (Ensifer)*, beside another nine genera of rhizobia-like bacteria, in the alpha, beta and gamma subclasses of Proteobacteria (Velázquez et al., 2017, Zahran 2017). The ability of rhizobia to fix nitrogen reduces significantly the use of chemical fertilizers in agriculture. In fact, nearly 90% of the nitrogen needs by plants are well satisfied and the soil is enriched with nitrogen that will be used by subsequent crops, with no need to add chemical fertilizers (Gopalakrishnan et al., 2015). In this study some rhizobial isolates were investigated for their N₂ fixation and growth-promoting activities and for possible use as a biofertilizer inoculant.

**MATERIALS AND METHODS**

**Colony morphology and biochemical tests**

Root-nodule bacteria were isolated from healthy nodules of *V. faba* plants collected from the farm of Agricultural Research Centre (ARC) at Sids (Beni-Suef Governorate). After nodules were surface-sterilized, individual nodules were crushed in sterile distilled water. One loopful of each nodule suspension was streaked into plates of yeast-extract mannitol agar (YMA) (Vessey and Chemining Wa 2006). This medium comprised the following constituents in (g/l): K₂HPO₄ 0.75, MgSO₄.7H₂O 0.2, NaCl 0.2, yeast-extract 0.2, mannitol 10, pH was adjusted to 6.8. The medium was supplemented with Congo red (0.0025 % w/v). Plates were incubated at 28 °C for 3-5 days then single colonies were selected and each isolate was re-streaked for purification. The bacterial isolates were stored on YMA slant tubes for further investigations. All bacterial isolates were maintained in YEM broth containing 20% glycerol at -70 °C. Bacterial identification of the isolates was confirmed by a series of morphological, physiological, biochemical, molecular tests.

**Assimilation of different carbon and nitrogen sources; and production of extracellular polysaccharides (EPS)**

The yeast-extract mannitol medium (YEM) was used as a control for estimating the effect of carbon sources on the growth of rhizobia isolates and production of EPS. Different carbon sources were used; they are glucose, fructose, sucrose, sodium acetate, maltose, mannose, lactose and xylose. They were supplemented at the rate of 1% to YEM medium instead of mannitol. Different nitrogen sources such as ammonium sulphate, sodium nitrate, ammonium phosphate, glycine, B-alanine, and glutamic acid were added separately to basal (YEM) medium containing the most suitable carbon source. The medium was inoculated with rhizobia isolates and grown at 28 °C for three days. The growth was measured spectrophotometrically (Jenway Model 6300) at 610 nm, then EPS was isolated and estimated. For quantitative determination of EPS, isolates were grown in 250 ml conical flasks containing 100 ml YEM medium incubated at 28 °C on rotary shaker for 72h. EPS was extracted by the method of Qurashi and Sabri (2011) with some modifications. After the incubation period, bacterial cultures were centrifuged at 10,000 rpm for 20 min at 4 °C. The EPS fraction from the bacterial supernatant was precipitated using three volumes of pre-chilled acetone to unit volume of bacterial supernatant. Precipitated EPS was separated by filtration with the help of Whatman filter paper. The filter paper was allowed to dry overnight at 50 °C and was reweighed after overnight drying. The increase in the weight of the filter paper represents the EPS produced.

**Assays for growth promoting abilities of isolates**

All bacterial isolates were screened for indole-3-acetic acid production, phosphate solubilization, antifungal activity, Siderophore production, and HCN production. The methods for evaluating plant growth-promoting rhizobacteria traits are recently

reviewed (Castellano-Hinojosa and Bedmar 2017).

Quantitative estimation of indole-3-acetic acid (IAA)

Five ml of filtered sterilized 0.5% L-tryptophan solution was added to autoclaved medium. Each flask was inoculated with bacterial strain after incubation at 28 °C for 48 h on shaking incubator. After incubation period, the content was filtrated through Whatman filter paper No 2, and then 3 ml of filtrate was mixed with 2 ml of Salkowski reagent (50 ml of 35% perchloric acid, 1 ml of 0.5 M FeCl₃ solution) in the ratio of 1:1. as described by Sarwar et al., (1992) and allowed to stand for 30 mint for color development. Pink color indicates the production of indole-3-acetic acid, and its optical density was recorded at 530 nm. Concentration of IAA produced was estimated against standard curve of IAA.

Ammonia production

Isolates were grown in peptone water broth for five days at 28 °C. A 0.2 ml culture supernatant was mixed with 1 ml Nessler’s reagent and volume of this mixture was made up to 8.5 ml by addition of ammonia free distilled water. Development of brown to yellow color is an indication for ammonia production (Cappucino and Sherman 1992).

Qualitative screening of hydrocyanic acid (HCN)

For the qualitative estimation of HCN production, picrate assay described by Castric (1975) was used. Bacterial isolates were streaked on nutrient broth. A Whatman filter paper No. 1 (soaked in solution of 2% Na₂CO₃ in 0.5% picric acid) was placed in-between base and lid of the culture-plate. Plates were sealed with parafilm and incubated at 28 °C for 96 h. Production of HCN was indicated by color change of filter paper from yellow to orange-brown.

Inorganic phosphorus (Pi) solubilization assay

Bacterial strains were tested for phosphate solubilization by an agar assay method using sterilized Picovskaya’s media (Nautiyal 1999). The medium was poured into sterilized Petri plates and after solidification was inoculated with bacterial strains. Plates were then incubated at 28 °C for 5 days. After incubation, phosphate solubilization activity was determined by the development of the clear zone around the bacterial colony.

Siderophore production

Siderophore productions of the bacterial strains were detected as described by Ahmad et al., (2008) using chrome azurol S (CAS) medium. The autoclaved CAS medium was poured into Petri plates and spot inoculated with bacterial isolates and incubated for 5 days at 28 °C. Development of a clear yellow zone around the bacterial colony was considered as positive for the production of Siderophore.

Inhibition of pathogenic fungi (antifungal activity)

Effect of the rhizobial strains against the following species of plant pathogenic fungi, R. solani and F. oxysporum was evaluated as indicated by Shoebitz et al., (2009). A 0.9 cm diameter agar plugs containing fungal mycelium was placed near the border of plate containing PDA medium (250 g potato; 10 g dextrose; 15 g agar per liter of water), then the rhizobial strain to be tested was streaked in a straight line to the opposite side of the plate. The inhibitory effect on fungal growth was checked every 2 days for 12 days at 28 °C, and the percentage of inhibition relative to the control (without bacteria) was evaluated. Inhibition of mycelial growth was estimated following the formula % Inhibition = [(Gc- Gs) / Gc] x 100, where, Gc= diameter of control mycelial growth and Gs = diameter of the bacterial growth.

Effect of salt on growth of bacteria

Tubes of YEM broth having the variable concentrations (0, 1, 2, 3,4,5,6, and 7%) of salt (sodium chloride) were inoculated with pure rhizobial culture suspension and incubated at 28 °C for 48 h with constant shaking at 120 rpm. Growth was measured as optical density at 610 nm using spectrophotometer (Jenway Model 6300).

Molecular characterization of rhizobial strains

DNA Extraction

Genomic DNA was isolated from bacterial cells (rhizobial isolates) using the Gene JET plant genomic DNA purification kit (Thermo Scientific) following the manufacturer’s instructions (Zahran et al., 2013). After 48 hours of incubation at 28°C in YEM medium, bacteria from 4 ml of the culture were collected by centrifugation at 12000 rpm for 3-4 minutes. The bacterial biomass was washed with TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and suspended in 300 μl TE buffer. Later, 100 μl
of 5% SDS and 100 μl proteinase E (2.5 mg ml⁻¹ in TE buffer) were added. After mixing, the solution was incubated over-night. The DNA was then mixed with 300 μl of Tris-equilibrated phenol solution. The molecular mixture was centrifuged at 15,000 rpm for 3 minutes. The DNA was further purified with 300 μl of isopropanol, and centrifuged at 15,000 rpm for 5 minutes. DNA from the aqueous phase was precipitated with 2.5 volumes of absolute ethanol. The samples were centrifuged for 10 minutes at 14,000 rpm at 4°C. The resulting DNA pellet was washed with 70% ethanol. After centrifugation for 15 minutes at 13,000 rpm at 4°C and removal of the liquid phase, the DNA pellets were suspended in 25 μl of sterile Milli-Q water, and finally kept at 4 °C for use and at -20 °C for long term work.

**PCR amplification of 16SrDNA sequencing and analyses**

The 16SrDNA was amplified using the primers 41F (5-GCTCAAGATTGACGCCGCG-3), and 1483r (5-CCGTTACCTT GTTA CGACTTCACC-3). PCR tubes consisted of 25 μl Emerald Amp GT PCR Master Mix (Takara Bio Inc.), 2 μl of each primer forward and reverse, 5 μl DNA template and the volume was completed to 50 μl with sterile distilled water. The amplification protocol was carried out as follows: denaturation at 94°C for three min 35 cycles each consists of the following: denaturation at 94 °C for 30 sec, 60°C for 30 sec; primer annealing for two min, according to GC ratio of each primer and incubation at 72°C for two min for DNA polymerization. Then, 72°C for 5 min, at the end, the PCR product kept at 4°C till analysis.

The purified PCR product was sequenced through Sanger sequencing technology, using the ABI prism sequencer 3730 (Applied Bio systems, USA), following the manufacturer's protocol. To identify the related strain, the obtained sequence was subjected to a BLAST search using the NCBI database. The sequence was aligned using Clustal W software ([Thompson et al. 1997] and a phylogenetic tree was constructed by a distance (neighbor-joining) method using MEGA version 6 software (Tamura et al., 2013). Phylogenetic tree was carried out to know the final highest percentage of similarity for each isolate with the type strains.

**Effect of salt concentration on growth, nodulation and N₂ fixation (Nitrogenase activity); and chlorophyll and nitrogen content of V. faba plants**

Seeds of broad bean V. faba (Giza 843), obtained from Agricultural Research Center were surface sterilized by dipping them in 95 % ethanol solution for 2 min, followed by immersion in a 0.2 % HgCl₂ solution for 3 min. The seeds were then rinsed with sterile deionized water. Surface-sterilized seeds were inoculated with each respective rhizobial strain RV1, RV2, RV3, and RV4, which isolated from nodules of broad bean, by dipping in broth inoculated with the rhizobial strain, for 5 min, and then, five seeds were sown in pots containing 4 kilograms sterile sand soil. After germination, the plants were thinned to three plants per pot. Pots were supplemented with about 1.5 mM KNO₃ which equivalent to half of the field dose (7.5 Kg KNO₃ for feddan of broad bean). The nitrate application was stopped after two weeks for the inoculated treatments and kept for the control (non-inoculated). Plants were fed with the relevant nutrient solution. The composition of the nutrient solution is as the following (g/l): K₂SO₄ 17.4, KH₂PO₄ 6.8, K₂HPO₄ 4.4, MgSO₄.7H₂O 12.3, CaSO₄ 0.12, H₂BO₃ 1, ZnSO₄.7H₂O 0.1, CuSO₄.5 H₂O 0.05, MnCl₂.4H₂O 0.05, Na₂MoO₄.2H₂O 0.01, FeCl₃ 0.1, ferric citrate 0.025 and pH at 6.8. To obtain desired levels of salinity in soil, (i.e., control without salt, 50 mM and 100 mM NaCl), the required amount of NaCl was dissolved in distilled water and added to each pot to soil saturation percentage before sowing. After 6 weeks plants were harvested and the numbers of nodules were determined, and acetylene reduction rate was measured as described by Ligero et al., (1996). Nodules, roots and shoots dry weight was measured for each seedling after oven drying at 80 °C for 48 hours, the height of seedlings and total N content of the seedlings at the final harvest was measured using Kjeldahl method as specified by Bergersen (1980).

**Statistical analysis.**

The collected data was subjected to analysis of variance (ANOVA) and treatment means were compared by Duncan’s New Multiple Range Test (DMRT) at P<0.05 using a computer-based statistical software package (IBM SPSS Statistics 21).
RESULTS

Colony characteristics
Rhizobial isolates were examined under light microscope. The bacteria are short rods and Gram negative. Some colonies were circular, elevated, semi-translucent, shiny and with slimy appearance, because these isolates produce extracellular polysaccharides. The colonies were opaque white or milky colour when grown on YMA medium. The colonies had diameters ranged between 2-5 mm after 3-5 days of incubation at 28 °C. The colonies appeared with white colour because they lack the ability to absorb Congo red from a yeast extract mannitol agar (YMA) medium. All bacterial isolates were suggested to be belong to rhizobia, because bacterial isolates demonstrating the ability to nodulated their original leguminous host plants known to be nodulated by rhizobial species (Data not shown).

Assimilation of different carbon and nitrogen sources; and production of extracellular polysaccharides (EPS)
All isolates grew well at medium supplemented with mannitol, glucose, and sucrose as a sole carbon source, and produced high mount of EPS (Table 1). The isolate RV1 produced 2.4 g/l of EPS when grew in media supplemented with mannitol as a carbon source, and yeast extract as a nitrogen source (Table 1). The RV2 isolate grew well with mannitol and sodium nitrate and produced high amount of EPS (Table 1). The rhizobial isolates showed good growth when yeast-extract was used as a sole nitrogen source. Ammonium sulphate, ammonium phosphate and glutamic acid stimulated the production of higher amount of EPS in RV1, RV2 and RV3, but RV4 did not produced EPS when ammonium sulphate was used as a nitrogen source.

Table 1: Effect of different carbon and nitrogen sources on growth (OD) and extracellular polysaccharides (EPS) production (g/l) by isolates

<table>
<thead>
<tr>
<th>Carbon and Nitrogen sources</th>
<th>RV1</th>
<th>RV2</th>
<th>RV3</th>
<th>RV4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A- Carbon sources</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.2</td>
<td>1.4</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.8</td>
<td>1.1</td>
<td>0.8</td>
<td>2.4</td>
</tr>
<tr>
<td>mannitol</td>
<td>1.4</td>
<td>2.4</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.8</td>
<td>1.0</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.8</td>
<td>1.2</td>
<td>0.6</td>
<td>1.04</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.4</td>
<td>0.8</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>B- Nitrogen sources</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.3</td>
<td>1.2</td>
<td>0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>1.2</td>
<td>2.1</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Ammonium phosphate</td>
<td>0.8</td>
<td>1.9</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>B-alanine</td>
<td>1.1</td>
<td>2.0</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.7</td>
<td>1.4</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>1.6</td>
<td>2.3</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast-extract</td>
<td>1.5</td>
<td>2.4</td>
<td>1.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 2: Biochemical characters of rhizobial isolates

<table>
<thead>
<tr>
<th>Biochemical characters</th>
<th>RV1</th>
<th>RV2</th>
<th>RV3</th>
<th>RV4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA (µg/ml)</td>
<td>134±15</td>
<td>90±9</td>
<td>154±17</td>
<td>75±14</td>
</tr>
<tr>
<td>HCN production</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ammonia production</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Siderophore production</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phosphate solubilization</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Means ± standard error
Biochemical characters of rhizobial isolates

In the presence of tryptophan, all isolates produced IAA (Table 2). RV3 showed the highest (154 µg/ml) and RV4 was the lowest (75 µm/ml) IAA production. RV1 and RV4 produced ammonia, but only isolate RV1 which produced siderophore. Three isolates (RV1, RV2, and RV4) solubilized inorganic phosphate. Only one isolate (RV1) produced HCN. The isolate RV1 showed positive response to all biochemical tests.

Growth inhibition of pathogenic fungi by rhizobia (Antifungal activity)

When fungi and bacteria were in contact, the isolates RV1, RV2, RV3 and RV4 inhibited the mycelial growth of F. oxysporum, with the following inhibition percentages (46%, 42%, 57%, and 35%), respectively, after 12 days incubation period. Strains RV1, RV2, RV3 and RV4 inhibited mycelial growth of R. solani with percentages of 36%, 25%, 28% and 20%, respectively, after 12 days incubation period (Fig. 1). The isolate RV3 showed the highest inhibition rate against the fungus F. oxysporum, and the isolate RV1 showed the highest inhibition rate against the fungus R. solani.

Effect of salt stress on growth, nodulation, N₂ fixation (Nitrogenase activity), and chlorophyll and nitrogen contents of V. faba plan

Growth of V. faba (height, root-dry weight and shoot-dry weight) was reduced after salt treatment (Fig. 3). Growth of V. faba plants showed varied growth with regards to the rhizobial strains, under control and salt treatment. Rhizobial strains RV1 and RV3 showed the best growth (height) under control and salt treatment. (Fig. 3a). Similar trend was found with the rest of growth parameters (dry weight of shoots and roots). The highest level of salt treatment (100 mM NaCl) dramatically reduced all growth parameters of V. faba (Fig. 3)

Figure (3): Effect of salt stress on (3a): plant height, (3b): shoot dry weight and (3c): root dry weight
DISCUSSION

Rhizobial cultural and morphological characteristics

The rhizobia are among the most intensively studied groups of microorganisms (Sessitsch et al. 2002), mainly due to their N\textsubscript{2}-fixing ability and their potential to replace N\textsubscript{2} fertilizers, with emphasis on their key role in achieving sustainability of N-poor soils (Zahran 2009). Growth pattern and the cultural characteristics of root-nodule bacterial isolates from the crop legume (\textit{V. faba}) were examined. \textit{Rhizobium} isolates formed colonies with convex elevation in YMA medium, these results agreed with the rhizobium characteristics reported in Bergey’s Manual of Systematic Bacteriology (Jordan 1984). The bacteria produced copious amount of exopolysaccharides. These features are common for rhizobia and agreed with the characteristics of isolates from faba bean plants (Zahran et al., 2012). \textit{Rhizobium} isolates were succeeded to renodulate their legume hosts and forming effective nodules.

Utilization of different carbon and nitrogen sources; and production of extracellular polysaccharides (EPS) by rhizobial isolates.

The colonies of all strains were large with a circular form, translucent and mucoid on YMA. Colonies which don't produce EPS are rough and strains producing EPS appear smooth. The EPS synthesis by microbial cells depends on the carbon and nitrogen availability in the culture medium. Czaczky and Wojcienchowska (2003) reported that most exopolymer-producing microorganisms utilize carbohydrates as their nutrient content in the four treatments (plants inoculated by rhizobia), however, under mild salt treatment (50 mM NaCl), rhizobial strains did not show obvious variations (Fig. 5)

Figure (5): Effect of salt treatment on nitrogen content

*Means sharing the same letter(s) are statistically non-significant according to Duncans Multiple Rang Test (DMRT)
carbon and energy source and either ammonium salts and amino acids as their nitrogen source. Thus, these sources (type and its concentration) have obvious influence on EPS productivity. The present study showed bacterial strains gave high amount of EPS (2.4 g/l) in media contains mannitol as a carbon source and yeast-extract as a nitrogen source (Table 1). These results agreed with previous studies. Sayyed et al., (2011) found that sucrose resulted in maximum biomass, ammonium sulphate and yeast-extract gave optimum yield of EPS, while urea inhibited both EPS, production and growth of the bacteria. Nirmala et al., (2011) reported that EPS production was maximum when medium was supplemented with mannitol and potassium nitrate, and among the ten strains isolated from root nodules of the leguminous crop (Vigna mungo), the maximum amount of EPS produced by Rhizobium strain was 1.68 g/l.

Quantitative estimation of indole-3-acetic acid (IAA)

IAA is responsible for the enhancement of plant growth by stimulation of apical dominance and development of a highly organized root system by which uptake of nutrients becomes more efficient (Goswami et al., 2014). In this study, four strains of Rhizobium isolated from root nodules of broad bean (V. faba) plant showed different amounts of IAA secretion in the presence of tryptophan (Table 2); the highest value of IAA was observed with isolate RV3 (150 µg/ml). Our results agreed with other studies, e.g., Samina et al., (2010) who found that Rhizobium etli (QR1) produced amount of IAA equal to 160 µg/ml. Hingole and Pathak (2016) found that all four isolates of bacteria produced a considerable amount of IAA in the presence of tryptophan.

Inorganic phosphorus solubilization assay

Phosphate solubilization is a very important trait of plant growth promotion as microorganisms solubilize insoluble phosphate making it available for plants (Azzam and Omran 2005, Bhattacharyya and Jha 2012). El-Azeem et al., (2007) reported that all isolates which solubilize tri-calcium phosphate (TCP) in liquid and solid medium could produce organic acids causing the pH of the medium acidic. Our results showed that three strains of R. leguminosarum (RV1, RV2, and RV4) have phosphate solubilization ability (Table 2). This phosphate solubilization ability, besides to the N2-fixation ability, offer these rhizobial strains growth promoting activity.

Siderophore production

Our result showed that only one isolate (RV1) produced Siderophore (Table 2). Iron is an essential micronutrient for almost all organisms in the biosphere, but iron is not readily assimilated by either bacteria or plants (Gupta et al., 2015). Microorganisms have evolved specialized mechanisms for the assimilation of iron, including the production of low molecular weight iron-chelating compounds known as Siderophores, which transport this element into their cells (Arora et al., 2013). Various studies had reported about isolation of Siderophore producing bacteria belonging to the genera Bradyrhizobium, Pseudomonas, Rhizobium, Serratia and Streptomyces from the rhizosphere (Sujatha and Ammani 2013). Siderophores produced by various bacteria having wide application in various fields, such as agriculture, to improve soil fertility and pest biocontrol (Ali and Vidhale 2013).

Inhibition of pathogenic fungi

In the present study, in vitro assessment of the anti-fungal activity against the test fungi (F. oxysporum and R. solani) was done. Four isolates from broad bean (V. faba) plants were found to suppress growth of test fungus with an inhibition rate of 57% (Fig. 1). Similar results showed that treatment with Rhizobium sp. significantly reduced growth of Fusarium sp by 54% in vitro (Sharif et al., 2003). Akhtar et al. (2011) found that Rhizobium sp. significantly reduced the Fusarium wilt on lentil plant. Jan et al. (2011) reported that several strains of Pseudomonas, which could suppress the growth of pathogenic F. oxysporum, possessed the ability to produce HCN, Siderophores and antibiotics. The presence of rhizobia in the rhizosphere presumably protects the host roots from pathogens, besides fixing atmospheric nitrogen.

Effect of salt on growth of rhizobia

In the current study, growth of rhizobial isolates was reduced with increasing salt concentration (Table 3) and this was consisted with the results of Ali et al., (2009) and Shoukry et al., (2013). The increasing salt concentration may have detrimental effects on rhizobial populations as a result of direct toxicity as well as through osmotic stress. In our study, we have strains grew at 6% NaCl about (1000 mM NaCl), this agree with Ali et al., (2009) who found that six isolates of Leucaena leucocephala showed tolerance at 4.5% salt concentration.
Bouhmouch et al., (2005) found that growth of *Rhizobium tropici* strain RP163 and *Rhizobium giardinii* strain RP161 was moderately decreased at 250 mM NaCl and was highly decreased at 342 mM NaCl. Shoukry et al., (2013) found that isolate (RL7) from faba bean plant can grow up to 200 mM NaCl (about 1.2%). It appears that rhizobia are more tolerant to salt stress than their host legumes (Laranjo and Oliveira 2011).

**Molecular characteristics**

In this study, rhizobial bacteria from root nodules *V. faba* were identified using 16SrDNA genes as *R. leguminosarum* (Fig 2), which is consistent with previous reports (Zahran et al., 2013, Benidire et al., 2017). Zahran et al., (2013) reported that sequences analysis of 16SrDNA indicated that the strains from *V. faba* grown in cultivated lands of Beni-Suef Governorate (Egypt) had 99.6 % identity with *R. leguminosarum*. Several studies done in other regions of the world indicate that faba bean-nodulating bacterial strains are relatively diverse. For example, Tian et al., (2007) found that there was a great diversity among 75 rhizobial isolates associated with faba bean from China and these isolates were identified as *R. leguminosarum*. Shamseldin et al., (2009) found that sequence analysis of 16SrDNA indicated that 57% of 28 faba bean rhizobial isolates from Egyptian soils were *R. leguminosarum*.

**Effect of salt concentration on growth, nodulation and *N*<sub>2</sub> fixation (Nitrogenase activity) of *V. faba* plants**

In this study, *V. faba* plants were inoculated by four *R. leguminosarum* strains to evaluate growth response and ability of these strains to form root nodules under salinity (50 and 100 mM NaCl). The effects of salinity were more pronounced on growth parameters at 100 mM NaCl (Figs. 3, 4). Salinity usually has detrimental effects on growth of most legumes (Benidire et al., 2017, Zahran 2017). *V. faba* plants subjected to higher salinity levels (100 mM NaCl) showed dwarfed plants, and growth of shoots and roots were reduced (Fig. 3a), shoots were more sensitive to salt than roots with regards to fresh and dry weight. Shoots of legumes has been reported to be more sensitive to salinity than roots (Benidire et al., 2017). The reduction of shoot growth can be linked to disturbance of growth regulators (cytokinins and abscisic acid) induced by salinity, or to a reduction of photosynthetic capacity following a decrease in stomatal conductance of CO₂ under salt stress (Yurekli et al., 2004). The symbiosis between *R. leguminosarum* and *V. faba* plants was also greatly affected by high salt level (nodule number and Nitrogenase activity were significantly reduced), and the most effective salt concentration was 100 mM NaCl (Fig. 4). Our results were in agreement with those obtained by Yasin et al., 2018. *R. leguminosarum* strains (RV1, RV2, RV3 and RV4), which were used in the symbiotic experiment are moderately salt-tolerant, being grew at about 5 % NaCl. RV1 and RV3 strains established good symbiosis with *V. faba* plants under salt stress than RV2 and RV4. At 100 mM NaCl, nodule number, and activity were only slightly reduced (Fig 4). Strains RV1 and RV3 were able to cause a significant response for seedling growth parameters, nodulation and nitrogen fixation, which may be due to an adaptation of these strains to environmental factors and salt stress than other strains. The total nitrogen content in *V. faba* plants was affected by salt concentration, the control treatment (no salt) had the highest total nitrogen content, while under salt treatment the total nitrogen per plant was decreased (Fig. 5). Similar results were reported in the work of Benidire et al., 2017, but other studies found no reduction in nitrogen content per plant under salt stress (Weil and Khalil 1986). Further studies are needed to investigate the growth promoting ability of the diverse indigenous rhizobial strains in the Egyptian soil.

**CONCLUSION**

All of *Rhizobium leguminosarum* strains (RV1 and RV3) has growth-promoting characters under...
salt stress and can be used as a biofertilizer inoculant beneficial for broad bean cultivation and other crops. Increasing salt stress decreased all growth parameter of broad bean. The most salinity-tolerant strains were RV1 and RV3 and the least strains tolerant to salt stress were RV2 and RV4.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT
Thanks and great appreciation are extended to all the stuff members of both Microbiology Department at the Soils, Water and Environment Research Institute, and Cell Research Department at the Field Crops Research Institute, Agricultural Research Center for their great help during this work and for giving us the opportunity to carry out this research work by providing the funding, as well as, all the stuff members of Botany and Microbiology Department, Faculty of Science, Beni-Suef University for all support and for their great help and encouragement.

AUTHOR CONTRIBUTIONS
MA and ESA chose the research point. RRI and MNAO designed, performed the experiments, carried out the practical experiment and wrote the manuscript. HHZ analyzed the data and wrote the manuscript. CRA designed and performed experiments and conducted research, wrote and reviewed the manuscript and submitted the manuscript to the publishing journal. All authors read and approved the final version.

Copyrights: © 2017 @ author(s).
This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author(s) and source are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

REFERENCES
Bouhmouch I, Souad-Mouhsine B, Brhada F,


Sessitsch A, Howieson JG, Peret X, Antoun H,


