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Phenotypic characteristics and genetic diversity of rhizobia nodulating soybean in Egyptian soils

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ABSTRACT

Twenty rhizobial strains isolated from the root nodules of soybean (*Glycine max* L.) were collected from nine governorates representing different agro-climatic and soil conditions in Egypt. The strains were characterized using a polyphasic approach, including nodulation pattern, phenotypic characterization, 16S rDNA sequencing, *nifH* and *nodA* symbiotic genes sequencing, and rep-PCR fingerprinting. Symbiotic properties assay revealed that all local rhizobial strains showed a wide spectrum of prolific nodulation and a marked increase in plant growth parameters compared to the un-inoculated control. Complete sequencing of 16S rRNA demonstrated that, native soybean nodulating rhizobia are phylogenetically related to *Bradyrhizobium*, *Ensifer* and *Rhizobium* (syn. *Agrobacterium*) genera. Study of tolerance ability to environmental stresses revealed that local strains survived in a wide pH ranges (pH 5–11) and a few of them tolerated high acidic conditions (pH 4). *Agrobacterium* strains were identified as the highest salt-tolerant and were survived under 6% NaCl, however *Ensifer* strains were the uppermost heat-tolerant and can grow at 42 °C. *Agrobacterium* strains have been shown to harbor *nifH* and *nodA* genes similar to those in other fast growing soybean symbionts and were largely distinct from symbiotic genes of slow growing bradyrhizobia. The symbiotic effectiveness stability of *Agrobacterium* strains to nodulate soybean roots was confirmed using plant nodulation assay.

1. Introduction

Soybean (*Glycine max* L. Merrill) is called a miracle golden bean because of its nutritive value, especially as a substitute or complement of protein. Soybean production was 223 million metric tons (MMT) in 2007 [13], accounted for a half of all the leguminous crops [32]. Within the past 60 years, soybean emerged as the dominant oilseed in world trade. In 2005, the USDA [47] estimated world soybean production at 218 MMT, representing about 56% of total global oilseed production.

Soybean like other legumes forms symbiotic relationship with soil bacteria commonly referred to rhizobia [5]. Biological Nitrogen Fixation (BNF) is a fascinating biological phenomenon, which involves some legumes, whether grown as pulses for food, as pasture, in agro-forestry or in natural ecosystems [19]. The process involves the reduction of atmospheric dinitrogen to ammonia (NH_3) [16]. Worldwide 44–66 MMT of N_2 are fixed every year by agriculturally important legumes [1], with another 3–5 MMT fixed by legumes in natural ecosystems [42]. Symbiotic nitrogen fixation by legumes plays an important role in sustaining crop productivity and maintaining soil fertility of the semi-arid lands, like most Egyptian land [56]. Nevertheless, it is sensitive to various environmental stresses such as soil salinity, acidity, alkalinity, temperature which leads to unsuccessful legume nodulation. Hence, identification of bacterial strains and host cultivars that are tolerant to these stresses [3,53] will give rise to a more sustainable agriculture and it would open the way for alternate, lower cost solutions to these problems.

Soybean-nodulating rhizobia are genetically diverse and are classified into different genera and species. The slow growing bradyrhizobia, that effectively nodulate soybeans are *Bradyrhizobium japonicum* [21], *Bradyrhizobium elkanii* [23], *Bradyrhizobium liaoningense* [52] and *Bradyrhizobium yuanmingense* [4]. Other symbionts of soybean are fast growing and classified as *Ensifer* (*Sinorhizobium*) *fredii* [39] and *Ensifer xinjiangense* [34]. In addition

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to the "traditional" rhizobial genera, strains of the genus *Agrobacterium* (now, rather controversially, included in the genus *Rhizobium* [14,55]) have been frequently isolated from root nodules of various legumes including *Phaseolus vulgaris* [30], *Sesbania* spp. [7] and *Vicia faba* [46]. However, little information has been published regarding to nodulation of soybean by *Agrobacterium* [6]. These strains have been identified as *Agrobacterium* based on biochemical studies, numerical taxonomy and 16S rRNA-restriction fragment length polymorphism (RFLP) [27].

Till now there is no report addressed the phylogeny of rhizobial population associated with soybean in Egypt. Therefore, this original research is aiming at exploration the biodiversity of rhizobial population that nodulate soybean under different Egyptian agro-climatic conditions using ribosomal gene (16S rRNA) and symbiotic genes (*nifH* and *nodA*) sequencing and rep-PCR fingerprinting (REP, ERIC and BOX).

2. Materials and methods

2.1. Germplasm collection

Soybean root nodules have been collected from nine governorates that grow soybean in Egypt (Table 1 and Fig. 1). The emerging nodules which showed nitrogen fixation activity by the presence of leghaemoglobin were collected from healthy plants 50 days after sowing. The collected nodules were carefully excised from the roots with scalpel, washed by water and stored on silica gel containing vials till isolation process.

2.2. Bacterial isolates and reference strains

Root nodules were surface sterilized by washing for 30 s with 95% ethanol, immersed in 3% sodium hypochlorite and finally were washed six times by sterile double distilled water. Following sterilization, nodules were crushed aseptically in 1 ml sterile double distilled water, the nodules extract were streaked on surface of Yeast Extract Mannitol Agar (YEM) plates supplemented with 0.025 g L^{-1} of Congo red and finally were incubated at 28 °C for 3–6 days [45]. The reference strains used in this study were obtained from The Biological Nitrogen Fixation Unit, Agricultural Research Center (ARC), Giza, Egypt and Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technology Applications, Alex., Egypt.

Fig. 1. GIS map of soybean nodulating rhizobia collection sites.

2.3. Plant nodulation test

All the 20 rhizobial isolates and two reference strains were tested for their ability to nodulate soybean plants under sandy soil condition in pot experiment. The soil was analyzed according to Page et al. [33]. The main physical and chemical properties of

Table 1
List of rhizobial local isolates used in this study and their geographical origin.

Isolate	Site/governorate	Longitude	Latitude	Soil texture
NGB-SR 1	Nubaria Area/Behaira	29 51 50.634	30 54 48.22	Sandy loam (calcareous)
NGB-SR 2				
NGB-SR 3				
NGB-SR 4	Sakha village/Kafr ElSheikh	30 56 49.652	31 5 38.543	Clay (fertile)
NGB-SR 5				
NGB-SR 6				
NGB-SR 7				
NGB-SR 8				
NGB-SR 9	Agricultural Research Center/Giza	31 12 49.272	29 58 55.046	Clay loam (fertile)
NGB-SR 10	Al Batanoon, Shbeen Al Koom/Menoufia	30 59 14.539	30 37 44.906	Clay loam (fertile)
NGB-SR 11	Tersa, Tookh/Qalyoubia,	31 11 41.472	30 18 25.516	Clay loam (fertile)
NGB-SR 12	Sedse village/Beni Suef	31 05 25.775	29 04 54.349	Clay (fertile)
NGB-SR 13	Behidah, Menia/Menia	31 13 2.0520	30 26 42.942	Clay (fertile)
NGB-SR 14	Samaloot/Menia	30 42 0.4500	28 17 85.896	Clay (fertile)
NGB-SR 15	Abo Qorqas/Menia	30 49 56.280	27 55 34.590	Heavy clay (highly fertile)
NGB-SR 16	Baroot, Beni Suef/Beni Suef	31 01 17.781	29 04 15.800	Clay (fertile)
NGB-SR 17	Fourikah, Bebba/Beni Suef	30 57 53.053	28 56 8.1410	Clay (highly fertile)
NGB-SR 18	Gemizah village/Gharbia	31 7 50.8430	30 44 53.297	Clay (fertile)
NGB-SR 19	Al Khargah, New Valley	30 33 38.726	25 26 6.565	Sandy (low fertile)
NGB-SR 20				

Table 2
Physical and chemical properties of the soil used in plant nodulation tests.

Property	Value
Particle size distribution	
Sand (%)	90.00
Silt (%)	3.13
Clay (%)	6.87
Texture grade	Sandy
$CaCo_3$ (%)	1.62
Saturation percent S.P (%)	21.90
pH	7.42
E.C. (dS m^{-1} at 25 °C)	0.31
Soluble cations (meq/L)	
Ca^{2+}	0.48
Mg^{2+}	0.28
Na^{+}	1.62
K^{+}	0.58
Soluble anions (meq/L)	
CO_3^{2-}	0.00
HCO_3^{-}	0.81
Cl^{-}	0.61
SO_4^{2-}	1.54
Total N (%)	0.020
Total soluble-N (ppm)	19.87
Available-P (ppm)	6.73
Available-K (ppm)	168.30
Available-K (ppm)	0.24
DTPA extractable (ppm)	
Fe	0.98
Mn	0.32
Zn	0.50
Cu	0.26

the soil used are presented in Table 2. Seeds of the Soybean cv. *Giza* 22 were surface sterilized by immersion in 95% ethanol, followed by rinsing in 1% sodium hypochlorite for 3 min and were exhaustively washed with sterile water [22]. Plastic pots (13 cm diameter) were filled with 2 kg of soil and arranged in a complete randomized block design with three replicates. Two un-inoculated controls were conducted including, Control 1 (un-inoculated + 0 ppm N) and Control 2 (un-inoculated + 20 ppm starter N dose). All treatments received the recommended dose of super phosphate (15.5% P_2O_5) and potassium sulfate (48.5% K_2O) at the rate of 0.4/pot and 0.2 g/pot, respectively. Three seeds were planted in each pot and were inoculated with 1 ml rhizobial culture of 10^9 cells ml^{-1}. After 35 days of planting, plants were uprooted and assayed for number and dry weight of nodules and shoots and roots dry weight of soybean plants. Data were analyzed using the MSTATC analysis software [44].

2.4. Phenotypic characterization and numerical taxonomy

The tolerance ability of different rhizobial isolates and reference strains to grow under stress conditions (alkalinity, acidity, salinity and high temperatures) were assessed by inoculating 10 μl of overnight culture (about 1×10^8 rhizobial cells ml^{-1}) on YEM agar plates [17]. The pH tolerance of the isolates was examined by growing rhizobia on YEM agar medium adjusted to a pH range from 4 to 11 [20]. The salt tolerance of rhizobia was tested on YEM agar medium containing 0.1%, 0.5%, 1%, 2%, 3%, 3.5%, 4%, 5%, 6% and 7% (w/v) NaCl according to Moschetti et al. [29]. Temperature tolerance of rhizobia was examined by inoculating rhizobia on YEM agar plates and incubating at 28, 35, 40, 42 and 45 °C [25]. After 24 h of incubation at 28 °C for fast growing rhizobia and 5 days for slow growing rhizobia, plates were examined for bacterial growth. All the collected data were combined in a final matrix presenting all tested phenotypic traits for rhizobial isolates and reference strains. Traits were coded 1 for positive and 0 for negative. The relationships between rhizobia with regard to their response to pH, salinity and high temperature were analyzed using Phoretix 1D software (TotalLab, UK). Phenograms were constructed by the Un-weighted Pair Group Method with Average (UPGMA) clustering method [10,43].

Table 3
List of the primers used in PCR reactions.

Primer	Target gene	Sequences	References
fD1	16S *rDNA*	5′-AGA GTT TGA TCC TGG CTC AG-3′	[51]
rD1		5′-AAG GAG GTG ATC CAG CC-3′	
Pol-F	*nifH*	5′-TGCGAYCCSAARGCBGACTC-3′	[35]
Pol-R		5′-ATSGCCATCATYTCRCCGGA-3′	
nodA-1	*nodA*	5′-TGCRGTGGAARNTRNNCTGGGAAA-3′	[18]
nodA-2		5′-GGNCCGTCRTCRAAWGTCARGTA-3′	
REP IR	*REP*	5′-IIIICGI CGI CAT CIG GC-3′	[49]
REP 21		5′-ICG ICT TAT CIG GCC TAC-3′	
ERIC 1R	*ERIC*	5′-ATGTAAGCTCCTGGGGATTCAC-3′	[49]
ERIC 2		5′-AAGTAAGTGACTGGGGTGAGCG-3′	
BOX A1R	*BOX*	5′CTACGGCAAGGCGACGCTGACG-3′	[48]

2.5. PCR amplification and sequencing of 16S rRNA gene

Genomic DNA of rhizobial cells was isolated using Wizard® Genomic DNA purification Kit (Promega® Corporation, Madison, USA). Primers fD1 and rD1 (Table 3) were used to amplify nearly full-length 16S rRNA gene (1500 bp). PCR was performed using the standard reaction mixture (50 μl) containing: 1× PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 mM of each dNTPs, 15 pmol of each primer, 1 U of Taq polymerase enzyme (Promega® Corporation, Madison, USA) and 50 ng of DNA template. PCR reaction conditions were: an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 45 s, and extension at 72 °C for 2 min [29]. Reactions were performed in a PCR Thermo cycler machine (Biorad®, Hercules, USA). PCR products were separated on 1% agarose gel. DNA fragments were further purified and cleaned up by QIAquick Gel extraction kit (Qiagen®, Hilden, Germany) according to the kit manual instructions and sequenced using primers rD1 and fD1 (Table 3).

Table 4
Evaluation the symbiotic effectiveness of different rhizobial isolates and reference strains on soybean plants under greenhouse conditions.

Rhizobial isolates/ strains	Nodules dry weight (mg/plant)	Roots dry weight (g/plant)	Shoots dry weight (g/plant)
NGB-SR 1	122	0.74	1.84
NGB-SR 2	140	0.85	1.95
NGB-SR 3	168	0.89	2.15
NGB-SR 4	154	0.86	2.01
NGB-SR 5	174	0.84	2.11
NGB-SR 6	130	0.84	1.84
NGB-SR 7	148	0.88	2.05
NGB-SR 8	128	0.82	1.89
NGB-SR 9	141	0.82	1.91
NGB-SR 10	116	0.69	1.80
NGB-SR 11	119	0.67	1.77
NGB-SR 12	113	0.64	1.73
NGB-SR 13	113	0.62	1.71
NGB-SR 14	156	0.88	2.17
NGB-SR 15	121	0.66	1.76
NGB-SR 16	118	0.66	1.87
NGB-SR 17	121	0.80	1.84
NGB-SR 18	113	0.76	1.78
NGB-SR 19	113	0.82	1.75
NGB-SR 20	121	0.77	1.78
B. japonicum 110	137	0.80	1.90
S. fredii HH303	144	0.82	1.97
Control + 0 ppm N	0	0.53	1.42
Control + 20 ppm N	0	0.59	1.65
LSD at 0.05 level	20.4	0.07	0.16

Table 5
Phenotypic characteristics of rhizobial isolates and reference strains nodulating soybean used in this study.

Rhizobial isolates/ strains	NaCl inhibiting concentrations	Low pH tolerated values	High pH tolerated values	Maximal tolerated temperatures °C	Morphological group patterns[a]
NGB-SR 1	6.0	4	<11	40	B
NGB-SR 2	2.0	5	<11	42	A
NGB-SR 3	2.0	5	<11	42	A
NGB-SR 4	0.5	5	<11	40	C
NGB-SR 5	0.5	5	<11	40	C
NGB-SR 6	5.0	4	<11	40	B
NGB-SR 7	5.0	5	<11	40	B
NGB-SR 8	2.0	5	<11	42	A
NGB-SR 9	2.0	5	<11	42	A
NGB-SR 10	6.0	4	<11	40	B
NGB-SR 11	0.5	5	<11	40	C
NGB-SR 12	5.0	4	<11	40	B
NGB-SR 13	7.0	5	<11	40	B
NGB-SR 14	0.5	5	<11	40	C
NGB-SR 15	0.5	5	<11	40	C
NGB-SR 16	5.0	4	<11	40	B
NGB-SR 17	5.0	4	<11	40	B
NGB-SR 18	5.0	4	<11	40	B
NGB-SR 19	6.0	5	<11	40	B
NGB-SR 20	5.0	5	<11	40	B
S. fredii HH303	2.0	5	<11	42	A
B. elkanii 94	2.0	5	<11	40	C
B. japonicum 110	2.0	5	<11	40	C
B. yuanmingense	2.0	5	<11	40	C

[a] A (white, moist and flat fast growing rhizobial colonies), B (transparent, mucoid and raised fast growing rhizobial colonies) and C (opaque, highly mucoid and highly raised slow growing rhizobial colonies).

2.6. PCR amplification and sequencing of *nifH* and *nodA* symbiotic genes

The PCR protocol to amplify *nifH* products were performed according to the method of Poly et al. [35] with primer pair PolF and PolR (Table 3), which amplify a 360-bp fragment. The PCR conditions used were, 2 min at 94 °C, 35 cycles consisting of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, and finally 7 min at 72 °C. The amplification of *nodA* products was performed using the methods described by Haukka et al. [18] with primers nodA-1 and nodA-2 (Table 3), which amplify approximately 666-bp fragment. The following PCR profile was used: 2 min at 94 °C, 35 cycles consisting of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C, and finally 7 min at 72 °C. Amplification products were visualized using electrophoresis in a 1.5% agarose gel. The amplified products were purified and sequenced directly using PolF and PolR primers for *nifH* gene and nodA-1 and nodA-2 primers for *nodA* gene (Table 3).

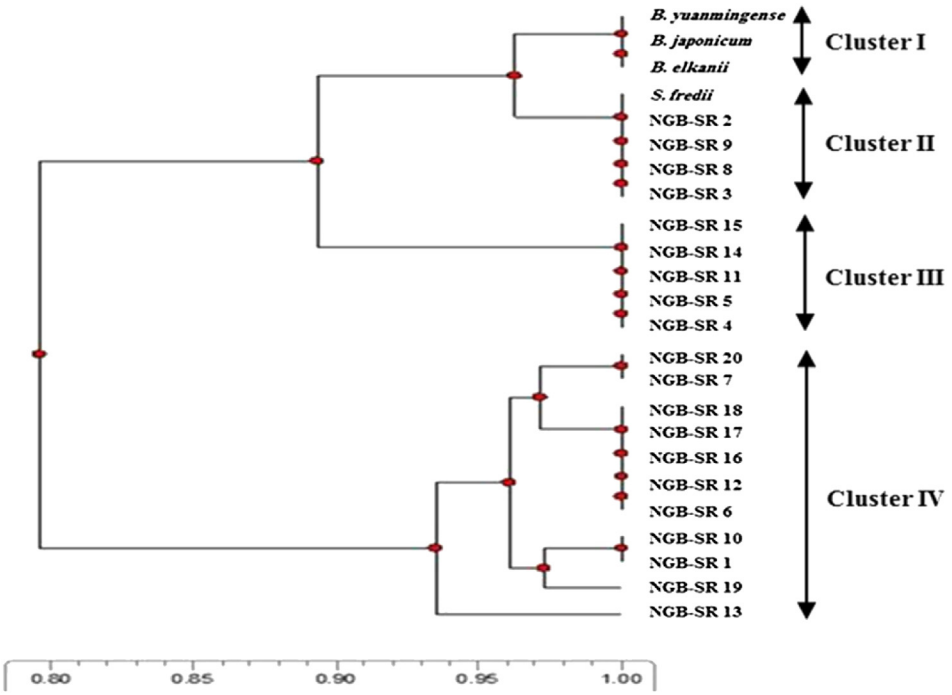

Fig. 2. UPGMA dendrogram showing relationships between the rhizobial isolates and reference strains based on phenotypic variation.

Table 6
Sequence analysis of 16S rDNA, *nifH* and *nodA* genes from Egyptian rhizobial strains nodulating soybean.

Isolate id.	16S rDNA					*nifH*		*nodA*	
	Sequence (bp)	Accession no.	Homology to the reference strains		Identity (%)	Sequence (bp)	Accession no.	Sequence (bp)	Accession no.
NGB-SR 1	1389	AB825989	*A. tumefaciens* IAM	NR_041396	99	330	AB825969	Nd	
NGB-SR 2	1403	AB825990	*E. fredii* HH303	AY260146	99	336	AB825970	626	AB827315
NGB-SR 3	1416	AB571228	*E. fredii* HH303	AY260146	99	338	AB825971	629	AB827316
NGB-SR 4	1420	AB571229	*B. yuanmingense* TSB7	FJ540949	99	335	AB825972	644	AB827317
NGB-SR 5	1397	AB825991	*B. yuanmingense* TSB7	FJ540949	99	340	AB825973	654	AB827318
NGB-SR 6	1406	AB825992	*A. tumefaciens* IAM	NR_041396	99	304	AB825974	Nd	
NGB-SR 7	1417	AB571230	*A. tumefaciens* IAM	NR_041396	100	317	AB825975	637	AB827638
NGB-SR 8	1387	AB825993	*E. fredii* HH303	AY260146	99	333	AB825976	637	AB827319
NGB-SR 9	1404	AB825994	*E. fredii* HH303	AY260146	99	332	AB825977	641	AB827320
NGB-SR 10	1407	AB825995	*A. tumefaciens* IAM	NR_041396	99	338	AB825978	Nd	
NGB-SR 11	1433	AB571231	*B. yuanmingense* TSB7	FJ540949	99	366	AB825979	640	AB827321
NGB-SR 12	1392	AB825996	*A. tumefaciens* IAM	NR_041396	99	210	AB825980	640	AB827322
NGB-SR 13	1414	AB571232	*A. tumefaciens* IAM	NR_041396	99	342	AB825981	Nd	
NGB-SR 14	1419	AB571233	*B. yuanmingense* TSB7	FJ540949	99	373	AB825982	643	AB827323
NGB-SR 15	1420	AB571234	*B. yuanmingense* TSB7	FJ540949	99	349	AB825983	645	AB827324
NGB-SR 16	1392	AB825997	*A. tumefaciens* IAM	NR_041396	99	284	AB825984	Nd	
NGB-SR 17	1416	AB571235	*A. tumefaciens* IAM	NR_041396	99	323	AB825985	511	AB827639
NGB-SR 18	1340	AB825998	*A. tumefaciens* IAM	NR_041396	99	362	AB825986	Nd	
NGB-SR 19	1399	AB825999	*A. tumefaciens* IAM	NR_041396	99	364	AB825987	Nd	
NGB-SR 20	1392	AB826000	*A. tumefaciens* IAM	NR_041396	99	328	AB825988	Nd	

Nd: Not detected.

2.7. Phylogenetic analysis

Sequence reads were edited and assembled using the DNASTAR software (Lasergene, Madison, WI). Sequence similarity searches were performed at the National Center for Biotechnology Information (NCBI) server using BLASTN (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned using Clustal W version 1.8 [2] and subjected to phylogenetic analysis. Phylogenetic trees in the context of 16S rRNA, *nifH* and *nodA* sequences were conducted using the Neighbor-Joining (NJ) method [36] and 1000 bootstrap replication to assess branching confidence.

2.8. rep-PCR fingerprinting

rep-PCR reactions were carried out using enterobacterial repetitive intergenic consensus primers ERIC 1R and ERIC 2, enterobacterial repetitive extragenic palindromic primers REP IR and REP 21 and enterobacterial repetitive sequences BOX A1 primer (Table 3). PCRs were performed using the standard reaction mixture [37]. PCR reaction conditions were: an initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min (ERIC-PCR), 53 °C for 1 min (BOX-PCR), and 40 °C for 1 min (REP-PCR) and extension at 65 °C for 8 min. PCR reaction was terminated by a final extension at 65 °C for 8 min.

3. Results

3.1. Plant nodulation assay

Twenty local rhizobial isolates were isolated from root nodules of field grown soybean in nine governorates representing all soybean cultivation areas in Egypt (Table 1). The symbiotic effectiveness of local rhizobial isolates was confirmed using plant nodulation test (Table 4). Nodules dry weight varied from 113 mg/plant for isolate NGB-SR 12–174 mg/plant for isolate NGB-SR 5. However, reference strains *B. japonicum* 110 and *S. fredii* HH303 gave 137 and 144 mg nodules/plant, respectively. On the other hand, the un-inoculated controls did not form any nodules. Shoot and root dry weight, as an indirect measure of the nitrogen fixation benefit, varied considerably among all tested isolates. Roots dry weight of soybean inoculated plants ranged from 0.62 to 0.89 g/plant. While, soybean plants inoculated by reference strains *B. japonicum* 110 and *S. fredii* HH303 gave root dry weight of 0.80 and 0.82 g/plant, respectively. The shoot dry weight of soybean inoculated plants (ranged from 1.71 to 2.17 g/plant) were significantly higher than the un-inoculated control + 20 ppm N (1.65 g/plant), indicating that all plants benefited from forming symbiosis with the rhizobial isolates.

3.2. Phenotypic characteristics

The isolated soybean rhizobia showed clear biodiversity by their growth characteristics. Seventy five percent of local rhizobial isolates were classified as fast growing rhizobia and 25% of them were slow growing. The phenotypic traits of the twenty soybean local isolates and four reference strains were summarized in Table 5. All strains grew in the optimum growth conditions (28 °C, pH 7.0 and 0.01% NaCl). Physiological traits deriver results declared a high degree of diversity between the isolated rhizobia. The Minimum Inhibitory Concentrations (MICs) of all rhizobial isolates and reference strains were estimated under different salt concentrations ranged from 0.1% to 7% NaCl (Table 5). The results showed that, the slow growing local isolates were the most sensitive and could not tolerate 0.5% NaCl, while slow growing reference strains tolerated up to 1% NaCl. On the contrary, fast growing rhizobia were more endurable and exhibited tolerance from 1% up to 6% NaCl. The most tolerant isolate was NGB-SR 13 by a MIC of 7% NaCl compared with 2% NaCl in case of *S. fredii* HH303 reference strain. All tested rhizobia were tolerant to a wide pH range started from pH 5 up to pH 11, while seven fast growing local isolates (NGB-SR 1, 6, 10, 12, 16, 17 and 18) were highly acidic tolerant up to pH 4. Regarding tolerance to high temperatures, only four fast growing local isolates (NGB-SR2, 3, 8 and 9) and *S. fredii* HH303 reference strain were tolerant up to 42 °C; however other rhizobial isolates and reference strains were sensitive to high temperatures. The dendrogram obtained from computer numerical analysis of all phenotypic traits grouped all tested rhizobia into four major clusters (Fig. 2). Slow growing rhizobia were grouped in two clusters (cluster I and cluster III). They grew at a pH between 5 and 11, at temperatures between 30 °C and 40 °C, and their growth was inhibited at 1% NaCl concentration. Fast growing rhizobia were placed in two clusters (cluster II and cluster IV).

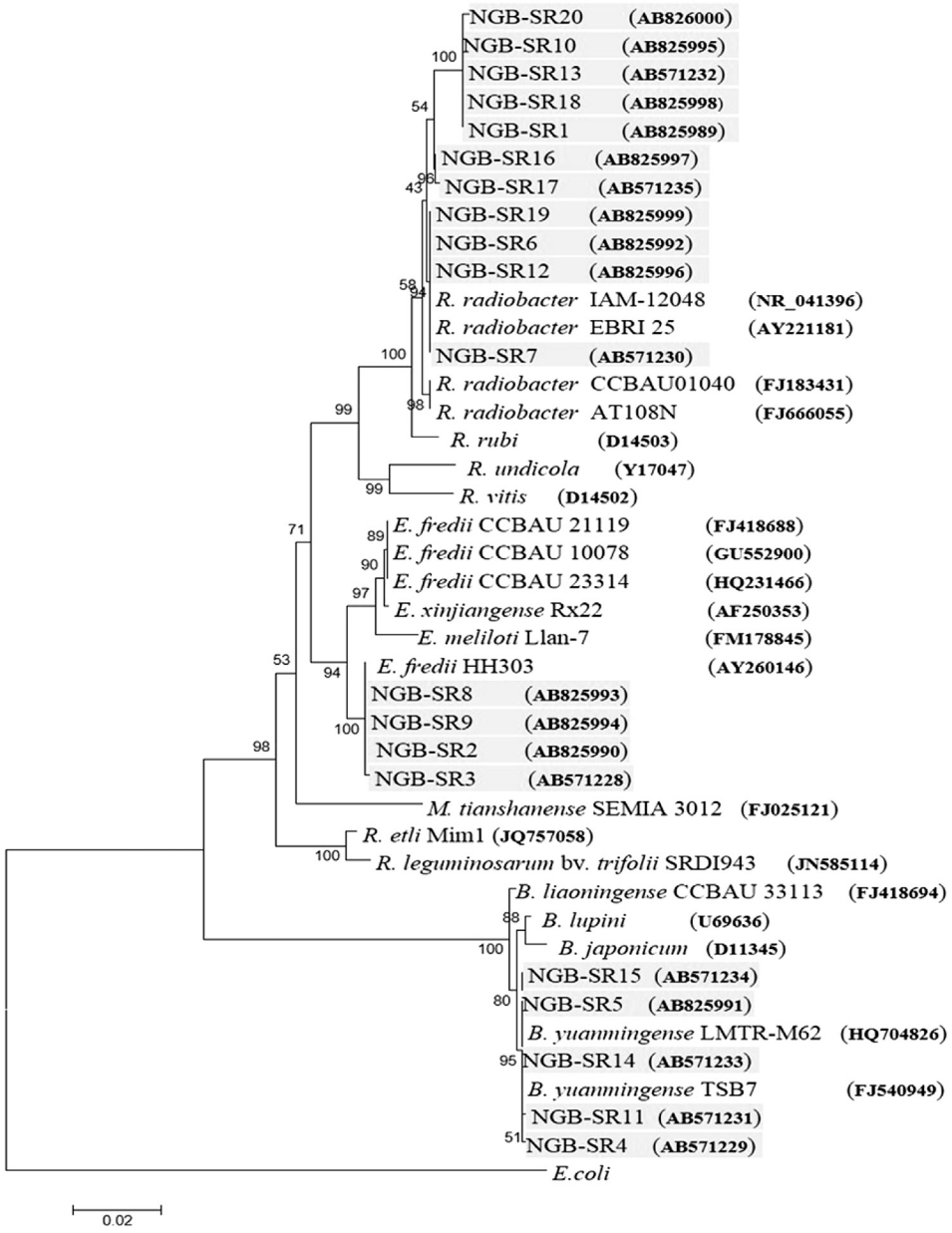

Fig. 3. Phylogenetic tree based upon the 16S rRNA sequences obtained by neighbor-joining method. The tree shows the phylogenetic positions of local soybean rhizobia isolated from this study (shaded) to reference type strains of different rhizobial species. GenBank accession numbers are indicated in parentheses. Bootstrap values were calculated from 1000 trees compared with the sequence of standard strains. Bootstraps are shown at the branching points.

Conversely to slow growing rhizobia, they were able to grow at highly acidic condition (pH 4), high temperatures (42 °C) and high levels of salinity with NaCl concentrations up to 6%.

3.3. 16S rRNA sequencing and phylogenetic analysis

Using the 16S rDNA specific primers [51], nearly full-length 16S rDNA gene regions were successfully amplified and sequenced. According to the sequences similarity, local rhizobial isolates are identified as members of *Ensifer*, *Rhizobium* (*Agrobacterium*) and *Bradyrhizobium* genera (Table 6). A phylogenetic tree representing different rhizobial reference species with local isolates was constructed (Fig. 3) by Neighbor-Joining (NJ). The fast growing rhizobia corresponding to *Rhizobium* (syn. *Agrobacterium*), *Ensifer* and *Mesorhizobium* genera were clearly separated from the slow growing *Bradyrhizobium* genus (Fig. 3). Rhizobial isolates NGB-SR 1, 6, 7, 10, 12, 13, 16, 17, 18, 19 and 20 were clustered on *Rhizobium* (syn. *Agrobacterium*) linage, as a close relative to *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) reference strains, compared to other *Rhizobium* (*Agrobacterium*) species; *Rhizobium rubi*, *Rhizobium vitis*, *Rhizobium undicola* and *Rhizobium rhizogenes*. Isolates NGB-SR 2, 3, 8 and 9 were assembled on *Ensifer* lineage and showed high degree of genetic resemblance to *E. fredii* strain HH303. On the other hand, local slow growing rhizobia NGB-SR 4, 5, 11, 14 and 15 were nested within *Bradyrhizobium* spp. cluster.

3.4. Identification and characterization of symbiosis-related genes (nifH and nodA genes)

The presence of the *nifH* and *nodA* genes within the genome of tested soybean nodulating rhizobia was confirmed using specific PCR primers. All tested isolates successfully amplified *nifH* gene.

The *nodA* gene was successfully amplified in only twelve rhizobial isolates, while eight *Agrobacterium* strains failed to amplify the target gene. PCR products for *nifH* (360 bp) and *nodA* (666 bp) genes were sequenced and aligned with reference sequences (Table 6). According to *nifH* and *nodA* derived phylogenetic trees (Figs. 4 and 5), local soybean nodulating rhizobia were grouped in two distinct clusters; cluster A and B. The *nifH* and *nodA* sequences isolated from *Agrobacterium* and *Ensifer* related strains were nested in cluster (A) containing a number of *Ensifer* accessions, but was most similar to *E. fredii*, which were also originally isolated from *G. max* or *Glycine soja*. Nevertheless, local slow growing *Bradyrhizobium* spp. were included in cluster (B), with other *Bradyrhizobium* reference accessions.

3.5. rep-PCR genetic diversity

rep-PCR fingerprinting was performed to assess the degree of similarity among local rhizobial isolates associated with *Rhizobium* (syn *Agrobacterium*) and *Bradyrhizobium* genera. The electrophoretic PCR patterns indicated a high level of genetic variation among slow growing *Bradyrhizobium* as well as fast growing *Agrobacterium* genotypes. The data of REP, ERIC and BOX of each genomic group were combined together, individually in order to obtain more detailed cluster analysis. The resulting dendrogram declared an enough degree of diversity to differentiate between tested *Rhizobium* (syn. *Agrobacterium*) isolates. They were grouped into three clusters at about 35% level of similarity (Fig. 6A). Cluster I included six *Rhizobium* isolates (NGB-SR 6, 7, 12, 18, 19 and 20), cluster II consisted of two isolates (NGB-SR 16 and 17) and cluster III involved three isolates (NGB-SR 1, 10 and 13). Likewise, rep-PCR derived-fingerprinting showed genetic variation within local bradyrhizobia compared to *B. japonicum* 110, *B. elkanii* 94 and *B. yuanmingense* strains. They were categorized in two major clusters at about 20% level of similarity (Fig. 6B). Cluster I included *B. elkanii* 94, however cluster II comprised local bradyrhizobia with *B. japonicum* 110 and *B. yuanmingense* reference strains.

4. Discussions

The history of soybean cultivation in Egypt may be back to 1858 according to a *Bulletin de la Societe d'Acclimatation*, 1858 [40]. As a nodulating legume, soybean forms a nitrogen-fixing symbiosis with both fast and slow growing rhizobial species [38,50]. Although, soybean has been grown for decades in Egypt, there is no a clear study addressing the biodiversity of soybean nodulating rhizobia under Egyptian soil conditions. The major objective of this research was to identify and explore soybean nodulating rhizobia and to study their phenotypic characteristics. Our study indicated that Egyptian soils do not have sufficient numbers soybean nodulating rhizobia, supporting the observation reported by Moawad

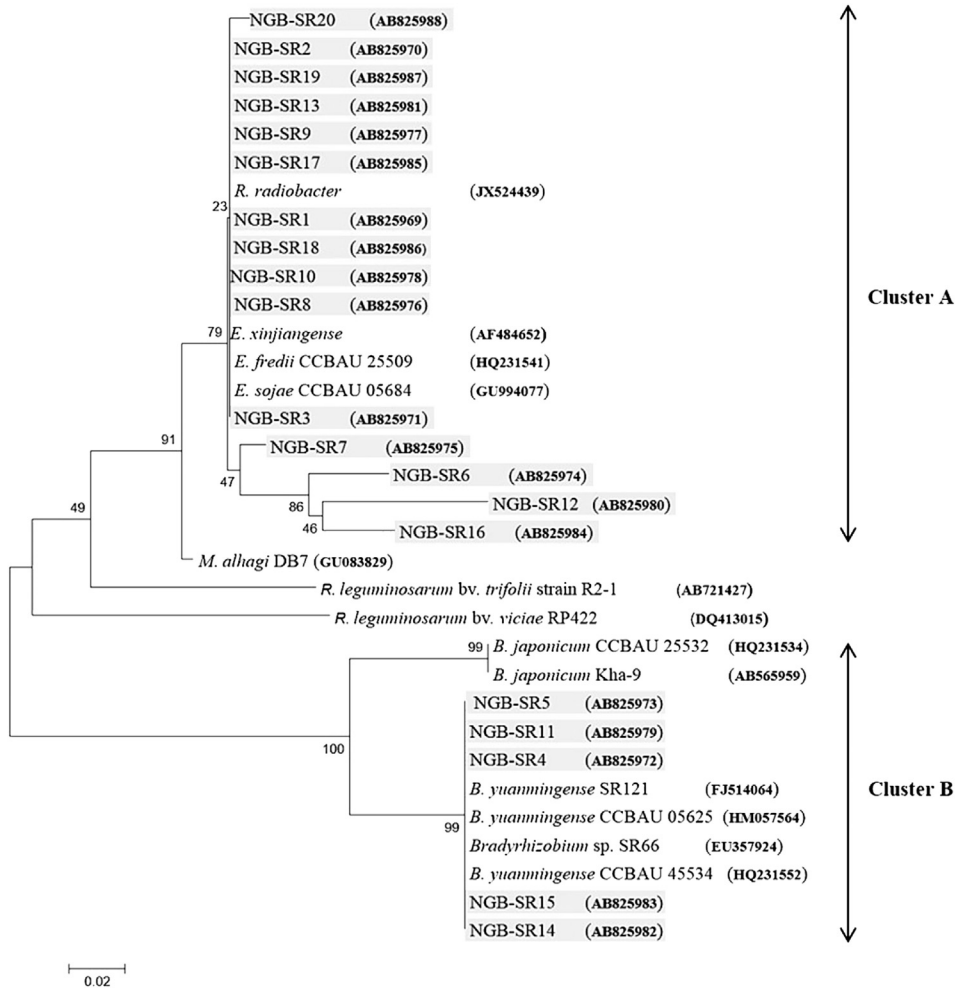

Fig. 4. Bootstrap neighbor-joining phylogenetic tree of local soybean nodulating rhizobia and reference strains, based on *nifH* gene sequences. The tree shows the phylogenetic positions of local soybean rhizobia isolated from this study (shaded) to reference type strains of different rhizobial species. GenBank accession numbers are indicated in parentheses.

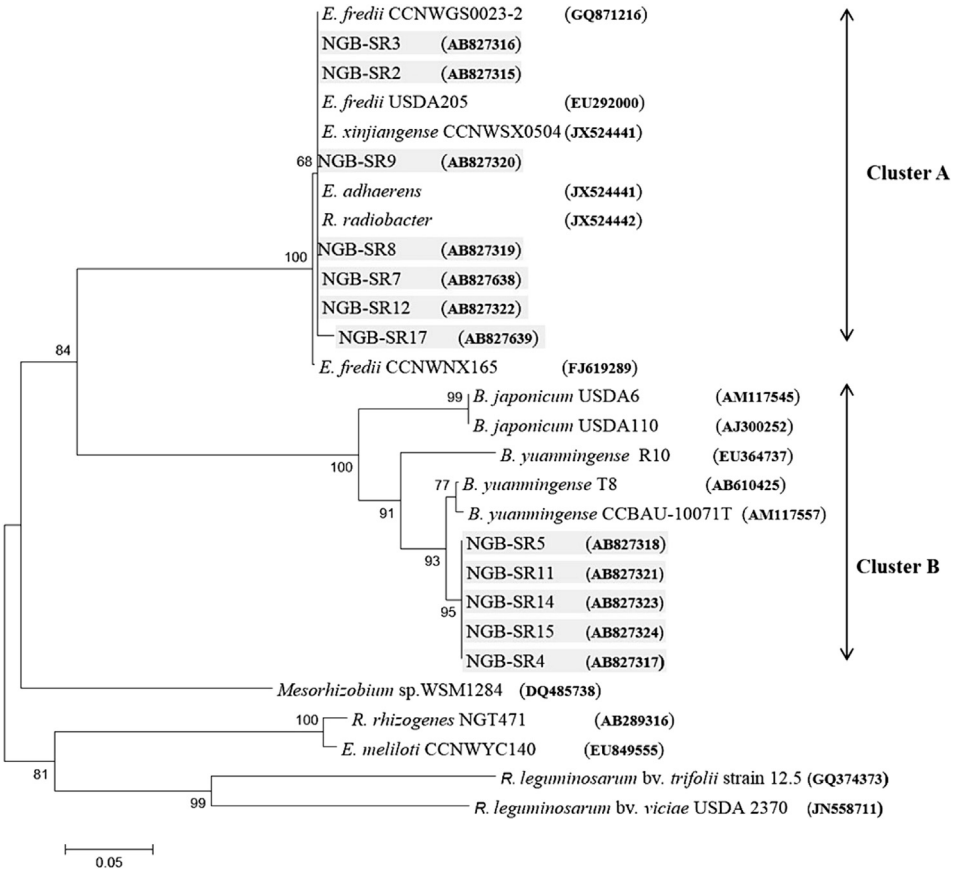

Fig. 5. Bootstrap neighbor-joining phylogenetic tree of local soybean nodulating rhizobia and reference strains, based on *nodA* gene sequences. The tree shows the phylogenetic positions of local soybean rhizobia isolated from this study (shaded) to reference type strains of different rhizobial species. GenBank accession numbers are indicated in parentheses.

[28]. Twenty local rhizobial isolates were collected from root nodules of field-grown soybean in nine governorates in Egypt (Table 1). Seventy five percent of local rhizobial isolates were classified as fast growing rhizobia and twenty five percent of them were slow growing rhizobia. Local rhizobial isolates showed significant performance on nodulation status and plant growth parameters of soybean inoculated plants compared to the uninoculated control treatments (Table 4).

Local rhizobial isolates were phenotypically characterized in order to assess their tolerance ability under stress conditions (Table 5). The obtained results declared that, fast-growing rhizobia, that grew up to 6% NaCl are more salt tolerant than slow-growing bradyrhizobia. These findings are consistent with other reports published by Elsheikh [11] and Hungria et al. [20]. Most of tested rhizobia grew at a wide pH range (pH 5–11), however seven *Agrobacterium* isolates were able to grow at pH 4. These results were in line with those obtained by Fujihara and Yoneyama [15] and Hungria et al. [20]. Most of tested local rhizobia grow at 37 °C, while NGB-SR 8 and 9 isolates could survive at 40 °C. These results are compatible with other data reported by Elsheikh and Wood [12].

Based on nearly full-length 16S rDNA sequences (1500 bp) of local soybean nodulating rhizobia and other species, a bootstrap phylogenetic tree was constructed (Fig. 3). Out of local rhizobial isolates, 25% of them were allocated under the genus *Bradyrhizobium*, whereas, 20% of them assigned within the genus *Ensifer* (*Sinorhizobium*). A phylogenetic overlapping between different species of *Bradyrhizobium* genus was found. This finding was consistent with the results published by Yao et al. [54]. The

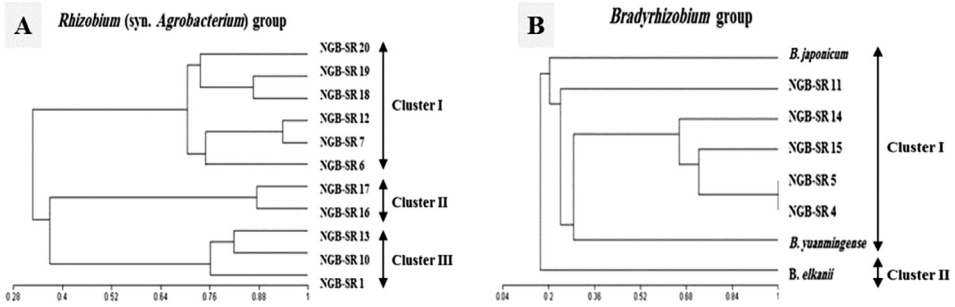

Fig. 6. UPGMA clustering constructed from combined rep-PCR fingerprints (REP, ERIC and BOX) of *Rhizobium (*syn. *Agrobacterium)* associated local isolates (A), and local bradyrhizobia with reference strains of *B. japonicum*, *B. elkanii* and *B. yuanmingense* (B).

obtained data declared the dominance of *Agrobacterium* spp. among tested soybean rhizobia. Isolation of *Agrobacterium* strains from soybean root nodules was previously reported [6,24].

The topology of the *nifH* phylogenetic tree (Fig. 4) was very similar to that of the *nodA* tree (Fig. 5). The studied strains were grouped in two clusters; cluster (A) includes *Rhizobium* (*Agrobacterium*) and *Ensifer* derived sequences and cluster (B) which contains *Bradyrhizobium* related sequences. It has been proposed that the nature of the Nod factor acyl group attached by *nodA* can contribute to the determination of host range [8]. Phylogenetic analyses of *nodA* sequences indicated that they had a distinct evolutionary history to the conserved 16S rRNA sequences. The observed dissimilarities between *nodA* and core gene phylogenies (ribosomal genes) were in line with previous reports [7,9]. The presence of effectively nodulating symbiosis-specific genes (e.g., *nodA*) in *Agrobacterium* was previously confirmed [7]. Nevertheless, identification of these Egyptian agrobacterial strains with *nifH* and *nodA* genes, that effectively nodulate soybean roots and fix N_2, is a novel and potentially valuable.

Local rhizobial strains belonged to *Agrobacterium* and *Bradyrhizobium* genera were analyzed by rep-PCR fingerprints. Genotyping with consensus sequences, such as REP, ERIC and BOX, has been widely used for the detection of rhizobial diversity at the strain level [1,26,41]. The results declared that, REP and ERIC-derived fingerprints showed a high genetic polymorphism compared to BOX-derived fingerprint (data not shown). This is in agreement with the results obtained by Ogutcu et al. [31]. Based on combined rep-PCR clustering, *Agrobacterium* and *Bradyrhizobium* local isolates (Fig. 6A and B) displayed a high degree of genetic diversity. Our results showed that the diversity among rhizobia was unrelated to the geographical sites of isolation. The results confirmed that, the rep-PCR is a powerful tool for the genetic analysis and strain differentiation of rhizobia.

5. Conclusion

The main target of this work was identification of soybean nodulating rhizobia in Egypt. Phenotypic characterization of local rhizobial isolates identified a highly tolerant Egyptian isolates to different abiotic stress conditions including salinity, pH and high temperature. Genotypic characterization showed a high degree of variation among local soybean nodulating rhizobia. The comparative 16S sequencing identified different genomic groups related to *Rhizobium* (syn. *Agrobacterium*), *Ensifer* and *Bradyrhizobium* genera. Identification of *Agrobacterium* strains that effectively nodulate soybean roots and harbored symbiotic genes (*nifH* and *nodA* genes), supporting the addition of soybean to the list of *Agrobacterium* nodulating hosts.

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