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Diversity and Environmental Stress Responses of Rhizobial Bacteria from Egyptian Grain Legumes

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Abstract: The aim of this work was to characterize and identify root-nodule bacteria isolated from different grain leguminous plants grow in the cultivated lands of Beni-Suef Governorate, Egypt. The bacteria (68 isolates) were obtained from five cultivated grain leguminous plants, namely: *Cicer arietinum*, *Lens esculentus, Phaseolus vulgaris*, *Pisum sativum*, and *Vicia faba*, and one naturally-grown herb legume, *Trifolium resupinatum*. After their isolation, the bacteria were confirmed as rhizobia by re-nodulating their specific host legumes. The phenotypic characteristics (e.g. utilization of carbon and nitrogen sources, tolerance to salt, acid, heat, and heavy metals, antibiotic resistance, etc.) were determined. The bacteria under investigation expressed noticeable ability to grow under various stress factors examined. These rhizobial isolates could be a good candidate to establish a successful symbioses with their compatible legumes under the environmental conditions prevail in Egypt. The bacterial features of the isolates were compared to those of strains from two reference rhizobial genera (*Rhizobium* and *Mesorhizobium*). Based on phenotypic characteristics and nodulation capacity, the rhizobia were grouped and classified as the following: *V. faba* bacteria as *R. leguminosarum* and *P. sativuma* bacteria as *R. leguminosarum* and *R. etli*. *P. vulgaris* bacteria were classified as *R. lusitanum*, *R. mongolense*, and *R. leguminosarum*. *L. esculentus* bacteria were classified as *R. leguminosarum* and *C. arietinum* bacteria as *M. amorphae*, *M. temperatum* and *M. mediterraneum.* The bacteria of the wild-legume, *T. resupinatum* were classified as *Ensifer meliloti and R. leguminosarum*. The results emphasized the fact that the cultivated legumes nodulate by the common and diverse root-nodule bacteria (rhizobia) indigenous in the Egyptian soil. However, a molecular and genetic identification is needed to precisely classify and identify these bacteria.

Key words: rhizobia, phenotypic tests, nodulation, N_2 fixation, environmental stress.

INTRODUCTION

Rhizobia are soil-inhabiting bacteria with the potential for forming specific structures on roots, called nodules. In effective nodules, the bacteria (bacteroids) fix the atmospheric nitrogen gas (N_2) into ammonia, which is assimilated by the plant and supports growth, particularly in nutrient-deficient soils. In return, the bacteriods are supplied with nutrients (predominantly dicarboxylic acids), and protected inside the nodule structure. The nitrogen-fixing symbiotic relationship has been exploited in agriculture to enhance crop and pasture growth without the addition of nitrogen fertilizers (Zahran 2005, 2006, 2009). For this reason, the majority of research in this field has focused on crop legumes of agricultural significance. In contrast, few studies have been made of rhizobial associations among non-crop (wild) legumes, which may be ecologically important in the natural landscape (Zahran 1999, Zahran *et al.* 2003, Al Sherif *et al.* 2005). The increased use of *Rhizobium* inoculants should help in achieving increased yields of food and forage legume crops in a most economical way. So, much attention is required to discover new leguminous species with high production capabilities and high symbiotic performance. More effective strains of rhizobia will have to be discovered or developed and these ″super″ competitive strains will be more acceptable to their particular hosts than those currently in use. Then, perhaps it will be possible to bring about increased nitrogen fixation even in soils which already harbor numerous highly infective strains of the microsymbionts. The term “rhizobia”, in the strictest sense, refers to members of the genus *Rhizobium*. Over the years, however, the term has become to be used for all the bacteria that are capable of nodulation and nitrogen fixation in association with legumes and that belong to a genus that was at one time part of the genus *Rhizobium* or closely related to it (Sahgal and Johri 2006, Willems 2006). With the introduction of more DNA-rRNA hybridizations, rRNA catalogues, rDNA sequencing, more diversity of rhizobia was discovered and their relationships with other groups of bacteria became apparent. This led to a gradual increase in the number of genera. In parallel, there has been a significant increase in the number of validly published species. This increase in the number of genera and species can be explained as the numbers of leguminous plants that are being studied for nodulation are increasing. Until now, only 20 % of the

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total of about 18,000 species and 57 % of about 650 genera of leguminous plants has been studied for nodulation (Sprent 1995). This leaves a large number of legume species to be studied and potentially many more species and genera of rhizobia to be described. This will undoubtedly have a further major impact on rhizobial taxonomy. Most recent taxonomic studies have made use of a polyphasic approach (Graham *et al*. 1991, Vandamme *et al*. 1996), with genetic, phenotypic, chemotaxonomic, and phylogenetic data, which combined to establish a comprehensive picture of the relationships of the bacteria, and to propose a suitable classification. Recently, what is known as rhizobial species have been identified in many genera, most of the species are in the genus *Rhizobium*. Current rhizobial taxonomy describes seven main genera within the α-proteobacteria (Garrity *et al*. 2004), although more recent studies have ascribed nodulating bacteria to other genera within the β-proteobacteria (Zakhia *et al*. 2004, Mantelin *et al*. 2006). Meanwhile, there are other legume-nodulating bacteria belonging to genera other than those listed here like *Devosia*, *Burkholderia*, *Herbaspirillum,* etc.

The phenotypic studies are necessary for characterization and selection of rhizobial strains adapted to marginal edaphoclimatic conditions and to provide preliminary information about their genetic diversity. Early studies made use of some cultural and morphological characteristics in the taxonomy of rhizobia, including growth rate and colony characteristics on yeast extract-mannitol mineral salts medium (Dreyfus *et al*. 1988, Graham *et al*. 1991, Somasegaran and Hoben, 1994, Hafeez *et al*. 1995, Mpepereki *et al*. 1997, Odee *et al*. 1997, Zahran *et al*. 2003). Carbohydrate utilization is one of the tests commonly used in the characterization and classification of rhizobia. Rhizobial species differ significantly in carbohydrate metabolism and substrate utilization. The utilization of carbohydrates such as glucose, sucrose, lactose, fructose, arabinose, and succinate, serve as a diagnostic test in the differentiation of currently recognized species of *Rhizobium* (Graham *et al*. 1991, Rohm and Werner 1992, Somasegaran and Hoben, 1994, El-Idrissi *et al*. 1996). Fast- and slow-growing rhizobia are variably utilized sugars and other carbohydrates (Hafeez *et al*. 1995, Mpepereki *et al*. 1997). Among nutritional requirements of rhizobia is the utilization of nitrogen sources. The nitrogen requirements of rhizobia can be satisfied by inorganic nitrogenous salts (e.g., nitrate and ammonium salts) and by many amino acids and short chain peptides (Jordan 1984, Zhang *et al.* 1991). Mohamed *et al.* (2000) found that asparagine and L-methionine utilized by *Acacia* rhizobia as a sole nitrogen source.

The different responses of rhizobial strains to stress factors could be considered as basic criteria for differentiation and identification of these bacteria. Rhizobia are fairly salt-tolerant microorganisms (Zahran 1991 a and b, Zahran *et al.* 2003), however, distinct variations in salt tolerance were observed for some rhizobia (Kumer *et al*. 1990, Moawad and Beck 1991, Swelim 1996, Zahran 2010). Hosny *et al*. (1991) conducted a survey on the salt tolerance of indigenous *R. leguminosarum* bacteria nodulating *V. faba* in the Nile Delta soils in Egypt. They found that 55 % grew at a salt level of 1 % and only 2 % survived at 6 % NaCl. *R. leguminosarum* was reported to be tolerant to 2 % NaCl (Helemish and El-Gammal 1987, Breedveld *et al.* 1991). The rhizobia isolated from salt-affected soils appear to be highly salt-tolerant (Li *et al.* 2011). *R. meliloti* (currently *E. meliloti*) strains, isolated from nodules of leucerne cultivated at salt-affected soils, grew at about 3 % NaCl (Kassem *et al*. 1985) and two bean isolates were tolerant to 3 % - 3.5 % NaCl because they were originally isolated from plants grown at salt-affected soil (Nassef 1995). The rhizobia nodulating *Leucaena,* that grow in slightly salt-affected soils in Egypt, are tolerant to about 4 % NaCl (Swelim 1996). For most rhizobia, the optimum temperature range for growth in culture was reported to be 28 to 31°C and many were unable to grow at 37 °C (Graham 1992). Mohamed *et al.* (2000) found that most of the *Acacia* root-nodule bacteria grew at 35 °C and 37 °C, only few strains grew above 40 °C. About 90 % of cowpea *Rhizobium* strains, obtained from hot and dry environments of the Sahel Savannah, grew well at 40 °C (Eaglesham and Ayanaba 1984) and high percentage of *R. phaseoli* strains persisted at 45 °C (Karanja and Wood 1988). The most heat-tolerant strains known so far were isolated from *Sesbania aculaeta* (Kulkarni *et al.* 2000). Two strains survived at 50 °C and 65 °C in YMB for up to 2 and 4 hours. Maatallah *et al*. (2002) reported that *C. arietinum* rhizobial isolates showed growth within a wide temperature range (5 °C to 45 °C). The pH can be a major factor limiting growth of a number of microorganisms in soil (Brockwell *et al.* 1991). A wide variety in the pH tolerance of *C. arietinum* rhizobia (Maatallah *et al*. 2002) and *Acacia* spp. rhizobia (Mohamed *et al.* 2000), had been reported. Fast-growing rhizobia are considered as less tolerant to acid pH than slow-growing rhizobia, however, some strains of fast-growing rhizobia, e.g., *R. loti, R. trifolii* and *R. tropici,* are highly acid-tolerant (Cunningham and Munns 1984, Cooper *et al.* 1985, Wood *et al.* 1988, Richardson and Simpson 1989, Graham *et al.* 1994). Several studies had shown that heavy metals might have been toxic to rhizobia when present in soil at moderate to high concentrations (El-Gamal 1988, McGrath *et al.* 1988, Giller *et al.* 1989, McGrath 1993, Zahran 1999). The lowest *R. meliloti* populations were present in the soil with the highest concentration of heavy metals (El-Aziz *et al.* 1991). However, Biomy (2000) reported that rhizobia from *V. faba,* grown in sewage sludge-contaminated soils, were highly resistant to heavy metals (650 ppm Zn and 350 ppm Pb) than other strains or isolates. Similarly, Vidal *et al.* (2009) identified a metal-resistant symbiont (*M. metallidurans* species) from the legume *Anthyllis vulneraria* growing on metallicolous soil in France. Sensitivity to different antibiotics at different ranges of concentrations varied between rhizobial species, it was suggested that such variation may be a useful taxonomic character (Date and Hurse 1992, Somasegaran and Hoben, 1994). Intrinsic antibiotic

resistance profiles was used to identify strains of *R. leguminosarum* bv. *viceae* (Sinclair and Eaglesham 1984) and chickpea rhizobial isolates (Maatallah *et al*. 2002). Rhizobial isolates from *V. faba* were sensitive to three antibiotics, neomycin, kanamycin, and streptomycin (Hosny *et al*. 1991), while those rhizobial strains from *P. vulgaris* were resistant to streptomycin, rifampicin, ampicillin, spectinomycin, nalidixic acid, genomycin, chloramphenicol, and tetracycline (Vargas *et al*. 1992). *Rhizobium* isolates from nodules of *Leucaena leucocephala* plants were intrinsically resistant to nalidixic acid and rifampicin (Swelim 1996). Intrinsic antibiotic resistance varied among fast-and slow-growing rhizobia (Young and Chao 1989, Mpepereki *et al*. 1997).

MATERIALS AND METHODS

Root-nodule Sampling and Rhizobial Isolation:

Root nodules were collected from five species of grain legumes (*C. arietinum, L. esculentus, P. vulgaris, P. sativum,* and *V. faba*) and one species representing wild herb legumes (*T. resupinatum*), the legumes grow at different sites of Beni-Suef Governorate, Egypt. Healthy representatives of each plant species were selected for nodule sampling. The plant roots were carefully cleaned from soil particles by gentle shaking, then washed under a gentle stream of tap water, and left for air drying (Somasegaran and Hoben 1985). Healthy nodules were excised from the roots leaving a small piece of root attached. Undamaged and apparently effective nodules (with pink colour), were surface sterilized for 5-10 min in 95 % ethanol, then soaked for 4-6 min in 3 % sodium hypochlorite solution, and rinsed several times with sterilized distilled water (Vincent 1970). The surface sterilized nodules were crushed in sterile test tube containing one ml of sterile distilled water with sterile glass rods, and a loopful of the nodule extract was used to streak sterile plates with yeast-extract-mannitol agar (YMA) medium (Vincent 1970) supplemented with Congo red to differentiate the bacterial contaminants from rhizobia. This medium comprised the following constituents in g/L; Mannitol 10, K_2HPO_4 0.5, $MgSO_4$. $7H_2O$ 0.2, NaCl 0.1, yeast extract 0.5, and 10 ml/L of a stock Congo red (1/400 aqueous solution) to colour media, and agar is added (20 g/L) to solidify media. This medium was used as a selective one for rhizobial isolation from the nodules, as well as, to evaluate the effect of particular factors (e.g., NaCl tolerance, antibiotic sensitivity, etc.) on growth of rhizobia under the laboratory conditions. Plates were incubated at 28 °C for 3 days. The purity of the cultures was verified by repeated streaking of a single-colony isolate onto YMA medium. In order to guarantee the surface sterilization process, aliquots of the sterile distilled water from the final rinse were plated on YMA medium and the plates were incubated at 28 °C for 4 days (Kuklinsky-Sobral *et al*. 2004). Each isolate was re-streaked for more purification, and then single colony was selected and streaked on yeast-extract-mannitol agar slants contain calcium carbonate 1g/L per liter and stored at 5 °C for further studies. All bacterial isolates were maintained at YEM broth containing 20 % (v/v) glycerol at - 20 °C and - 80 °C. The colony characteristics of rhizobial isolates were examined on YMA medium in plates and compared to those of rhizobial references obtained from Agriculture Research Centre (ARC), Giza, Egypt (Table 1). The rhizobial isolates were compared for phenotypic characteristics with these reference rhizobia strains.

Authentication of Isolates Via Re-Inoculation of Legume Hosts:

All isolates were tested as being one of the rhizobial bacteria. Such a confirmation was dependent on demonstration of nodule-forming ability of each isolate on the compatible host legume under bacteriololgically-controlled conditions. Seeds of the host legumes were surface-sterilized with a 2.5 % (v/v) sodium hypochlorite after soaking for 5 min, and finally rinsed in five changes of sterile water. Sterilized seeds were germinated in sterile Petri dishes containing damp sterile filter paper. After being inoculated with 48 h-old YEM broth culture of the corresponding rhizobia, seedlings were transplanted into sterilized plastic pots containing autoclaved sand. Pots were kept at randomized blocks in a greenhouse under non-sterile conditions. Seedlings were watered with sterile nutrient solution of the following composition (g/L): $CaCl_2$ 0.1, $MgSO_4.7H_2O$ 0.12, KH_2PO_4 0.1, $Na_2HPO_4.2H_2O$ 0.15, Ferric citrate 0.005, and 1.0 ml of trace elements stock solution. The trace elements stock solution contains the following components (g/L): H_3BO_3 2.86, $MnSO_4.7H_2O$ 2.03, $ZnSO_4.7H_2O$ 0.22, $CuSO_4.5H_2O$ 0.08, and $NaMoO_2.2H_2O$ 0.14. pH of the nutrient solution was adjusted to 6.8-7.0 with NaOH. Pots of uninoculated seedlings were used as controls and received weekly 0.5 % (KNO_3) as nitrogen source. Nodule formation was recorded after 25 days.

Nitrogenase Activity:

The acetylene reduction technique was used to measure the nitrogenase activity (Hardy *et al*. 1968). Cleaned (soil-free) nodulated-roots or rootlets were placed immediately in 150 ml bottles capped tightly with rubber septa. Root systems were not washed as this has been shown to decrease nodule activity (Sprent 1969). About 15 ml of acetylene was injected into each bottle. Before that and in order to adjust the internal pressure inside each bottle, 15 ml (about 10 % of the vial volume) of air was withdrawn from the bottles. After injection, bottles were sealed against possible leakage and were incubated for 1 hour at 28 °C. The reaction was terminated with 1 ml of 5N HCl. Triplicate samples were prepared for gas chromatography detection. About

0.125 ml of the gas sample was withdrawn and assayed for ethylene production using Hewlett-Packard model 5890 gas chromatograph equipped with a HP-Plot Al_2O_3 capillary column, a flame ionization detector, and a Hewlett-Packard model Vectra 486133 VL computer. Peaks were automatically integrated and the ethylene amounts calculated.

Utilization of Carbon and Nitrogen Sources:

All isolates, along with the reference strains, were tested for their ability to utilize some carbon sources, e.g., glucose, fructose, sucrose, lactose, inositol, starch, and mannitol. The medium used for this test was a carbohydrate-free medium **(**Somasegaran and Hoben 1994**),** yeast extract was reduced to 0.05 g/L. Separately-autoclaved sugars were added to the modified YMA basal medium (depleted of mannitol) to a final concentration of 1 % (W/V). Triplicate plates were used for each different sugar. Two control media were used for comparison, the standard medium containing mannitol and the C-free YMA basal medium. All the plates were streaked with the freshly prepared liquid culture of each of the rhizobial isolates, and then incubated at 28 °C for 3-5 days. Growth response of different isolates was recorded positive (visible growth) or negative (no growth**)**. A modified mannitol medium (Stowers and Eaglesham 1984), at which yeast-extract was replaced by 0.5 g/L of the tested amino acid, was used to investigate the utilization of nitrogen compounds. Isolates were tested to use each of the amino acids L-alanine, L-methionine, L-phenylalanine, L-serine, L-tryptophan and asparagin as a sole nitrogen source. N-free modified mannitol medium (devoid of any nitrogen source) was used as a control. After inoculation, plates were incubated at 28 °C for 3-5 days and visual growth was recorded.

Response to Environmental Stress Factors:

Response of all rhizobial isolates to various environmental stresses were monitored and compared together with reference rhizobial strains. YMA plates, each containing 1, 2, 3, 4, 5, and 6 % NaCl, were streaked with a loopful of each of different rhizobial isolates freshly grown in yeast-extract-mannitol broth (YEM). After incubating the plates for 3-5 days at 28 °C, bacterial growth was recorded as positive (visible growth) or negative (no growth**)**. The concentration 0.1 % NaCl was used as control, which was the concentration of NaCl in the basal YMA medium. Rhizobial isolates were inoculated into YMA medium incubated at different temperatures (10 °C - 55 °C) to test their maximum growth temperatures. After 3-5 days of incubation, bacterial growth was recorded by visual observation compared to control treatments incubated at 28 °C. Rhizobial isolates were tested for their ability to grow at different pH values (6-10) by inoculation into YMA medium. 1M HCl and 1M NaOH were used to adjust pH values in the acidic and alkaline media, respectively. After 3-5 days of incubation, bacterial growth was recorded by visual observation compared to control treatments incubated at pH 7. The rhizobial isolates were tested for their resistance to the final concentrations of 0.01 % and 0.1 % of Cd ($CdCl_2$), Zn ($ZnCl_2$), Cu ($CuCl_2$), Pb [$Pb(CH_3COO)_2$], and Ni ($NiCl_2$). Heavy metals solutions were filtered-sterilized and added to the YMA medium. Plates were inoculated by a 48 h-old YEM broth cultures and growth was observed and results were recorded after incubation at 28 °C for 3-5 days.

Intrinsic Antibiotic Resistance:

Discs of the following antibiotics (µg/disc): neomycin (30), kanamycin (30), streptomycin (20), geramycin (20), tetracycline (30), and tropamycin (30), were used to test the ability of each isolate to resist the given antibiotic doze. Discs were mounted over the YMA medium after which plates were incubated at 28 °C for 3-5 days. Resistance or sensitivity of each isolate to the used doze of antibiotics was recorded, a transparent halo in the vicinity of the antibiotic discs indicates the isolate sensitivity to the given doze and thus the growth was inhibited in such an inhibition zone around the disc. The growth around the disc means that the isolate showed apparent resistance to the antibiotic doze.

Enzymatic Activities:

The production of catalase enzyme was determined on YMA medium inoculated with each rhizobial isolates in plates, then bacterial cultures were incubated at 28 °C for 3-5 days. The formation of air bubbles, after the addition of few drops of 3 % H_2O_2 to each colony, was recorded as positive result. Plates containing YMA medium supplemented with gelatin were used to test the ability of each isolate to catalyze gelatin. After incubation at 28 °C for 3-5 days, plates were flooded with acidified $HgCl_2$ which precipitate the gelatin into a white precipitate. The transparent halo around the colony indicates that the isolate produces gelatinase and catalyzes gelatin. The ability of each isolate to produce amylase and catalyze starch was also determined on YMA medium supplemented with starch. After incubation at 28 °C for 3-5 days, plates were flooded with iodine solution. The transparent halo around the colony indicates a positive result, while the dark-blue precipitate around the colony indicates a negative result. To investigate urea hydrolysis, urea (2 %) was sterilized by filtration before its addition to the YMA medium supplemented with 0.012 % phenol red as pH indicator. After incubation at 28 °C for 3-5 days, the colour of the pH indicator changed from the yellow to the red colour, which indicates a shift to the alkaline side and a positive result.

Numerical Analysis of Phenotypic Variables:

A computer cluster analysis of phenotypic variables was carried out using a similarity coefficient. A dendrogram was constructed by the unweighed pairwise group method with the average (UPGMA) clustering method. The method was done using the statistical package for social science (SPSS). A dendrogram was constructed for each set of strains isolated from the same host plant, and in each dendrogram, the reference strains (mentioned in Table 1) were used.

Table 1: Reference rhizobial strains obtained from Agriculture Research Centre, Giza, Egypt.

Host Legumes	Rhizobia	Strain	References abbreviations
Vicia faba (Faba bean)	*R. leguminosarum* bv. *viceae*	ICARDA 441	VF. Ref
Trifolium alexandrinum (Berseem, Clover)	*R. leguminosarum* bv. *trifolii*	ARC 103	TA. Ref
Cicer arietinum (Chickpea)	*Mesorhizobium ciceri*	ICARDA 36	CA. Ref
Pisum sativum (Field pea)	*R. leguminosarum* bv. *viceae*	ARC 203	PS. Ref
Phaseolus vulgaris (Phaseolus)	*R. leguminosarum* bv. *phaseoli*	ARC 310	PV. Ref
Lens culinaris (Lentil)	*R. leguminosarum* bv. *viceae*	ICARDA 31	LE. Ref

Results:

Colony Characteristics, Nodulation and Nitrogen Fixation of Rhizobia:

Rhizobial isolates displayed various colony characteristics. Some colonies were circular, elevated, shiny, and milky or opaque when grown on YMA medium. The formed colonies had diameters ranged between 2-5 mm after 3-5 days of incubation at 30 °C. The root-nodule bacteria in this work exhibited colony characteristics very similar to those of the rhizobial references. All bacterial isolates were confirmed as being rhizobia. Such a confirmation was dependent on demonstrating the ability of the isolates to nodulate their original leguminous host plants known to be nodulated by rhizobial species. The majority of isolates formed nodules on their hosts (Table 2) except two isolates, one (BSLE2) from *L. esculentus* and the other (BSTR8) from *T. resupinatum*. A randomally-selected group of isolates (one from each host plant) was tested for its ability to form effective, N_2-fixing, nodules. Acetylene reduction technique was carried out to measure N_2-fixation (nitrogenase activity) of the formed nodules after inoculation of the legume roots. The selected samples were relatively efficient in N_2 fixation as shown in Table (3).

Table 2: Results of the nodulation test of all rhizobial isolates.

Isolate	Nodulation Result	Isolate	Nodulation Result	Isolate	Nodulation Result	Isolate	Nodulation Result
BSCA1	+	BSPV8	+	BSVF2	+	BSLE10	+
BSCA2	+	BSPV9	+	BSVF3	+	BSLE11	+
BSCA3	+	BSPV10	+	BSVF4	+	BSTR1	+
BSCA4	+	BSPV11	+	BSVF5	+	BSTR2	+
BSCA5	+	BSPV12	+	BSVF6	+	BSTR3	+
BSCA6	+	BSPS1	+	BSVF7	+	BSTR4	+
BSCA7	+	BSPS2	+	BSVF8	+	BSTR5	+
BSCA8	+	BSPS3	+	BSVF9	+	BSTR6	+
BSCA9	+	BSPS4	+	BSVF10	+	BSTR7	+
BSCA10	+	BSPS5	+	BSVF11	+	BSTR8	-
BSCA11	+	BSPS6	+	BSLE1	+	BSTR9	+
BSPV1	+	BSPS7	+	BSLE2	-	BSTR10	+
BSPV2	+	BSPS8	+	BSLE3	+	BSTR11	+
BSPV3	+	BSPS9	+	BSLE4	+	PV.REF	+
BSPV4	+	BSPS0	+	BSLE5	+	CA.REF	+
BSPV5	+	BSPS11	+	BSLE6	+	PS.REF	+
BSPV6	+	BSPS12	+	BSLE7	+	TA.REF	+
BSPV7	+	BSVF1	+	BSLE8	+	LE.REF	+
				BSLE9	+	VF.REF	+

The abbreviations: CA. Ref, PS. Ref, TA. Ref, LE. Ref, and VF. Ref., are for references.

Table 3: Nitrogenase activity of root-nodules from five legumes.

Isolates tested	Host legumes	Nitrogenase activity nmoles C_2H_4 plant^{-1} h^{-1}
BSPV9	*P. vulgaris*	450
BSVF8	*V. faba*	315
BSPS7	*P. sativum*	112
BSLE4	*L. esculentus*	098
BSCA9	*C. arietinum*	054

Utilization of Carbon and Nitrogen Sources:

All isolates from various hosts utilized glucose and mannitol as a sole carbon source. None of the 11 isolates from *L. esculentus* utilized inositol as a carbon source. All isolates from *P. sativum* and *V. faba* utilized sucrose, while lactose was utilized by the whole isolates from *P. sativum*. Inositol and fructose were the lowest carbon sources utilized. All isolates utilized the tested amino acids as a sole nitrogen source. All isolates from *C. areitinum* utilized serine and tryptophan. The whole isolates from *V. faba* utilized L-alanine, asparagin, and glycine. All isolates from *L. esculentus* utilized tryptophan, while those from *T. resupinatum* only utilized asparagin. All isolates from *P. sativum* utilized serine as a sole nitrogen source. The data for carbon and nitrogen sources did not shown, and instead, numerical taxonomy data are presented (Table 4).

Table 4: Clusters obtained from phenotypic characterization.

Label	Isolate code	Clusters
* Clusters of isolates from *V. faba*		
S2	BSVF2	1
S1	BSVF1	"
S3	BSVF3	"
S4	BSVF4	"
S6	BSVF6	"
S7	BSVF7	"
S5	BSVF5	"
S15	VF. Ref	"
S10	BSVF10	2
S11	BSVF11	"
S9	BSVF9	"
S8	BSVF8	"
S17	PV. Ref	"
S16	LE. Ref	"
S14	PS. Ref	"
S13	TA. Ref	3
S12	CA. Ref	related to cluster 3
* Clusters of isolates from *C. arietinum*		
S7	BSCA7	1
S10	BSCA10	"
S5	BSCA5	"
S6	BSCA6	"
S9	BSCA9	"
S15	VF. Ref	"
S13	TR. Ref	"
S1	BSCA1	2
S2	BSCA2	"
S16	LE. Ref	"
S3	BSCA3	3
S4	BSCA4	"
S8	BSCA8	"
S11	BSCA11	"
S12	CA. Ref	"
S14	PS. Ref	4
S17	PV. Ref	"
* Clusters of isolates from *P. vulgaris*		
S6	BSPV6	1
S7	BSPV7	"
S13	CA. Ref	"
S16	VF. Ref	related to clus. 1
S17	LE. Ref	2
S18	PV. Ref	"
S11	BSPV11	3
S12	BSPV12	"
S9	BSPV9	"
S8	BSPV8	4
S10	BSPV10	"
S4	BSPV4	"
S5	BSPV5	"
S2	BSPV2	"
S3	BSPV3	"
S1	BSPV1	"
S14	TA. Ref	"
S15	PS. Ref	related to clus. 4

Label	Isolate code	Clusters
* Clusters of isolates from *P. sativum*		
S11	BSPS11	1
S12	BSPS12	"
S10	BSPS10	"
S17	LE. Ref	"
S15	PS. Ref	"
S18	PV. Ref	"
S8	BSPS8	2
S9	BSPS9	"
S7	BSPS7	"
S5	BSPS5	"
S6	BSPS6	"
S1	BSPS1	"
S2	BSPS2	"
S3	BSPS3	"
S4	BSPS4	"
S14	TA. Ref	3
S13	CA. Ref	related to clus. 3
S16	VF. Ref	related to clus. 3
* Clusters of isolates from *L. esculentus*		
S9	BSLE9	1
S10	BSLE10	"
S11	BSLE11	"
S2	BSLE2	"
S13	TR. Ref	"
S6	BSLE6	2
S7	BSLE7	"
S3	BSLE3	"
S4	BSLE4	"
S5	BSLE5	"
S1	BSLE1	"
S8	BSLE8	"
S17	PV. Ref	"
S16	LE. Ref	"
S14	PS. Ref	"
S15	VF. Ref	"
S12	CA. Ref	related to clus. 2
* Clusters of isolates from *T. resupinatum*		
S10	BSTR10	1
S11	BSTR11	"
S9	BSTR9	"
S3	BSTR3	"
S6	BSTR6	"
S1	BSTR1	"
S5	BSTR5	"
S2	BSTR2	"
S16	LE. Ref	"
S17	PV. Ref	"
S14	PS. Ref	"
S4	BSTR4	"
S7	BSTR7	"
S13	TA. Ref	"
S15	VF. Ref	related to clus. 2
S8	BSTR8	3
S12	CA. Ref	related to clus. 3

Response to Environmental Stress Factors:

All bacterial isolates survived at 1 % and 2 % NaCl. All isolates from *T. resupinatum* grew at 3 % NaCl, while half number of them (6 isolates) grew at 4 % NaCl. Four isolates from *L. esculentus* resisted 3 % NaCl and only a single isolate from the same plant grew at 4 % NaCl. Only two isolates from *P. vulgaris* grew at 3 % NaCl while none of the isolates of *V. faba* grew beyond 2 % NaCl. Only one isolate (BSTR8) from *T. resupinatum* was tolerant to the highest NaCl (5 %) level. The optimum temperature for growth of all isolates was in the range 20-35 ºC. Below and beyond this range, the isolates showed variations in their growth. All isolates from *C. arietinum*, nine from *P. vulgaris*, three from *P. sativum*, seven from *V. faba*, four from *L. esculentus*, and three from *T. resupinatum* grew at 40 ºC. The majority of the isolates that grew at 45 ºC came from *C. arietinum* (9 isolates) and *P. vulgaris* (seven isolates), however, only two isolates from each of *V. faba* and *T. resupinatum* grew at 45 ºC. All isolates grew at a pH range from 6 to 8. All isolates from *C. arietinum* and *P. vulgaris*, 9 from *T. resupinatum*, and 7 from *L. esculentus* grew at pH 9. Only 2 isolates from *L. esculentus*, 8 from *C. arietinum*, and 10 from *P. vulgaris* showed growth at pH 10. None of the isolates grew over pH 10. All isolates from *C. arietinum* grew at 0.01 % of both Zinc and Iron. The majority (9 isolates out of 11) tolerated 0.01 % Lead, Nickel, and Cupper. The 0.1 % Nickel treatment inhibited growth of *C. arietinum* isolates, since only four isolates survived at this concentration. Isolates from *P. vulgaris* resisted 0.01 % Pb, Cu, and Iron. Nine isolates (out of 12) tolerated 0.1 % Iron, while 0.1 % Zn was inhibitory to all isolates from this plant. Ni, Cu, and Pb at 0.1 % came next to the 0.1 % Zn in its inhibitory action on isolates from *P. vulgaris*, where only two isolates from this plant grew at this level of these heavy metals. All isolates from *P. sativum* grew at 0.01 % Pb, Cu, and Fe. The majority (9 isolates out of 12) resisted 0.1 % of both Fe and Pb. Meanwhile, only 3 isolates grew at 0.1 % of Cu, Zn, and Ni. All *V. faba* isolates tolerated 0.01 % of all heavy metals tested except Fe. Nine isolates were resistant to 0.1 % Cu, 7 isolates resisted 0.1 % Fe, while only 4 isolates survived at 0.1 % Zn. Growth of *L. esculentus* isolates was inhibited by 0.1 % Zn, while the whole isolates grew at 0.01 % of Ni, Fe, and Pb. A high number of the same plant isolates (10 isolates out of 11) tolerated 0.1 % Fe, while only 3 isolates survived at 0.1 Ni. Isolates from *L. esculentus* were largely inhibited (only single isolate survived) by 0.1 % Pb. *T. resupinatum* isolates tolerated 0.01 % and 0.1 % Fe. Besides, nine isolates (out of 11 isolates) grew at both 0.01 % and 0.1 % of both Pb and Cu. *T. resupinatum* isolates were largely inhibited by 0.1 % Zn and each of 0.01 % and 0.1 % Ni. The data for stress factors did not shown, and instead, numerical taxonomy data are presented (Table 4).

Intrinsic Antibiotic Resistance:

Isolates from different plants showed different resistance to the selected antibiotics (data not shown but included in numerical analysis, Table 4). All isolates from *C. arietinum* resisted neomycin (30 µg/disc) and geramycin (20 µg/disc). All isolates from *P. vulgaris* resisted Geramycin (20 µg/disc), while only three isolates from the same plant resisted tetracycline (30 µg/disc). Eleven isolates (out of 12 isolates) from *P. sativum* resisted neomycin (30 µg/disc). All *V. faba* isolates resisted tetracycline (30 µg/disc). Growth of isolates from *L. esculentus* greatly inhibited by streptomycin (20 µg/disc) and tetracycline (30 µg/disc). Growth of *T. resupinatum* isolates was greatly inhibited by tetracycline (30 µg/disc) and streptomycin (20 µg/disc), only two isolates survived the effects of these two antibiotics.

Enzymatic Activities:

All isolates from various hosts showed a positive catalase test. *L. esculentus*, *T. resupinatum* isolates, and 11 isolates (out of 12 isolates) from *P. vulgaris* showed a positive urease test. All isolates from *P. sativum*, *L. esculentus*, and *T. resupinatum* showed a gelatinase activity. Data of enzymatic activities were not shown but included in numerical analysis data (Table 4).

Numerical Analyses:

Based on the data of the above phenotypic characteristics, dendrograms were constructed by the unweighed-pair wise group method with the average (UPGMA) clustering method using the statistical package for social science (SPSS). A dendrogram was constructed for each plant set of isolates, i.e. six dendrograms were obtained for the isolates from the six host plants, and in each dendrogram the reference isolates were included. Dendrograms did not shown but clustering analysis data are presented in Table (4). *V. faba* isolates grouped in 2 clusters. The majority of *V. faba* isolates (7 out of 11) grouped at 82 % similarity in cluster 1 along with *R. legumunosarum* reference strain VF (S15). The rest of the isolates grouped at cluster 2 harbouring the references PV(S17), LE (S16) and PS (S14). The majority (9 out of 12) of the isolates from *P. sativum* grouped at 80 % similarity in cluster 2, which is not including any of the reference strains. The rest of the isolates (3 isolates) grouped in cluster 1 along with *R. leguminosarum* reference strains PS (S15), LE (S17), and PV (S18). *C. arietinum* isolates grouped at 78 % similarity in 3 clusters of which cluster 1 comprised five, out of eleven, isolates, in addition to *R. leguminosarum* reference strains VF (S15) and TR (S13). Only two isolates grouped in cluster 2 along with *R. leguminosarum* reference strains LE (S16), while four isolates were grouped in cluster 3

with *Mesorhizobium* reference strain CA (S12). *L. esculentus* isolates classified at 78 % similarity into 2 clusters. Cluster 2 comprised the majority of isolates (7 isolates out of 11) along with *R. leguminosarum* reference strains PV (S17), LE (S16), VF (S15), and PS (S14), while four isolates were grouped in cluster 1. Isolates from *P. vulgaris* grouped at 80 % similarity into 4 clusters. Most of the isolates (7 isolates out of 12) grouped in cluster 4 along with the *R. leguminosarum* reference strain TR (S14), two isolates in cluster 3 with none of the reference strains, and two isolates in cluster 1 with *R. leguminosarum* VF (S16) and *Mesorhizobium* CA (S13) reference strains. *T. resupinatum* isolates from this plant grouped at 79 % similarity into 3 clusters. The majority (8 isolates out of 11) of the isolates grouped in cluster 1 along with *R. leguminosarum* reference strains PV (S17), LE (S16), and PS (S14). Two isolates were grouped in cluster 2 along with *R. leguminosarum* reference strains and only a single strain was grouped in cluster 3 along with *Mesorhizobium* reference strain CA (S12).

Discussion:

Rhizobia are soil bacteria that colonize the rhizosphere of legumes and other plants. They are rather diverse group of bacteria than might be supposed, but are united by their ability to form nodules on legumes and occasionally non-legume plants, e.g., *Parasponia (*Ulmaceae). These bacteria are among the most intensively studied groups of microorganisms (Sessitsch *et al*. 2002), mainly due to their N_2-fixing ability and their potential to replace N-fertilizers, with emphasis on their key role in achieving sustainability of N-poor soils (Zahran 2006, Zahran 2009). However, many of these rhizobia remain unidentified (Novikova *et al*. 1994).

Growth pattern and cultural characteristics of root-nodule rhizobial isolates in this study, whether being from crop legumes or from the wild herb legume (*T. resupinatum*), were in agreement with the general characteristics of fast-growing rhizobia summarized in Bergey's Manual of Systematic Bacteriology (Jordan 1984) and also in other reports (e.g., De Lajudie *et al*. 1994). Colonies developed within one to three days and produce extracellular polysaccharide slime and acid when grown on YMA medium. The results obtained in this study were in agreement with that obtained by Hafeez *et al*. (1995) and Mpepereki *et al*. (1997). The isolates from root nodules of *Macroptilium atropurpureum* (Siratro) formed gummy and translucent colonies (Hafeez *et al*. 1995). Cowpea (*Vigna unguiculata*) rhizobial isolates from 14 Zimbabwean soils had colony morphologies (colony diameter) ranged from small (<1-2 mm) dry or gummy to large (>3 mm) "wet" watery/slimy types (Mpepereki *et al*. 1997). The bacterial isolates in this study were related to rhizobium groups, being exhibited colony characteristics resembling those of reference rhizobia, and by their success to re-nodulate their legume hosts and forming effective nodules (Tables 2 and 3). The representative isolates selected from each legume plant showed measurable nitrogenase activity (up to 0.5 μ moles C_2H_4 plant-^{1} hr-^{1}) similar to the rate given in early studies (Zahran *et al.* 2003, Al Sherif *et al.* 2005) by plants raised in greenhouse experiments.

Rhizobia are quite variable in their nutritional requirements and, accordingly, were divided into fast or slow-growing species (Glenn and Dilworth 1981, Stowers and Elkan 1984, Ahmad and Smith 1985). Utilization of carbon and nitrogen sources is one of these nutritional requirements and nutritional surveys contributed to the earlier classification of rhizobia. Zabaloy and Gomez (2005), used the carbon source utilization as one of the taxonomic markers to discriminate rhizobial isolates. Isolates in this study utilized the monosaccharide sugars (glucose and fructose), the disaccharide sugars (sucrose and lactose), the polysaccharide (starch), as well as mannitol, which is the traditional energy source of most rhizobia. These results were in agreement with the previous studies (Singh *et al*. 1980, Glenn and Dilworth 1981, Stowers and Elkan 1984, Waldon *et al*. 1989, Zahran 1991b, Zhang *et al*. 1991, Helemish *et al*. 1993, Novikova *et al*. 1994). The present study showed that the tested isolates utilized a variety of carbon sources. This coincided with reports on some rhizobial isolates from chickpea (Maatallah *et al*. 2002), most of the isolates were able to catabolize a large variety of carbon sources including D-fructose, sorbitol, mannitol, trehalose, and cellobiose. Also, Kucuk *et al*. (2006) tested the ability of rhizobial isolates from *P. vulgaris* to utilize a variety of carbon sources, all isolates were able to grow well in the presence of D-fructose, D-galactose, D-glucose, D-mannitol, and sucrose. One of the nutritional needs of rhizobia that can be used as a phenotypic character is the utilization of amino acids as a sole nitrogen source. Many amino acids were tested as a sole nitrogen source to the rhizobial isolates (Stowers and Elkan 1984, Kucuk *et al*. 2006). *P. vulgaris* isolates showed diversity in their utilization of amino acids as sole nitrogen sources (Kucuk *et al*. 2006), the majority of these isolates utilized the amino acids L-alanine and L-serine. None of the rhizobial strains studied by Zhang *et al*. (1991) utilized the amino acids L-asparagine and L-methionine. However, other studies (Mohamed *et al*. (2000) reported the utilization of these two amino acids. The variation in utilization of different nutrients obviously reflects the possible taxonomic diversity of these isolates in this study.

Rhizobia are fairly salt-tolerant organisms. It was reported in many works that free-living rhizobia are more tolerant to salt stress than their host legumes (Zahran and Sprent 1986, Sprent and Zahran 1988, Mohammad *et al*. 1989, Zahran 1999, Zahran *et al.* 2007, Laranjo and Oliveira 2011). Most of rhizobial isolates in the present work showed high capacity for salt tolerance ranging from 1-5 % NaCl. This NaCl tolerance range agreed with previous reports (Hamdi and Al-tai 1981, El-Sheikh and Wood 1990, Breedveld *et al*. 1991, Zhang *et al*. 1991,

Mpepereki *et al.* 1997, Maatallah *et al*. 2002, Zahran *et al*. 2003, Al-Shaharani and Sheetta 2011). Nevertheles, other *Rhizobium* strains from various collections from arid and saline areas are highly salt-tolerant and withstand at high NaCl levels up to 5-10 % (Mohammad *et al*. 1991, Zahran *et al*. 2003). Rhizobia are predominately mesophilic and have optimum temperatures for growth in culture at the range of 28-31 °C (Graham 1992). Maximum temperature degrees (T_{max}) for free-living rhizobia ranged between 35-45 °C (Josephson and Pepper 1984, Silsbury *et al*. 1984, Hosny *et al*. 1991, Zhang *et al*. 1991, Zahran *et al*. 1994). However, other studies (Kulkarni *et al*. 2000) showed that rhizobia strains from *Sesbania aculaeta*, survived at 50 °C and 65 °C on YMA at pH 7 for up to 2 and 4 hours. Rhizobial strains obtained in the present study showed a relatively high (T_{max}), a large number of isolates showed growth at 40 and 45 °C and one strain (BSTR8) from *T. resupinatum* showed growth at 50 °C. This finding agreed with the results of previous studies on *R. leguminosarum* strains isolated from Nile Valley of Egypt, which showed tolerance to temperatures between 35-40 °C (Moawad and Beck 1991) and *C. arietinum* rhizobial isolates, which grew at 45 °C (Maatallah *et al*. 2002). The general responses to temperature stress in rhizobia are recently reviewed (Alexandre and Oliveira 2012). Abundance of microorganisms (e.g. rhizobia) in soil can be limited with soil acidity and alkalinity (Brockwell *et al*. 1991). The majority of the isolates in this study grew well at pH ranges from 6 to 8. About 70 % and 28 % of the isolates in the present study tolerated alkaline pHs (9 and 10), respectively. The growth of our isolates at pH 6 and the alkaline pH 9, agreed with the results of earlier studies on rhizobial strains (Yadav and Vyas 1973), which showed that even pH 10 was not inhibitory to rhizobial strains and these strains were sensitive to acidic conditions (pH 3.5-4.0). In addition, Hemphil and Jackson (1982) found that pHs of 5.6-6.4 were optimal for bean rhizobia. Rhizobial strains from *C. arietinum* showed similar behavior to our isolates, as 90 to 100 % of the isolates grew at slightly acidic and neutral pH and 50 to 70 % of the isolates grew at alkaline pHs (Maatallah *et al*. 2002). The growth and biological nitrogen fixation of rhizobia and their legume hosts under stressed environments were recently reviewed (Mabrouk and Belhadj 2012).

The intrinsic antibiotic resistance (IAR) pattern analysis was also adopted in the present study as a useful complementary tool for characterization and discrimination of rhizobial isolates. Several studies (Beynon *et al*. 1980, Jenkins and Bottomley 1985, Brockman and Bezdicek 1989, Shishido and Pepper 1990, Maatallah *et al*. 2002) had been used to employ (IAR) as a crucial phenotypic parameter for rhizobial characterization. Sensitivity to antibiotics differs among and within rhizobial species (Josey *et al*. 1979). A similar variation in the (IAR) pattern has been reported for chickpea rhizobial isolates (Maatallah *et al*. 2002), 65 % of the isolates showed high resistance to nalidix acid, kanamycin, and erythromycin, and 14-25 % of the isolates were resistant to ampicillin, streptomycin, tetracycline, and chloramphenicol. Similarly, the isolates in this study showed variation in their resistance to the tested antibiotics. Most of our isolates were sensitive to tetracycline, kanamycin, and streptomycin, but were resistant to neomycin, geramycin, and tropamycin. Tetracycline and streptomycin were generally the most active antibiotics against rhizobia.

In conclusion, the isolated rhizobial strains are of good traits, e.g., tolerant to high salt levels, resistant to antibiotics, resistant to alkaline pH, showed positive reactions to the enzymes tested, and utilized wide sources of carbon and nitrogen. The rhizobia are diverse, as shown from phenotypic characterization and numerical analysis. *P. vulgaris* was shown to be a broad host for more than a single rhizobium species such as *R. leguminosarum*, *R. mongolense,* and *R. lusitanum.* The isolates (BSPS7 and BSPS10) from *P. sativum* were classified as *R. etli*, though it has not been reported that *R. etli* nodulate *P. sativum*. Consequently, these rhizobial strains also have a much broader host range than originally thought.

REFERENCES

Ahmad, M.H., E. Smith, 1985. Utilization of carbon and nitrogen sources and acid/alkali production by cowpea rhizobia. Plant and Soil, 86: 279-282.

Alexandre, A., S. Oliveira, 2012. Response to temperature stress in rhizobia. Critical Reviews in Microbiology, DOI: 10.3109/1040841X.2012.702097.

Al-Shaharani, T.S., N.D. Shetta, 2011. Evaluation of growth, nodulation and nitrogen fixation of two *Acacia* species under salt stress. World and Applied Sciences Journal, 13: 256-265.

Al-Sherif, E.A., H.H. Zahran, A.M. Atteya, 2005. Nitrogen fixation and chemical composition of wild annual legumes at Beni-Suef Governorate, Egypt. Egyptian Journal of Biology, 6: 32-38.

Beynon, J.L., J.E. Beringer, A.W.B. Johnston, 1980. Plasmid and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. Journal of General Microbiology, 120: 421-429.

Biomy, A.M., 2000. Symbiosis between rhizobia and legumes as affected by heavy metals. Ph. D. Thesis, Cairo University, Giza, Egypt.

Breedveld, M.W., L.P.T.M. Zevenhuizen, A.B. Zahnder, 1991. Osmotically-regulated trehalose accumulation and cyclic beta-(1,2) glucan excreted by *Rhizobium leguminosrum* bv. *trifolii* TA-L. Archives of Microbiology, 156: 501-506.

Brockman, F.J. and D.F. Bezdicek, 1989. Diversity within serogroups of *Rhizobium leguminosorum* bv. *viceae* in the Palouse region of Eastern Washington as indicated by plasmid profiles, intrinsic antibiotic resistance, and topography. Appllied and Environmental Microbiology, 55: 109-115.

Brockwell, J., A. Pilka and R.A. Holliday, 1991. Soil pH is a major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non cultivated soils of New South Wales. Australian Journal of Agriculture, 31: 211-219.

Cooper, J.E., M. Wood and A.J. Bjourson, 1985. Nodulation of *Lotus pedunculatus* in acid rooting solution by fast- and slow-growing rhizobia. Soil Biology and Biochemistry, 17: 487-492.

Cunningham, S.D. and D.N. Munns, 1984. The correlation between extracellular polysaccharides prodution and acid tolerance in *Rhizobium*. Soil Sci. Soc. Am. Journal, 48: 1213-1226.

Date, R.A. and L.S. Hurse, 1992. Growth, competitiveness and effectiveness of spontaneous antibiotic resistant strains of *Bradyrhizobium* for *Desmodium intortum* cv. greenleaf. Soil Biology and Biochemistry, 24: 33-39.

De Ljudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M.D. Collins, B. Dreyfus, K. Kersters and M.F. Gillis, 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. lnt. J. Syst. Bacteriology, 44: 715-733.

Dreyfus, B., Y.L. Garcia, M. Gillis, 1988. Characterization of *Azorhizobium caulinodans* gen. nov. sp. nov., a stem-nodulating nitrogen - fixing bacterium isolated from *Sesbania rostrata.* Int. J. Syst. Bacteriology, 38: 89-98.

Eaglesham, A.R.J., A. Ayanaba, 1984. Tropical stress ecology of rhizobia, root-nodulation and legume nitrogen fixation. In: Current Developments in Biological Nitrogen Fixation, Subba Rao, N.S. (ed.), Edward Arnold Publishers, London, U.K, pp: 1-35.

El-Aziz, R., J.S. Angle, R.L. Chaney, 1991. Metal tolerance of *Rhizobium meliloti* isolated from heavy-metal contaminated soils. Soil Biology and Biochemistry, 23: 751-758.

El-Gamal, M.S., 1988. Effect of cadmium compounds on growth of *Rhizobium sesbani* and dinitrogen fixation of *Sesbania sesban.* Egyptian. Journal of Microbiology, 23: 343-355.

El-Idrissi, M.M., N. Aujjar, A. Belabed, Y. Dessaux, A. Filali-Maltouf, 1996. Characterization of rhizobia from a carob tree (*Ceratonia siliqua*). Journal of Applied Bacteriology, 80: 165-173.

El-Sheikh, E.A.E., M. Wood, 1990. Salt effects on survival and multiplication of chickpea and soybean rhizobia. Soil Biology and Biochemistry, 22: 343-347.

Garrity, G.M., J.A. Bell, T.G. Lilburn, 2004. Taxonomic outline of the prokaryotes, Bergey's Manual of Systematic Bacteriology, second edition, Release 5.0 May 2004. Bergey's Manual Trust. http://dx.doi.org/10. 1007/bergeysoutline.

Giller, K.E., S.P. McGarth, P.R. Hirsch, 1989. Absence of nitrogen fixation in clover grown on soil subjected to long-term contamination with heavy metals is due to survival of only ineffective *Rhizobium.* Soil Biology and Biochemistry, 21: 841-848.

Glenn, A.R. and M. Dilworth, 1981. The uptake and hydrolysis of disaccharides by fast- and slow-growing species of *Rhizobium*. Archives Microbiology, 129: 233-239.

Graham, P.H., 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can. Journal of Microbiology, 138: 475-484.

Graham, P.H., K. Draeger, M.L. Ferrey, K.J. Coneoy, B.E. Hammer, E. Martinez, S.R. Naarons, C. Quinto, 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance in *Rhizobium tropici* UMB 1899. Canadian Journal of Microbiology, 40: 198-207.

Graham, P.H., M.J. Sadowsky, H.H. Keyser, Y.M. Barnet, R.S. Bradley, J.E. Cooper, D.J. Deley, B.D.W. Jarvis, E.B. Roslycky, B.W. Strijdom, J.P.W. Young, 1991. Proposed minimal standards for the description of new genera and species of root-and stem- nodulating bacteria. Int. J. Syst. Bacteriology, 41: 582-587.

Hafeez, F.Y., S. Asad, T. Ahmad, K.A. Malik, 1995. Host-specificity and characterization of fast-growing rhizobia from *Macroptilium atropurpureum* cv. *siratro* in Pakistan. Soil Biology and Biochemistry, 27: 729-733.

Hamdi, Y.A., A.M. AI-tai, 1981. Salt tolerance of strains of *Rhizobium meliloti* and *R. trifolii* to the chlorides of sodium, calcium and magnesium. Egypt. Journal of Microbiology, 16: 1-7.

Hardy, R.W.F., R.D. Holsten, E.K. Jackson, R.C. Burrns, 1968. The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. Plant Physiology, 43: 1185-1207.

Helemish, F.A., S.M.A. El-Gammal, 1987. Salt and pH tolerance of *Rhizobium leguminosarum* TAL 271. Zentrablbl. MiKrobiol., 142: 211-214.

Helemish, F.A., M.T. EI-Mokadem, S.H. Abou-Zekry, 1993. Nutritional requirements and invertase activity of *Rhizobium* nodulating *Sesbania sesban* roots. Zentralbl. Mikrobiol., 148: 582-587.

Hemphill, D.J., T.L. Jackson, 1982. Effect of soil acidity and nitrogen on yield and elemental concentration of bush bean (*Phaseolus vulgaris*) carrot (*Ducas carrota*) and lettuce (*Lactuca sativa*). Am. Soc. Hortic. Science, 5: 740-744.

Hosny, I.M., L. Zohdy, A. Turky, 1991. Prevalence in Egyptian soils of *R. leguminosarum* bv. *viceae* and their physiological properties. Egypt. Journal of Microbiology, 26: 209-222.

Jenkins, M.B., P.J. Bottomley, 1985. Composition and field distribution of the population of *Rhizobium meliloti* in root-nodules of uninoculated field-grown alfalfa. Soil Biology and Biochemistry, 17: 173-179.

Jordan, D.C., 1984. Family III. Rhizobiaceae Conn. 1938. In: Bergey's Manual of Systematic Bacteriology, Krieg, N.R and Holt, I.G. (eds.), Vol. I, Williams and Wilkins, Baltimore, pp: 234-244.

Josephson, K.L., I.L. Pepper, 1984. Competitiveness and effectiveness of strains of *Rhizobium phaseoli* isolated from the Sonoran desert. Soil Biology and Biochemistry, 16: 651-655.

Josey, D.P., J.L. Benyon, A.W.B. Johnston, J.E. Beringer, 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. Journal of applied Bacteriology, 46: 343-350.

Karanja, N.K. and M. Wood, 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya. Tolerance of high temperature and antibiotic resistance. Plant and Soil, 112: 15-22.

Kassem, M., A. Capellano, A. Gounot, 1985. Effect of sodium chloride on *in vitro* growth and infectivity and effectiveness of *Rhizobium meliloti.* Micren J. Appl. Microbiology, 1: 63-76.

Kucuk, C., M. Kivanc, E. Kinaci, 2006. Characterizaion of *Rhizobium* sp. isolated from bean. Turk. Journal of Biology, 30: 127-132.

Kulkarni, S., S. Surange, C.S. Nautiyal, 2000. Crossing the limits of *Rhizobium* existence in extreme conditions. J. current Microbiology, 41: 402-409.

Kuklinsky-Sobral, J., W.L. Araújo, R. Mendes, I.O. Geraldi, A.A. Pizzirani-Kleiner, J.L. Azevedo, 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environmental Microbiology, 6: 1244-1251.

Kumer, V., K. Vimal, R. Bala, N. Nafees, 1990. Effect of salinity, alkalinity and acid conditions on growth of cowpea *Rhizobium*. Acta Botanica Indica, 18: 290-292.

Laranjo, M., S. Oliveira, 2011. Tolerance of *Mesorhizobium* type strains to different environmental stresses. Antonie van Leeuwenhoek, 99: 651-662.

Li, Q.Q., E.T. Wang, Y.L. Chang, Y.M. Zhang, X.H. Sui, W.H. Chen, W.X. Chen, 2011. *Ensifer sojae* sp. nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils. Int. J. Syst. Evolut. Microbiology, 61: 1981-1988.

Maatallah, J., E. Berraho, J. Sanjuan, C. Lluch, 2002. Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Moroccoan soils. Agronomie, 22: 321-329.

Mabrouk, Y., O. Belhadj, 2012. Enhancing the biological nitrogen fixation of leguminous crops grown under stressed environments. African Journal of Biotechnology, 11: 10809-10815.

Mantelin, S., M. Fisher-Le Saux, F. Zakhia, G. Bena, S. Bonneau, H. Jeder, P. De Lajudie, J.C. Cleyet-Marel, 2006. Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassiccearum* sp. nov. Int. J. Syst. Evolut. Microbiology, 56: 827-839.

McGrath, S.P., 1993. Effect of heavy metals from sewage sludge on soil microbes in agricultural ecosystems. In: Toxic Metals in Soil-Plant Systems, Ross, S.M. (ed.), Wiley, Chichester, New York, pp: 247-274.

McGrath, S.P., P.R. Hirsch, K.E. Gillar, 1988. Effects of heavy metals contamination on the genetics of nitrogen fixing populations of *Rhizobium legumonosarum* bv. *trifolii.* In: Proceedings of the International Conference of Environmental Contamination, Orio, A.A. (ed.), Venice, Italy, pp: 164-166.

Moawad, H., D.P. Beck, 1991. Some characteristics of *Rhizobium leguminosarum* isolates from uninoculated field grown lentil. Soil Biology and Biochemistry, 23: 933-937.

Mohammad, R.M., A. Akhauan-Kharazian, W.F. Campbell, M.D. Rumbaugh, 1991. Identification of salt- and drought-tolerant *Rhizobium meliloti* strains. Plant and Soil, 134: 271-276.

Mohammad, R.M., W.F. Campell, M.D. Rumbaugh, 1989. Acetylene reduction in salt-tolerant alfalfa and *Rhizobium.* Arid Soil Rehabilitation, 3: 469-476.

Mohamed, S.H., A. Smouni, M. Neyra, D. Kharchat, A. Filali MaItouf, 2000. Phenotypic characteristics of root-nodulating bacteria isolated from Libyan *Acacia* spp. Plant and Soil, 224: 171-183.

Mpepereki, S., F. Makonese and A.G. Wollum, 1997. Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. Symbiosis Rehovot., 22: 275-292.

Nassef, M.A.A., 1995. Selection of effecient strains of *Rhizobium leguminosarum* with special traits and studying their serological relationship. Ph.D. Thesis, Faculty of Agriculture, Ain Shams University, Egypt.

Novikova, N.I., E.A. Pavlova, N.I. Vorobjev, E.V. Lim_shcbenko, 1994. Numerical taxonomy of *Rhizobium* strains from legumes of temperate zone. Int. J. Syst. Bacteriology, 44: 734-742.

Odee, D.W., J.M. Sutherland, E.T. Makatiani, S.G. McInroy and J.I. Sprent, 1997. Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. Plant and Soil, 188: 65-75.

Richardson, A.E., R.J. Simpson, 1989. Acid-tolerance and symbiotic effectiveness of *Rhizobium trifolii* associated with *Trifolium subterraneum* L. based pasture growing in acid soil. Soil Biology and Biochemistry, 12: 1213-1226.

Rohm, M., D. Werner, 1992. *Robinia pseudoacacia-Rhizobium* symbiosis: isolation and characterization of a fast nodulating and efficiently nitrogen fixing *Rhizobium* strain. Nitrogen Fixing Tree Res. Reports, 10: 193-197.

Sahgal, M., B.N. Johri, 2006. Taxonomy of rhizobia: current status. Current Science, 90: 486-487.

Sessitsch, A., J.G. Howieson, X. Perret, H. Antoun, E. Martinez-Romero, 2002. Advances in *Rhizobium* Research. Critical Review Research Plant Science, 21: 323-378.

Shishido, M., I.L. Pepper, 1990. Identification of dominant indigenous *Rhizobium meliloti* by plasmid profiles and intrinsic antibiotic resistance. Soil Biology and Biochemistry, 22: 11-16.

Silsbury, J.H., D. Zuill, P.H. Brown, 1984. Effect of temperature on germination and early seedling growth of swards of cultivar Mt Barker of subterraneaum clover *T. subterraneum* plants grown with and without nitrate. australian J. Agriculture Research, 35: 539-246.

Sinclair, M.J., A.R.J. Eaglesham, 1984. Intrinsic antibiotic resistance in relation to colony morphology in three populations of West African cowpea rhizobia. Soil Biology and Biochemistry, 16: 247-251.

Singh, R., P.S. Sidhu, S. Vadhera, J.S. Sital, I.S. Bhatia, 1980. Extracellular invertase of *Rhizobium japonicum* and its role in free sugar metabolism in developing root nodules of *Sesbania grandiflora*. Physiologia Plantarum, 48: 504-508.

Somasegaran, P., H.J. Hoben, 1985. Methods in legume-*Rhizobium* technology, University of Hawaii NIFTAL Project and Mircen, Hawaii, USA.

Somasegaran, P., H.J. Hoben, 1994. Handbook for rhizobia, Springer-Verlag, New York, USA.

Sprent, J.I., 1969. Prolonged reduction of acetylene by detached soybean nodules. Planta, 88: 372-375.

Sprent, J.I., 1995. Legume trees and shrubs in the tropics: N_2 fixation in perspective. Soil Biology and Biochemistry, 27: 401-407.

Sprent, J.I., H.H. Zahran, 1988. Infection, development and functioning of nodules under drought and salinity. In: Nitrogen Fixation by Legumes in Mediterranean Agriculture, Beck, D. P. and Materon, L. A. (eds.), Martinus Nijhoff/Dr. W. Junk, Dordrecht, Netherland, pp: 145-151.

Stowers, M., A.J. Eaglesham, 1984. Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum.* Plant and Soil, 77: 3-14.

Stowers, M.D., G.M. Elkan, 1984. Growth and nutritional characteristics for cowpea rhizobia. Plant and Soil, 80: 191-200.

Swelim, D.M., 1996. Environmental and nutritional studies on *Rhizobium* spp. (*Leucaena*). Ph.D. Thesis, Faculty of Agriculture, Cairo University, Cairo, Egypt.

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Keresters, J. Swings, 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Review, 60: 407-483.

Vargas, A.A.T., N.D. De Nardin, P.V. Berkum, P. Van-Berkum, 1992. Tolerance of indigenous bean rhizobia to antibiotic and possible relationship with soil acidity factors. Revista-Brasileira-de-Ciencia-do-Solo, 16: 331-336.

Vidal, C., C. Chantreuil, O. Berge, L. Mauré, J. Escarré, J. Béna, B. Brune, J.C. Cleyet-Marel, 2009. *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of *Anthyllis vulneraria* growing on metallicolous soil in Languedoc, France. Int. J. Syst. Evolution. Microbiology, 59: 850-855.

Vincent, J.M., 1970. A manual for the practical study of the root-nodule bacteria. IBP15. Blackwell Scientific Publications, Oxford and Edinburgh. U.K.

Waldon, H.B., M.B. Jenkins, R.A. Virginia, E.E. Harding, 1989. Characteristics of woodland rhizobial populations from surface- and deep-soil environments of the Sonoran Desert. Applied and Environmental Microbiology, 55: 3058-3064.

Willems, A., 2006. The taxonomy of rhizobia: an overview. Plant and Soil, 287: 3-14.

Wood, M., J.E. Cooper and A.J. Bjourson, 1988. Response of *Lotus* rhizobia to acidity and aluminum in liquid culture and in soil. Plant and Soil, 107: 227-231.

Yadav, N.K. and S.R. Vyas, 1973. Salts and pH tolerance of rhizobia. Folia Microbiologica, 3: 242-247.

Young, C.C. and C.C. Chao, 1989. Intrinsic antibiotic resistance and competition in fast- and slow-growing soybean rhizobia on a hybrid of Asian and US cultivars. Biology and Fertility of Soils, 8: 66-70.

Zabaloy, M.C. and M.A. Gomez, 2005. Diversity of rhizobia isolated from an agricultural soil in Argentina based on carbon utilization and effects of herbicides on growth. Biology and Fertility of Soils, 42: 83-88.

Zahran, H.H., 1991a. Conditions for successful *Rhizobium*-legume symbiosis in saline environments. Biology and Fertility of Soils, 12: 73-80.

Zahran, H.H., 1991b. Cultural and physiological properties of some root-nodule bacteria indigenous in the salt-affected soils of Egypt. Bulletin of the Faculty of Science (Assiut University, Egypt), 20: 85-99.

Zahran, H.H., 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews, 63: 968-989.

Zahran, H.H., 2005. Rhizobial nitrogen fixation in agriculture: biotechnological perspectives. In: Microbial biotechnology in agriculture and aquaculture, Volume 1, Ray, R.C. ed., Science Publishers, Inc., Enfield, USA, pp: 71-100.

Zahran, H.H., 2006. Nitrogen (N_2) fixation in vegetable legumes: biotechnological perspectives. In: Microbial biotechnology in Horticulture. Volume 1, Ray, R.C. Ward, O.P., eds., Science Publishers, Inc., Enfield, USA, pp: 49-82.

Zahran, H.H., 2009. Enhancement of rhizobia–legumes symbioses and nitrogen fixation for crops productivity improvement. In: Microbial Strategies for Crop Improvement, M.S. Khan et al. (eds.), Springer-Verlag, Berlin, Heidelberg, pp: 227-254.

Zahran, H.H., 2010. Legumes–microbes interactions under stressed environments. In: Microbes for legume improvement, M. S. Khan et al. (eds.), Springer-Verlag, Berlin, Heidelberg, pp: 353-387.

Zahran, H.H., Abdel-Fattah, M., Ahmad, M.S., Zaki, A.Y. 2003. Polyphasic taxonomy of symbiotic rhizobia from wild leguminous plants growing in Egypt. Folia Microbiologica, 48: 510-520.

Zahran, H.H., M.C. Marin-Manzano, A.J. Sanchez-Raya, E.J. Bedmar, Venema., Kees, Rodriguez-Rosales 2007. Effects of salt stress on the expression of *NHX*-type ion transporters in *Medicago intertexta* and *Melilotus indicus* plants. Physiologia Plantarum, 131: 122-130.

Zahran, H.H., L.A. Rasanen, M. Karsisto and K. Lindstrom, 1994. Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. World Journal of Microbiology and Biotechnology, 10: 100-105.

Zahran, H.H., J.I. Sprent, 1986. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta, 167: 303-309.

Zakhia, F., H. Jeder, O. Domergue, A. Willems, J.C. Cleyet-Marel, M. Gillis, B. Dreyfus and P. de Lajudie, 2004. Characterisation of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Systematic and Applied Microbiology, 27: 380-395.

Zhang, X., R. Harper, M. Karsisto and K. Lindstrom, 1991. Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. International Journal of Systematic Bacteriology, 41: 104-113.