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Molecular Characteristics of Rhizobia Isolated from *Arachis hypogaea* Grown under Stress Environment

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Abstract: The phenotypic and genotypic characterization of eight rhizobial isolates obtained from *Arachis hypogaea* nodules grown under stress environment was performed. Isolates were screened for their ability to tolerate different abiotic stresses (high temperature (60° C), salinity (1–5% (w/v) NaCl), and pH (1–12). The genomic analysis of 16S rRNA and housekeeping genes (*atp*D, *rec*A, and *gln*II) demonstrated that native groundnut rhizobia from these stress soils are representatives of fast growers and phylogenetically related to *Rhizobium sp*. The phenotypic characterization (generation time, carbon source utilization) also revealed the isolates as fast-growing rhizobia. All the isolates can tolerate NaCl up to 3% and were able to grow between 20 and 37 °C with a pH between 5 to 10, indicating that the isolates were alkali and salt-tolerant. The tested isolates effectively utilize mono and disaccharides as carbon source. Out of eight, three rhizobial isolates (BN-20, BN-23, and BN-50) were able to nodulate their host plant, exhibiting their potential to be used as native groundnut rhizobial inoculum. The plant growth promoting characterization of all isolates revealed their effectiveness to solubilize inorganic phosphate (56–290 μg mL^{-1}), synthesize indole acetic acid (IAA) (24–71 μg mL^{-1}), and amplification of nitrogen fixing *nifH* gene, exploring their ability to be used as groundnut biofertilizer to enhance yield and N$_2$-fixation for the resource poor farmers of rainfed Pothwar region.

Keywords: *Arachis hypogaea*; abiotic stress; *Rhizobium*; 16S rRNA; housekeeping genes; phenotypic characterization; biofertilizer

1. Introduction

Salinity is one of the most brutal among abiotic stresses limiting groundnut (*Arachis hypogaea* L.) productivity. The nitrogen fixing bacteria played a vital role in crop production and soil health owing to their ability to fix atmospheric nitrogen, and proved to be environmentally friendly by minimizing pollution problems concerned with the application of chemical fertilizers even under stress conditions. Similarly, application of chemical fertilizers are a big cause of climate change as they have a strong connection with greenhouse gas (GHG) emissions. It has been reported that 10–20% of global GHG (Carbon dioxide, Methane, and Nitrous oxide) emissions are from the agricultural sector. Nitrous oxide (N$_2$O) is a potent greenhouse gas that contributes to the destruction of the stratospheric ozone. The level of N$_2$O has been increased from 270 ppb to 328 ppb since 1750 to 2015. This higher level contributed a lot to climate change and the destruction of the ozone layer. Since more than half

of the global N_2O emissions originate from agricultural soils because of nitrogen (N) fertilizer use in crop production, it is imperative to minimize these losses by replacing chemical fertilizers with microbial fertilizers. This will also help to reduce the carbon footprint [1]. Symbiotic bacteria belonged to genus *Allorhizobium* [1,2], *Azorhizobium* [3], *Bradyrhizobium* [4], *Mesorhizobium* [5], *Rhizobium* [6], and *Sinorhizobium* [7], having the ability to establish a symbiotic relationship with legumes by forming root nodules [8]. Symbiotic N_2-fixation is a significant source of plant available nitrogen and several legume species can fix up to 300 kg nitrogen N ha^{-1} [9]. Inoculation of legumes with efficient rhizobia can enhance their ability to fix atmospheric nitrogen, especially when indigenous rhizobial strains are lacking in the soil or they are inefficient [10].

Groundnut (*Arachis hypogaea* L.) is an important leguminous crop cultivated in tropical, subtropical, and temperate zones, and sandy loam soils are ideal for obtaining the best pod yield. The major groundnut producing districts of Pakistan include Chakwal, Attock, and Rawalpindi in Punjab, Karak, and Sawabi in Khyber Pakhtunkhwa (KPK) and Sanghar in Sindh province. Different varieties of spreading and erect groundnut are grown on an area of 1 million hectare with an average yield of 1.1 t ha^{-1} as compared to the 4 t ha^{-1} average yield of USA and China [8]. The major constraints of low groundnut yield are unavailability of high yielding genotypes, abiotic stresses, unavailability of biofertilizer for groundnut in the country, and no/inefficient specific rhizobial inoculation. As a legume, groundnut is also considered as an efficient N_2-fixer when coupled with efficient rhizobial host, fixes about 100–130 kg N ha^{-1} y^{-1}, yet there is potential to increase nitrogen fixation and yield by manipulation of specific inoculants [11]. The nodulation of groundnut could be possible by the indigenous *Rhizobia* present in the soil, however; inoculation of groundnut seeds with specific *Rhizobium* would be helpful in attaining better yields and improving N_2-fixation [12]. Previously, groundnut has been reported to establish effective nodulation with slow-growing *Bradyrhizobium* [13–15], although effective fast-growing *Rhizobium* have also been recently described [8,12,16]. Effective symbiosis of groundnut with *Rhizobium giardini* and *Rhizobium tropici* have also been described by Taurian et al. [17].

Nowadays, polyphasic taxonomic approaches comprising phenotypic and genotypic characterization are recommended as a rhizobial identification and classification tool [18]. By using these techniques, the diversified characteristics of the rhizobial population has been validated [19], implying the potential for identification of many novel rhizobial taxa. The adaptation of rhizobia to saline conditions confirms that symbiont is more resistant to salinity than the host crop. Keeping in view the importance of rhizobia characterization from stress environment at the molecular and cellular level, the present study was carried out with objectives to isolate rhizobial strains from groundnut nodules under naturally saline conditions, particularly as they relate to salt tolerance, high temperature, moisture stress, and examined their systemic taxonomy for future validation of *Rhizobium* species. Our results have revealed that isolated rhizobial strains have 16S rRNA and housekeeping genes (*atpD*, *recA* and *glnII*) similarity with the members of fast-growing genus *Rhizobium*. We had also published and further validated a novel rhizobium species, BN-19^T, from groundnut nodules, indicating the potential of existence of many novel *Rhizobium* species from the diverse ecology of Pakistan.

2. Materials and Methods

2.1. Isolation of Rhizobia and Nodulation Test

The rhizobial strains were isolated from root nodules of groundnut (*Arachis hypogaea* L.) grown under saline soil with EC 8.2 dSm^{-1}, pH 7.8, SAR 7.2 meqL^{-1}. Host plants were sampled by using 1 m^2 quadrat from semi-arid soils of Attock district, Pakistan (latitude 32°93′ N and longitude 72°86′ E). Different farmers' fields were visited and plants were sampled. Healthy, unbroken, and pink nodules indicating active nitrogen-fixation were selected from each plant. The nodules were first washed thoroughly with tap water and then carefully detached from the roots by using sterilized forceps. Intact and undamaged nodules were immersed in 95% ethanol (v/v) for 10 s and surface sterilized

in 3% hydrogen peroxide (H_2O_2) for 3 min and washed six times in autoclaved distilled water. Sterilized nodules were crushed with a glass rod in a drop of sterile distilled water, and the suspension was streaked on Yeast Mannitol Agar (YMA) [20]. Plates were incubated for 3–7 days at 28 °C. Pure bacterial colonies were obtained by repeatedly streaking the individual colony on YMA and preserved in 20% glycerol at –80°C for further use.

A total of eight *Rhizobium sp.* were obtained and their nodulation ability was tested by inoculating seedlings of *Arachis hypogaea* L. (Var. BARI 2011). One seedling was planted in plastic pots containing autoclaved vermiculite and inoculated with bacterial suspension of each rhizobial isolate. Four replicates per isolate were established and seedlings were irrigated with nitrogen-free nutrient solution [21]. A negative control (uninoculated seedling) was also included. Roots were visually examined for nodulation after six weeks of inoculation.

2.2. Screening of Isolates for Abiotic Stress Tolerance

The ability of the isolates to grow at different salt concentrations was tested by streaking each isolate on YMA medium containing 1–5% (w/v) NaCl. The tolerance of the isolates to acid or basic pH was examined in liquid tryptone yeast extract (TY) medium with pH adjusted between 2 (1 N HCl) and 12 (I N Na_2CO_3), at an increment of 1 pH unit. Temperature tolerance of all strains was tested on solid YMA plates by incubating at 4, 10, 15, 28, 37, 50, and 60 °C.

2.3. Morphology and Phenotypic Characterization

For morphological characterization, individual colonies were examined for color, size, and shape after 3–7 days of growth on YMA. Motility was determined by piercing of a soft-agar medium. Gram staining was performed using a commercial kit according to the manufacturer's instructions (bioMérieux, France). For Carbon-source utilization, all isolates were tested by using a Gram-negative (GN2) MicroPlate (Biolog) according to the manufacturer's instruction. Single colonies were subcultured on YMA plates for optimum growth at 28 °C and were read at 6, 24, and 48 h with a computer-controlled MicroPlate reader. Antibiotic resistance of *Rhizobium* isolates was determined using seven antibiotics at four (5, 50, 100, 300 µg mL^{-1}) concentrations in YMA medium: tetracycline, chloramphenicol, ampicilin, neomycine sulphate, erythromycin, and kanamycine sulphate. Catalase and Oxidase activities were determined by using API Color Catalase and API Oxidase reagents (bioMérieux, France). The generation time of all isolates was calculated following the protocol of Gao, et al. [22] The growth curve of each isolate was determined by cultivation in liquid TY medium at 28 °C and 150 rpm and measuring the optical density (OD) after every two hours. The mean generation time (MGT) was estimated from the logarithmic phase of growth, as the average time required for the bacterial culture to double.

2.4. Extraction of Total DNA for Amplification of 16S rRNA and Housekeeping Genes

For molecular characterization, total bacterial DNA of each isolate was extracted following the procedure as described by Terefework, et al. [23] to be used as a template in the amplification of the *nifH*, 16S rRNA gene, *atpD* (ATP synthase subunit beta), *recA* (recombinase A protein), and *glnII* (glutamine synthetase II) housekeeping genes. The 16S rRNA gene was amplified and sequenced according to the procedure of Tan et al. by using the primers P1 (AGAGTTTGATCCTGGCTCAGAACGAACGCT) and P6 (TACGGCTACCTTGTTACGACTTCACCCC). The presence of the nitrogen fixing *nifH* gene in the bacterial isolates was determined through Polymerase Chain Reaction (PCR) using forward (Pol F 5′TGCGAYCCSAARGCBGACTC3′) and reverse Pol R (5′ATSGCCATCATYTCRCCGGA3′) *nifH* gene primers [24]. PCR amplification and sequencing of partial *atpD* (510 bp), *recA* (530 bp), and *glnII* (600bp) was performed according to Gaunt et al. [25] and Turner and Young [26]. The almost complete sequences of 16S rRNA, *atpD*, *recA*, and *glnII* genes were submitted in DNA Data Bank of Japan (DDBJ) using the Sakura Nucleotide Data Submission System to obtain accession number of each strain.

The sequences obtained were aligned with the relevant *Rhizobium* sequences retrieved from Gene bank by using CLUSTAL W program in the MEGA 5.2 software [27]. Aligned sequences were analyzed using the same software to construct Jukes–Cantor distance [28] and unrooted phylogenetic trees by using the neighbor joining [29] method, with bootstrap values based on 1000 replications [30].

2.5. Plant Growth Promoting Assay

The rhizobial strains were evaluated for their ability to enhance plant growth by solubilizing inorganic phosphate, production of indole acetic acid (IAA), and amplification of the *nifH* gene. The quantitative phosphorus solubilization capacity of *Rhizobium* isolates was determined in Nautiyal broth containing 0.5% tri-calcium phosphate (pH 7) on a rotary shaker incubate at 28 °C for 8 days [31]. The drop in pH of the medium was recorded and the available phosphorous was analyzed using the protocol of Watanabe and Olsen [32] and solubilization efficiency was calculated.

Isolated *Rhizobium* strains were tested for the production of IAA following the procedure as adopted by Hayat, et al. [33]. Strains were inoculated in Luria broth (LB) medium with or without adding tryptophan (500 µg mL^{-1}). Bacterial cultures were placed for 48 h on an incubator shaker at 6000 rpm for 10 min. The supernatant (2mL) was mixed with two drops of orthophosphoric acid (10mM) and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M FeCl$_3$ solution). Development of pink color indicated IAA production, which is measured on a spectrophotometer at 520 nm [34]. The ability of the bacterial isolates for nitrogen fixing *nifH* gene was determined through PCR by using forward PolFb (TGC GAY CCS AAR GCB GAC TC) and reverse PolRb (ATS GCC ATC ATY TCR CCG GA) *nif*H gene primers [24].

3. Results

3.1. Isolation and Growth Characteristics of Rhizobium Strains

A total of eight (8) rhizobial strains were obtained from nodules of *Arachis hypogaea* and designated as BN-6, BN-12, BN-19, BN-20, BN-23, BN-25, BN-39, and BN-50. All isolates formed circular, smooth, convex, and off-white colonies on YMA medium. The isolates were non-motile, rod-shaped, non-spore forming, and Gram-negative. Mean generation time (MGT) of all isolates was observed between 3 and 4 h. On the basis of the growth rate, rhizobia that have an MGT less than 6 h are considered a fast grower [20].

The three isolates (BN-20, BN-23, and BN-50) confirmed their ability to nodulate groundnut by the formation of typically reddish nodules at the concentration of 50 mM NaCl. The other isolates were unable to nodulate the host plant. This lack of nodulation on other isolates could be related to salt sensitiveness or the loss of nodulating genes. The genes that encode nitrogen-fixation and nodulation (*nifH*, *nod*ACD) are clustered on plasmids, which can be easily transferred between different strains, causing their loss from bacteria [35].

3.2. Phenotypic Characterization and Abiotic Stress Tolerance

The phenotypic characterization of the isolated *Rhizobium* strains is presented in Table 1. The table depicted that all strains are able to grow at 28 and 37 °C in the pH range of 5–10, but cannot grow at 4, 10, and 50 °C and at pH 2, 4, and 11. All strains can tolerate NaCl concentration of 0.5–3.0%, but cannot tolerate concentration of 4.0 and 5.0%. All strains were susceptible to tetracycline hydrochloride and neomycine sulfate at 100 and 300 µg L^{-1}; chloramphenicol and ampicilin at 300 µg L^{-1}. For kanamycine sulfate, only the strain BN-19^T is resistant to all four tested concentrations (5, 50, 100 and 300 µg L^{-1}).

Table 1. Differential stress tolerance features and antibiotic resistant test of isolated *Rhizobium* strains.

			BN-6	BN-12	BN-19	BN-20	BN-23	BN-25	BN-39	BN-50
	Growth at/in:									
	4 °C		−	−	−	−	−	−	−	−
	37 °C		+	+	+	+	+	+	+	+
	50 °C		−	−	−	−	−	−	−	−
Salt Tolerance	NaCl (1%)	Temperature	+	+	+	+	+	+	+	+
	NaCl (3%)	Tolerance	+	+	+	+	+	+	+	+
	NaCl (4%)		−	−	−	−	−	−	−	−
	pH4		−	−	−	−	−	−	−	−
	pH6		+	+	+	+	+	+	+	+
	pH10		+	+	+	+	+	+	+	+
	Tetracycline hydrochloride (5)		+	−	+	−	−	−	−	+
	Tetracycline hydrochloride (50)		−	−	+	−	−	−	−	−
	Chloramphenicol (100)	pH	+	+	+	+	−	+	+	+
	Streptomycin sulfate (300)	Tolerance	+	+	+	−	+	+	+	+
Antibiotic Resistance	Ampicilin (50)		+	+	+	−	−	−	+	+
	Ampicilin (100)		−	−	+	−	−	−	−	−
	Neomycin sulfate (50)		+	+	+	+	+	+	+	+
	Erythromycin (300)		−	−	+	−	−	−	−	−
	Kanamycin sulfate (100)		−	−	+	−	−	−	−	−
	Kanamycin sulfate (300)		−	−	+	−	−	−	−	−

+ Positive; − Negative.

All strains were catalase positive and grew in NaCl concentration of 0–3.0%, and optimum growth occurs between 0–1.5%, strengthening the fact that fast growing rhizobia are more tolerant and salt concentration than slow growing ones [8,36,37]. All the isolates were able to grow between 20 and 37 °C and pH 5–10, with optimum growth at 28 °C and at pH 7. None of the isolate was able to grow at 40 °C. The eight isolated bacterial strains were tested for seven antibiotics and the result is demonstrated in Table 1. All tested isolates were resistant to chloramphenicol and neomycine sulfate at 5 and 50 µg L^{-1}, streptomycine sulfate and erythromycin at 5, 50, and 100 µg L^{-1}, and for ampicilin all strains were resistant at 5 µg L^{-1}. In contrast, all strains were susceptible to tetracycline hydrochloride and neomycine sulfate at 100 and 300 µg L^{-1}; chloramphenicol and ampicilin at 300 µg L^{-1}. For kanamycine sulfate only the strain BN-19^T is resistant to all four tested concentrations (5, 50, 100, and 300 µg L^{-1}), whereas the other 7 isolates were susceptible.

Carbon source utilization (Biolog) can determine the ability of an organism to live symbiotically or saprophytically in the soil [38]. The biochemical results of rhizobial isolates (Table 2) demonstrated that all strains can utilize adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fructose, D-galactose, α-D-glucose, m-inositol, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, L-alanine, and glycyl-L-glutamic acid; whereas all the isolated *Rhizobium* sp. were not able to utilize α-cyclodextrin, i-erythritol, D-glucosaminic acid, α-keto butyric acid, L-phenylalanine, thymidine, phenyethyl-amine, and putrescine.

Table 2. Carbon source utilization of Rhizobium strains isolated from groundnut nodules by using BIOLOG.

Carbon-Source Utilization	BN−6	BN−12	BN−19	BN−20	BN−23	BN−25	BN−39	BN−50
α−Cyclodextrin	−	−	−	−	−	−	−	−
Dextrin	−	+	+	+	+	+	+	+
Glycogen	−	+	w	−	−	+	+	−
Tween 40	−	−	−	+	−	+	−	−
Tween 80	−	w	−	+	−	w	w	−
N−acetyl−D−galactoseamine	−	+	+	+	+	+	+	+
N−acetyl−D−glucoseamine	−	+	+	+	+	+	+	+
Adonitol	+	+	+	+	+	+	+	+
L−Arabinose	+	+	+	+	+	+	+	+
D−Arabitol	+	+	+	+	+	+	+	+
D−Cellobiose	+	+	+	+	+	+	+	+
i−Erythritol	−	−	−	−	−	−	−	−
D−Fructose	+	+	+	+	+	+	+	+
L−Fructose	+	+	+	+	+	+	+	+
D−Galactose	+	+	+	+	+	+	+	+
Gentibiose	−	+	+	+	+	+	+	+
α−D−Glucose	+	+	+	+	+	+	+	+
m−Inositol	+	+	+	+	+	+	+	+
α−D−lactose	+	+	w	+	+	w	+	+
Lactulose	−	+	−	+	+	−	+	+
Maltose	+	+	+	+	+	+	+	+
D−Mannitol	+	+	+	+	+	+	+	+
D−Mannose	+	+	+	+	+	+	+	+
D−melibiose	−	+	+	+	+	−	+	+
D−psicose	−	+	+	−	+	+	+	−
D−raffinose	−	+	+	+	+	−	+	+
L−Rhamnose	+	+	+	+	+	+	+	+
D−Sorbitol	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
D−Trehalose	+	+	+	+	+	+	+	+
Turanose	+	+	+	+	+	+	+	+
Xylitol	+	+	+	−	+	−	+	+
Pyruvic acid methyl ester	+	+	+	+	+	+	+	+
Succinic acid mono−methyl−ester	+	+	+	−	w	−	+	+
Acetic acid	−	+	+	+	+	+	+	+
Cis−aconitic acid	−	+	+	+	+	−	+	+
Formic acid	−	+	w	−	+	+	+	−

Table 2. *Cont.*

Carbon-Source Utilization	BN−6	BN−12	BN−19	BN−20	BN−23	BN−25	BN−39	BN−50
D−galactonic acid	−	+	−	−	+	+	+	−
D−galacturonic acid	+	−	−	−	−	−	−	−
D−gluconic acid	+	+	+	−	+	+	+	−
D−Glucosaminic acid	−	−	−	−	−	−	−	−
D−glucuronic acid	+	+	−	−	+	+	+	+
α−hydroxybutyric acid	−	+	+	−	+	+	+	+
β−hydroxybutyric acid	+	−	−	−	−	−	−	−
p−hydroxyphenylacetic acid	+	+	−	−	−	−	−	−
Itaconic acid	+	−	−	−	−	−	−	−
α−keto butyric acid	−	−	−	−	−	−	−	−
α−keto glutaric acid	−	+	+	−	w	+	+	−
α−keto valeric acid	−	+	−	−	−	−	−	−
D,L−Lactic acid	−	+	+	+	+	+	+	+
Malanoic acid	−	+	−	−	−	−	−	−
Propionic acid	−	+	+	−	+	+	+	+
Quinic acid	−	+	+	+	+	+	+	+
D−saccharic acid	+	+	−	−	−	−	−	−
Sebacic acid	−	+	−	−	−	−	−	−
Succinic acid	−	+	+	+	+	+	+	+
Bromosuccinic acid	−	+	+	+	+	+	+	+
Succinamic acid	−	+	w	−	+	−	w	+
Glucuronamide	−	+	−	−	−	−	−	−
L−alaninamide	−	+	−	−	+	+	+	+
D−alanine	−	−	+	−	+	−	+	+
L−alanine	+	+	+	+	+	+	+	+
L−alanyl−glycine	−	+	+	−	+	+	+	+
L−asparagine	−	−	+	+	+	+	+	+
L−aspartic acid	−	+	+	−	+	−	+	+
Glycyl−L−glutamic acid	+	+	+	+	+	+	+	+
L−glutamic acid	−	+	+	+	+	+	+	−
L−histidine	+	+	+	−	+	+	+	+
Hydroxy−L−proline	−	+	+	+	+	+	+	+
L−ornithine	−	−	+	−	+	−	+	+
L−phenylalanine	−	−	−	−	−	−	−	−
L−proline	−	−	+	+	+	+	+	+
L−pyroglutamic acid	−	+	−	−	+	+	+	+
L−serine	+	+	+	−	+	+	+	+
L−threonine	w	−	w	−	+	+	+	+
D,L−carnitine	+	+	−	−	−	−	−	−
γ−amino butyric acid	+	+	w	−	+	+	+	+
Urocanic acid	+	+	−	−	w	+	+	+
Uridine	+	+	+	−	+	+	+	+
Thymidine	−	−	−	−	−	−	−	−
Phenyethyl−amine	−	−	−	−	−	−	−	−
Putrescine	−	−	−	−	−	−	−	−
2−aminoethanol	−	+	w	−	w	+	+	−
2,3−butanediol	−	w	−	−	−	+	+	−
D,L−α−glycerol phosphate	w	−	−	−	−	−	−	−
D−glucose−6−phosphate	+	+	−	−	+	−	+	+

All strains were positive for carbon source utilization from adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fructose, D-galactose, α-D-glucose, m-inositol, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, L-alanine and glycyl-L-glutamic acid; but negative for α-cyclodextrin, i-erythritol, D-glucosaminic acid, α-keto butyric acid, L-phenylalanine, thymidine, phenyethyl-amine, and putrescine; + Positive; − Negative; w Weak.

3.3. Genotypic Characterization

Nearly the entire length of the 16S rRNA gene was amplified and all the eight *Rhizobium* isolates (BN-6, BN-12, BN-19, BN-20, BN-23, BN-25, BN-39, and BN-5) yielded a single band amplification product of approximately 1200 bp. The obtained sequences were basic local alignment search tool (BLAST) in EzBiocloud database (http://ezbiocloud.net/eztaxon) to obtain sequence percent similarities and possible identities with their closely related species. All identified strains fall in genus *Rhizobium* and exhibited more than 97% similarity with the closely validated species except BN-19, confirming

the isolates as fast-growing rhizobia (Table 3). The strain BN-19[T] exhibited 97.5% similarity with *R. alkalisoli* and identified and validated as *R. pakistanensis* sp. nov. [8]. The nearly complete 16S rRNA gene sequence of the rhizobial islaotes were submitted in DDBJ to obtain accession numbers.

Table 3. Sequence similarities (%) and accession numbers for *atpD*, *recA*, and *glnII* genes of *Rhizobium* isolates.

Strain ID	Sequence Similarity (%)				DNA Data Bank of Japan (DDBJ) Accession Numbers				16S Rrna Gene Identification
	16S rRNA	*atpD*	*recA*	*glnII*	16S rRNA	*atpD*	*recA*	*glnII*	
BN-6	99.7	95.5	99.1	100	AB969782	LC061200	AB971367	AB971366	*R. alkalisoli*
BN-12	100	97.8	99.4	99.1	AB969783	LC061201	LC061206	LC061211	*R. massiliae*
BN-19	97.5	93.3	89.5	89.0	AB854065	AB856324	AB855792	AB856325	*R. loessense*
BN-20	99.9	100	89.2	99.2	AB969784	AB970799	AB970801	AB970800	*R. huautlense*
BN-23	99.9	98.2	99.6	100	AB969785	LC061202	LC061207	LC061212	*R. pusense*
BN-25	99.3	100	100	98.7	AB969786	LC061203	LC061208	LC061213	*R. herbae*
BN-39	100	95.7	99.5	99.6	AB969787	LC061204	LC061209	LC061214	*R. massiliae*
BN-50	99.7	100	99.7	99.4	AB969788	LC061205	LC061210	LC061215	*R. alkalisoli*

A polyphasic study including multi-gene amplification was carried out to elucidate their taxonomic position in genus *Rhizobium*. A housekeeping gene sequencing of *atpD*, *recA*, and *glnII* genes was performed to confirm the phylogenetic status of the above mentioned strains (Table 3). The sequence obtained were BLAST using National Center for Biotechnology Information (NCBI) database and the results confirmed more than 96% similarity of the seven strains except BN-19[T] with closely related identified *Rhizobium* species. The strain BN-19[T] exhibited less than 94% sequence similarity with the closely related identified *Rhizobium* species; hence, it was validated as a novel species [8]. Housekeeping gene sequence of three genes were submitted in DDBJ and Accession Numbers of the genes were acquired (Table 3).

A neighbor-joining tree of the isolates for 16S rRNA gene was constructed (Figure 1), and the results depicted that each identified species lies in the subclade of genus *Rhizobium*.

For housekeeping genes (*atpD*, *recA*, *glnII*) of the *Rhizobium sp.*, sequences were obtained from NCBI GenBank, and neighbor-joining trees clarifying the positions of each strain in the genus *Rhizobium* were constructed (Figure 2).

*3.4. Plant Growth Promoting Ability of Isolated Strains*

All the eight rhizobial isolates were positive for phosphorus solubilization (Table 4), and solubilized inorganic phosphate (tricalcium phosphate) in the range of 56–290 µg mL^{-1}, decreasing the pH of the broth medium from 7.0 to 4.5 after 8 days of incubation, indicating the ability of the isolates to synthesize organic acids responsible for creating acidic conditions in the media. The ability of the rhizobial isolates for synthesis of Indole-3-Acetic Acid (IAA) was determined by incubating isolates in pure culture (LB broth) in the presence (500 µg mL^{-1}) and absence of L-tryptophan (precursor of IAA production). Without tryptophan, IAA was synthesized in the range of 7–21 µg mL^{-1}, whereas significant differences in IAA production were recorded with the addition of tryptophan (24–71 µg mL^{-1}), indicating the ability of tryptophan to act as a precursor in IAA production. Nitrogen fixing ability (*nifH* gene) was coded in all isolated rhizobial strains.
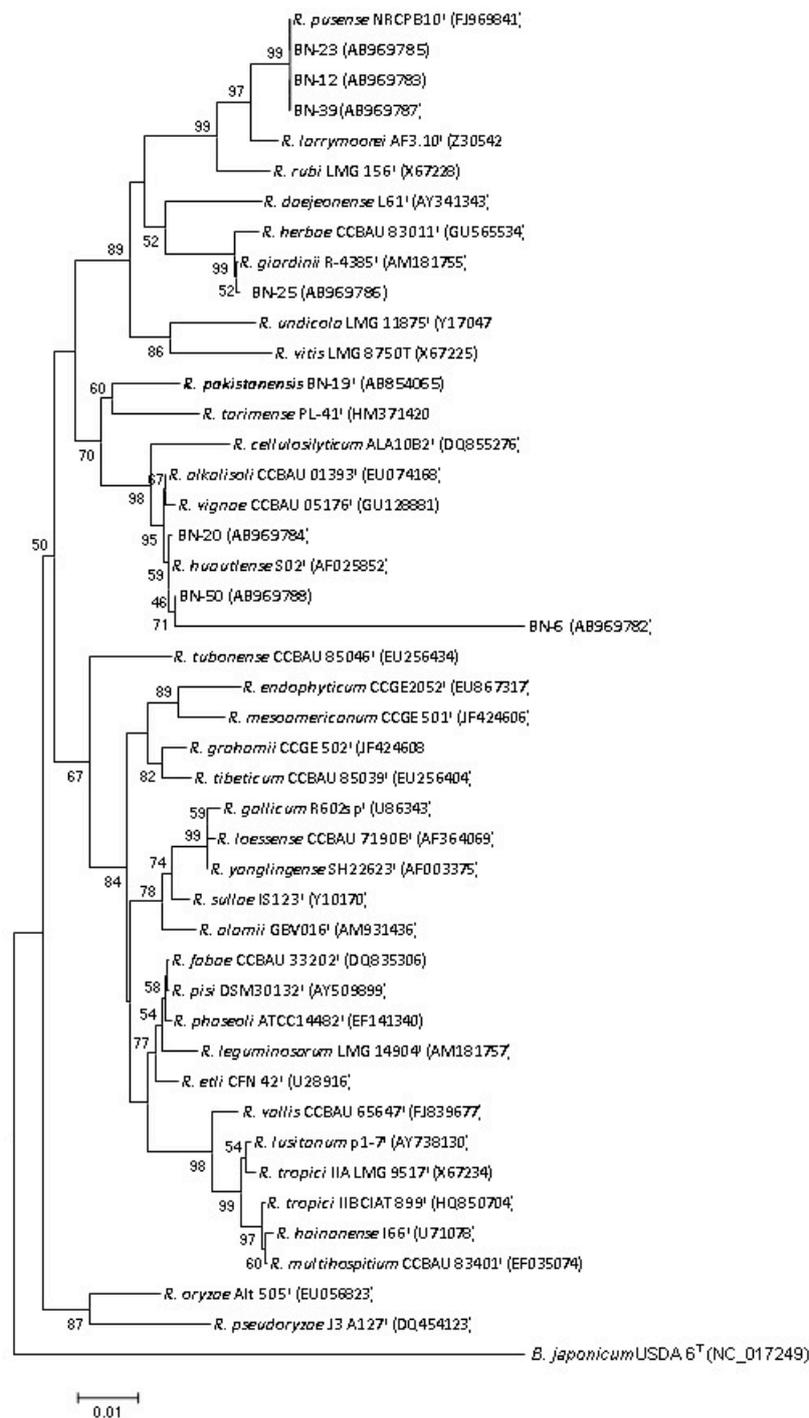
Figure 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences of isolated *Rhizobium* strains and the recognized species of genus *Rhizobium*. Bootstrap values >50% are indicated at the nodes. The sequence of *Bradyrhizobium japonicum* (USDA 6^{T}) was used as an outgroup.
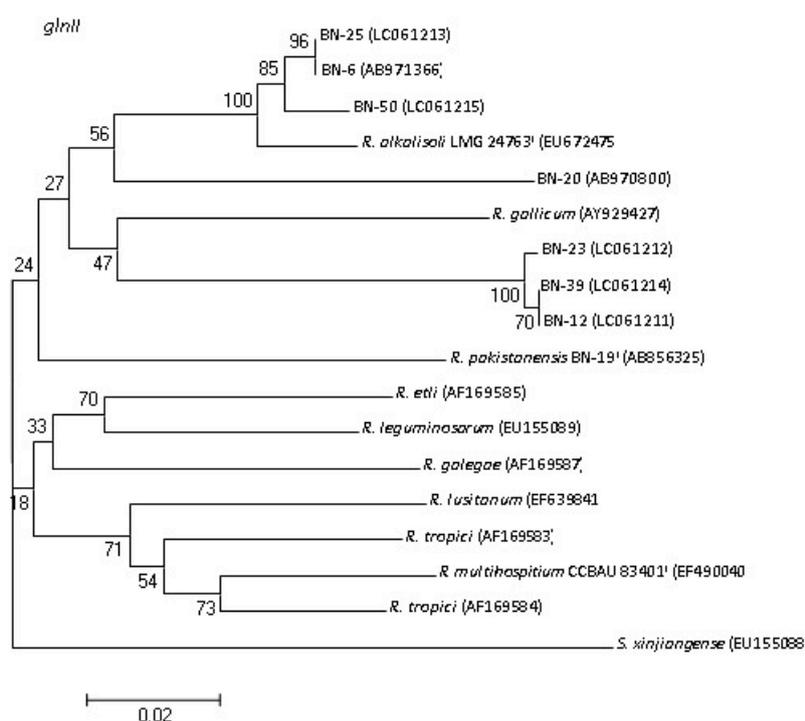
(**a**)

(**b**)

Figure 2. *Cont.*

(**c**)
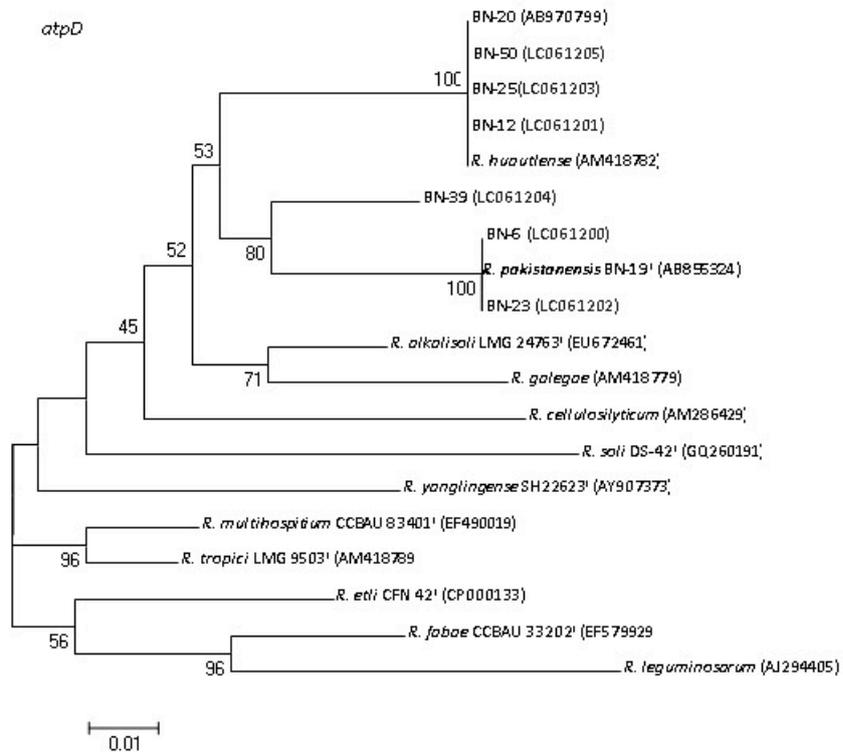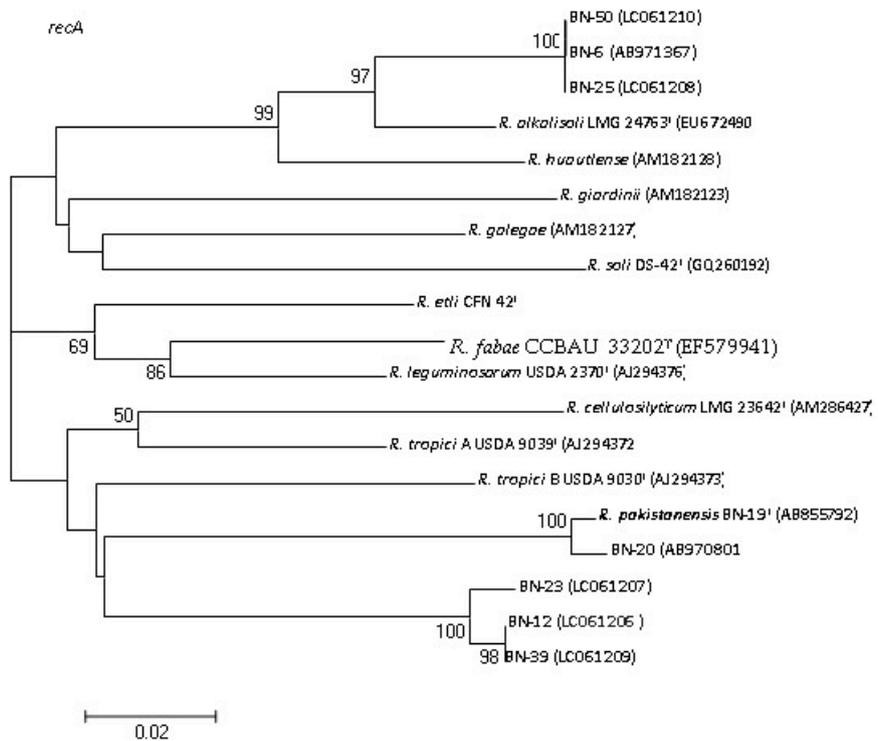
Figure 2. Phylogenetic trees constructed from *atpD* (**a**), *recA* (**b**), and *glnII* (**c**) gene sequences showing the relationship between the isolated strains and the recognized *Rhizobium* species. Trees were constructed by using the neighbor-joining (NJ) method with a Kimura two-parameter distance matrix. Bootstrap values (%) were based on 1000 replicates.

Table 4. Plant growth promoting characteristics of *Rhizobium* isolates.

Strain ID	P-solubilization	pH *	IAA with Tryptophan	IAA without Tryptophan	*nifH* Gene
			(μg mL^{-1})		
BN-6	152 ± 1.3	4.67	35.9 ± 1.5	20.5 ± 1.1	+
BN-12	79.9 ± 2.7	4.89	71.0 ± 2.2	21.2 ± 0.8	+
BN-19	152 ± 5.0	4.50	54.6 ± 1.9	10.0 ± 1.0	+
BN-20	56 ± 3.0	5.34	65.7 ± 2.2	11.6 ± 1.3	+
BN-23	290 ± 3.5	6.32	48.6 ± 2.4	12.8 ± 1.5	+
BN-25	58 ± 3.8	4.95	55.0 ± 1.7	18.3 ± 1.6	+
BN-39	278 ± 5.6	5.85	24.2 ± 1.8	13.1 ± 0.7	+
BN-50	162 ± 2.1	5.91	45.2 ± 2.4	7.0 ± 1.1	+

+ Positive; Values indicate the mean ± SE for three replications. * Initial pH 7.

4. Discussion

In this study, we have characterized 8 rhizobial isolates from root nodules of groundnut grown under stress environment by using polyphasic approach. The characteristics covered physiological, biochemical, genetic, and resistance to antibiotics, which indicated that the isolates belonged to the genus *Rhizobium*. The diversity within the rhizobial population made them capable to adapt to stress environments for survival and nodulation. The legume-rhizobia symbiosis is affected by factors such as changes in temperatures, antibiotic resistance, pH, and soil salinity, which restrict symbiotic nitrogen fixation, and the strains capable of tolerating the extreme conditions would survive efficiently. All the isolates can tolerate NaCl up to 3% and were able to grow between 20 and 37 °C with a pH

between 5 and 10 indicating that the isolates were alkali- and salt-tolerant, and exhibiting survival adaptations against the stressful soil and environmental conditions. The studies on groundnut indicated that it mostly forms effective nodules with slow-growing rhizobia belonging to the genus *Bradyrhizobium*, such as the species *B. japonicum*, *B. elkanii*, *B. lablabi*, *B. yuanmingense*, and *B. iriomotense* [13,14,36,39–43], but rarely with fast-growing *Rhizobium* species [12,17,36]. Santos, et al. [44] reported the dominance of fast-growing rhizobia that form effective nodules on groundnut in Northeastern Brazil soils. In the present study, all the isolates were able to grow on YEM agar medium after incubation at 28 °C for three days. The mean generation time (3 hours) and the growth on YMA proved the status of the isolated rhizobia to belong to fast-growing *Rhizobium*.

Alkali tolerance of fast growing strains has previously been reported by El-Akhal, Rincon, Mourabit, Pueyo, and Barrijal [36], Taurian et al. [45], and Graham [46]. Antibiotic resistance is a widely used parameter for rhizobium classification [47], and the variability among isolates in terms of tested antibiotics confirmed this test as a useful criterion for strain selection [48]. The results of carbon source utilization by isolated rhizobia strengthen the fact that fast-growing rhizobia are capable to utilize a wide array of carbon substrates [43]. These results are also in line with the studies of Jordan [4] and Stowers [49] who stated that fast-growing rhizobia utilize disaccharides most often as compared to slow-growing rhizobia.

In different previous works, rhizobia isolated from groundnut and established symbiosis belonged to genus *Bradyrhizobium* [13–15,42,43]; however Santos, Stamford, Borges, Neves, Rumjanek, Nascimento, Freitas, Vieira, and Bezerra [44] and Taurian, Ibanez, Fabra, and Aguilar [17] have reported the predominance of fast-growing rhizobia isolated from groundnut nodules. The occurrence of fast-growing rhizobia in this study could be because of the reason that fast growers are abundant in arid and semi-arid regions, which is the basic climatic pattern of sampled sites. In these climatic zones, rhizobia have adapted to multiply fast within short rains and are more tolerant to stress conditions than slow-growing strains. Currently, the housekeeping gene analysis of multiple protein-encoding genes has become an extensively applied method for the confirmation of taxonomic relationships between species of the same genus [50].

The drop in pH is associated with the synthesis of organic acids by bacteria; for example, gluconic acid, α-2-ketogluconic acid, lactic, isovaleric, isobutyric, acetic, malanoic, and succinic acids resulting in the release of a H^+ ion [51]. These results for P-solubilization and IAA production correlate the growth promoting activity and the potential of the rhizobial isolates to be used for plant growth promotion [52]. The nitrogenase enzyme responsible for biological nitrogen fixation has a dinitrogenase reductase subunit encoded by the *nifH* gene [53], which is used as the biomarker to study the ecology and evolution of nitrogen-fixing bacteria [54,55].

Our study confirmed the presence of fast-growing rhizobia in groundnut growing in stress environment and revealed that groundnut can be nodulated with fast-growing *Rhizobium* species; which release organic acids in Nautiyal medium and synthesize IAA exhibiting their potential to be used as Plant Growth Promoting Rhizobacteria (PGPR). The genotypic characteristics confirm the status of the isolates as belonging to genus *Rhiozbium*, whereas the phenotypic characteristics reveal that there is little diversity among the isolated rhizobia. The soil with abiotic stresses has a rich diversity of microbes and there is a potential to obtain novel taxa. Pot and field experiments are required to confirm the efficiency of the isolated *Rhizobium* to enhance growth and yield in groundnut especially under saline conditions and there is a need to develop the effective rhizobial innocula that can be used to nodulate groundnut under stress on farmer's field level.

5. Conclusions

It is now well-known fact that chemical fertilizers are a big cause of climate change; thus, the replacement of chemical fertilizers with microbial fertilizers could be a good source to mitigate ill effects of climate change. Meanwhile, eight rhizobial isolates as microbial fertilizers from root nodules of groundnut in this study exhibited survival adaptations against the stressful soil and

environmental conditions. Thus, they have good potential to be considered for the stressful environments. Among eight, three of them were able to nodulate the host plant, showing their potential to be used as native groundnut rhizobial inoculum. All isolates have shown plant growth promoting characterization through helping in solubilizing inorganic phosphate, synthesizing IAA, and amplification of nitrogen fixing *nifH* gene. Hence, they have the ability to be used as biofertilizer to enhance yield and N$_2$-fixation.

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