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RESEARCH ARTICLE

Morpho-physiochemical and genotypic characterization of rhizobia from chickpea (*Cicer arietinum*) root nodules

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Abstract

Rhizobia can enhance legume crop productivity, reduce nitrogen fertilizer use and promote sustainable agriculture. This study focused on isolating, identifying and characterizing rhizobia strains from chickpea root nodules. Forty strains were isolated from chickpea root nodules collected from nine districts in Bangladesh, which were cultured on Congo red yeast mannitol agar medium to obtain single colonies and study their morpho-physiological and genetic characteristics. Various physicochemical tests, including gram-staining, bromothymol blue test, temperature, salt and pH tolerance, phosphate solubilization and indole-3-acetic acid (IAA) production tests, were conducted using the isolated strains. These strains formed milky white colonies with spherical convex surfaces with 1.77 to 8.33 mm diameters. Physicochemical results indicated that strains were gram-negative, fast-growing and acid producers. Maximum strains thrived at pH levels 6 to 8, temperatures ranging from 28 to 35 °C and 5 % sodium chloride (NaCl). Seven strains exhibited strong phosphate solubilization capabilities and 30 % performed better in IAA production. In aseptic conditions, the inoculated plants showed higher dry matter percentage and higher nitrogen concentration in shoot than non-inoculated ones. Nodulation-positive strains possess *nif*H gene, indicating nitrogen-fixing capability of these strains. High genetic diversity was observed among the studied rhizobial strains, as determined by enterobacterial repetitive intergenic consensus sequence-based fingerprinting and they formed two major groups. Two strains, CRB-20 and CRB-28, were more diverse than others. Rhizobia isolated from different regions varied in morpho-physiological, genetic characters and symbiotic performances. These strains can be used for field trials to assess their suitability for efficient biological nitrogen fixation and growth of chickpeas.

Keywords: enterobacterial repetitive intergenic consensus sequence; isolated strains; *nifH* gene; rhizobia

Introduction

The global food system of 21st century faces challenges due to increasing population and resource competition. With the world's population predicted to reach 9.2 billion by 2050, ensuring food security has become a paramount concern (1). Consequently, sustainable methods to enhance crop productivity are urgently needed to meet the escalating demands. Legumes provide a viable alternative to cereal crops with nutrient richness and adaptability to diverse conditions. Chickpea (*Cicer arietinum*), the third most important pulse crop globally, is a protein-rich (20-22 %) supplement with high fiber, minerals (P, Ca, Mg, Fe, Zn), β-carotene and unsaturated fatty acids (2, 3). Chickpea is cultivated on 18.1 million hectares worldwide but its production, especially in Bangladesh, is declining compared to other South Asian countries like India, Myanmar and Pakistan (4). Legumes enhance soil fertility through symbiosis with rhizobia, which fixes atmospheric nitrogen through biological nitrogen fixation (BNF). Rhizobacteria excel in this process, converting atmospheric nitrogen into plant-usable form through nitrogenase enzyme and using plant photosynthesis-derived carbon substrates (5, 6). They also solubilize phosphate, produce growth hormones and improve plant yield (7).

The symbiosis between Fabaceae and rhizobia offers significant benefits for agriculture by reducing the amount of fertilizer used in fields. Chemical fertilizers have been found to have substantial negative effects on soil, water and the environment. Their imbalanced use degrades soil properties, causes nutrient leaching, reduces microbial activity and leads to environmental pollution (8). Rhizobia in root nodules contributes to the conversion of around 20 million tons of atmospheric nitrogen (N_2) into ammonia, which covers 50-70 % of global BNF (9). Chickpeas enhance soil fertility by fixing up to 106 Kg nitrogen (N)/ha from the air and meet 4-85 % of N needs through symbiotic association with effective rhizobia strains (10-12). Chickpea nodulating rhizobia, including species such as *Mesorhizobia amorphe, M. loti, M. tianshanense, M. ciceri, M. muleiense* and *M. mediterranum* are found worldwide (13, 14).

Appropriate rhizobial strain selection is essential for better symbiosis and BNF, which involves isolating strains from diverse environments and chickpea varieties to ensure their efficiency and survival in field conditions.

The survival, diversity and distribution of native rhizobia populations depend on genetic and physiological traits in addition to environmental and edaphic factors such as soil pH, phosphorous level, temperature, salinity and soil moisture content (15, 16). Evaluating rhizobial strains through tests for salt tolerance, pH adaptability, bromothymol blue (BTB), temperature resistance, phosphate solubilization and IAA production is important for optimizing their agricultural use. After isolation and characterization, they are inoculated into chickpeas to assess their impact on growth and yield under controlled conditions, pot experiments and field trials to ensure the selection of the most effective strains. This suggests potential to increase production by utilizing effective nitrogen-fixing bacterial strains.

Symbiosis is a complex, multi-step process that can be highly specific for some rhizobia strains. One bacterial strain might form effective nodules on several *Cicer* species, while another may only be compatible with a single plant species. However, certain species show less specificity to their host plants (17). Rhizobia are highly diverse, so effective classification methods are needed to identify strains with strong nitrogen-fixing abilities (18). Molecular techniques have simplified this process, enabling scientists to quickly distinguish rhizobia at the genus, species and even strain levels (18, 19). In recent years, the taxonomic classification of rhizobia has changed, among various tests, genetic marker-based techniques are now employed for strain characterization and differentiation (20). One of these methods, enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) analysis is a rapid and effective fingerprint method and separates strains accurately. ERIC sequences are mobile deoxyribonucleic acid (DNA) fragments that appear across rhizobia genomes and are especially helpful for identifying and classifying strains and studying how environmental factors affect rhizobia populations (20). Knowledge of the morpho-genetic characterization of the indigenous rhizobia will help them survive in current environmental conditions and address the impacts of future climate change. Despite the abundance of chickpea nodulating rhizobia and their geographical distribution around the world, the phenotypic and genotypic diversity of these rhizobia is still not well understood in Bangladesh. Therefore, the present study aimed to isolate rhizobial strains from chickpea root nodules from different areas of Bangladesh and characterize them based on morpho-physiological and genotypic characteristics.

Materials and Methods

Collection and surface sterilization of nodules

Nodules from chickpea plants were collected in this study from nine (Barisal, Chapainawabganj, Gopalganj, Pabna, Jessore, Jhenaidah, Magura, Natore and Rajshahi) districts of Bangladesh. Nodules were taken from flowering plants that were six to eight weeks old and preserved in a falcon tube with silica gel until the isolation of bacterial strains (21). After washing with sterile water, collected nodules were soaked overnight at 4 °C. They were then surface-sterilized with 70 % ethanol for 1 min and 5 % sodium hypochlorite (NaOCl) for 3 min, followed by washing with sterile distilled water.

Isolation and preservation of rhizobial strains

Nodules were crushed and bacterial suspension was streaked onto CRYEMA (Congo red yeast extract mannitol agar) plates and incubated at 28 °C (21). Single colonies were identified through repeated streak. One isolated colony was grown on CRYEMA medium for the study and maintained at 4 °C. For long-term preservation, another colony was grown on YEM (yeast extract mannitol) liquid medium and stored at -80 °C in a 50 % glycerol solution.

Morpho-physiological characterization

On CRYEMA plates, a single loop of bacterial culture was streaked and incubated at 28 °C for 72 hr. Colony diameters were measured (mm) and averaged. Gram staining was done with the isolated strains (22). For the BTB test, 5 µL of overnight-grown cultures were pipetted onto BTB plates, dried and incubated at 28 °C for 3 days. Isolated strains were tested in media at various pH levels (3, 4, 5, 6, 7, 9 and 10). For pH 3, YEM liquid culture was used. Salt tolerance was assessed on YEMA media with 1-6 % NaCl. For temperature tolerance, 5 µL of overnight cultures were inoculated on YEMA plates and incubated at 4, 28, 35, 37 and 40 °C for 3 days, with the 4 °C plates kept in the freezer.

Isolated rhizobial strains were screened and assessed for phosphate solubilization on National Botanical Research Institute's Phosphate (NBRIP) agar media comprising 0.2 g glucose, 5 g tricalcium phosphate ($Ca_3(PO_4)_2$), 5 g magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$), 0.25 g magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$), 0.2 g potassium chloride (KCl) and 0.1 g ammonium sulphate ($[NH_4]_2SO_4$) and 15 g bacteriological agar in 1 L distilled water (23). Two µL of overnight-grown rhizobia culture was pipetted on plates and incubated at 28 °C for 7 days. Phosphate solubilization was assessed by measuring the clear halo zone around bacterial growth which was assumed as positive (+) results. Phosphate solubilization index was calculated using the following equation (24).

Phosphate solubilization index (PSI) =

[Total diameter (halo + colony zone)] / Diameter of colony

(Eqn. 1)

Each isolate was grown in YEM liquid media supplemented with 40 µg/mL L-tryptophan for IAA evaluation, then incubated for 24 hr in a shaker (30 °C and 180 rpm). After centrifuging the culture at 8000 rpm for 15 min, 1 mL of the supernatant was combined with 4 mL of Salkowski's reagent [15 mL of 0.5 M ferric chloride ($FeCl_3$), 500 mL of distilled water and 300 mL of concentrated Sulphuric acid (H_2SO_4)] and allowed to stand for 30 min at room temperature (25). The presence of a reddish-pink color indicated IAA production by the isolate.

Nodulation test under *in-vitro* conditions and determination of shoot nitrogen

All rhizobial isolates were tested for nodule formation with a chickpea variety BINA SOLA-8. Plants were grown in conical flasks (250 mL) containing nitrogen-free agar medium (26). Overnight-grown bacterial cultures in YEM liquid medium (5 mL/plant) were used to inoculate 3-5 days-old chickpea

seedlings based on their root growth. After harvesting at 45 days, plant parameters were recorded (27). The dry matter percentage of the plants was calculated using the following formula:

Dry matter (%) = (Oven dry weight × 100)/Fresh weight (Eqn. 2)

The total nitrogen (N) of chickpea shoots grown in aseptic conditions after 45 days was estimated by Kjeldahl digestion method, where the amount of total N % was calculated using the following formula (28):

Total N (%) = [(T- B) × N × 0.014 × 100] / S (Eqn. 3)

Where, T = sample titration value (ml) of standard H_2SO_4, B = blank titration value (mL) of standard H_2SO_4, N = strength of H_2SO_4, S= weight of soil sample in gram

Amplification of nitrogen-fixing *nifH* gene

Bacterial DNA was isolated following a standard protocol (29). DNA concentration and purity were determined using Nanodrop spectrophotometry. Primers were used to amplify and sequence the *nifH* gene (30). The polymerase chain reaction (PCR) products were analyzed using agarose gel electrophoresis. The amplified product from each sample was separated by electrophoresis on 1.5 % agarose gel containing 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer at 80 V for 55 min, then stained with ethidium bromide for 30 min. DNA bands were documented under a high-performance ultraviolet light box (UV-trans illuminator). The *nifH* gene was observed as bands and the records were saved.

Genomic fingerprinting by ERIC-PCR

For high-resolution ERIC-PCR, the primers and PCR conditions were followed as previously described (31). The 25 µL reaction mixture contained 12.5 µL of PCR master mixture from Promega, USA, 2.5 µL dimethyl sulfoxide (DMSO), 30 pmol of each primer and approximately 100 ng of genomic DNA. The cycling conditions for ERIC-PCR comprised of initial cycle at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 45 sec, annealing at 52 °C for 90 sec and extension at 65 °C for 8 min, with a single final extension step at 65 °C for 16 min. Primers used for amplification of ERIC-sequences were ERIC 1R (5'-ATGTAAGCTCCTGGGGAT-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGGT GAGC-3'). DNA fragments were separated by vertical PAGE (polyacrylamide gel electrophoresis) using a base acer sequencer at 65 W for 3.5 hr. The gel was stained with ethidium bromide for 30 min and DNA bands were documented under gel documentation system.

Statistical analysis and dendrogram construction

The data on various plant characters grown at *in vitro* conditions were statistically analyzed. Analysis of variance (ANOVA) with statistical package R (version 4.3.1) and post-hoc tests were performed to separate differences among treatments. The fingerprint bands of ERIC-PCR were visualized manually and a score of '1' was given as the presence of bands and a score of '0' as the absence of bands. A dendrogram was constructed with these '0, 1' binary scores using the software package R (version 4.3.1).

Results

Morpho-physiological characterization

In this study, 40 strains grew quickly on CRYEMA plates within 3 days. A general description of the strains is given in Table 1. The colonies of the isolates were round to oval, creamy, gummy and spherical convex in elevation having nearly entire margins (Fig. 1). The diameters of the colonies varied from 1.17 to 8.33 mm (Table 1). Isolate CRB-13, CRB-32 and CRB-40 formed the highest colony diameter (8.33 mm) while isolates, CRB-1 and CRB-76 formed the lowest colony diameter (1.17 mm).

The rhizobial isolates were Gram-stained and appeared red and rod-shaped under the microscope (Fig. 2a). In bromothymol blue test, all the isolates demonstrated yellow coloration, indicating their ability for rapid growth and acid production (Fig. 2b). All the rhizobial strains showed vigorous growth on 1 to 4 % NaCl. In the case of 5 % NaCl, twenty-five strains showed moderate growth and four could not tolerate concentration, but the remaining eleven sustained (Table 2 and Fig. 3a). At 6 % salt concentration, none of the strains survived. The vigorous growth of all the rhizobial strains was observed at pH 6 and 7 (Fig. 3b and Table 2). At pH 9, most of the strains showed vigorous growth except CRB-1, 19 and 76 which showed moderate growth. In case of pH 10, twenty-three strains moderately grew but the remaining grew vigorously. At pH 5, twenty-seven strains showed vigorous growth but thirteen strains exerted moderate growth. Again, all the strains showed moderate growth at pH 4, but three strains (CRB-19, 61 and 75) only sustained. No strains could grow at pH 3.

In the current study, no growth of rhizobial strains occurred at 4 °C temperature (Fig. 4a and Table 3). At 28 and 35 °C temperatures, all strains grew vigorously. At 37 °C temperature, thirty-one strains showed moderate growth,

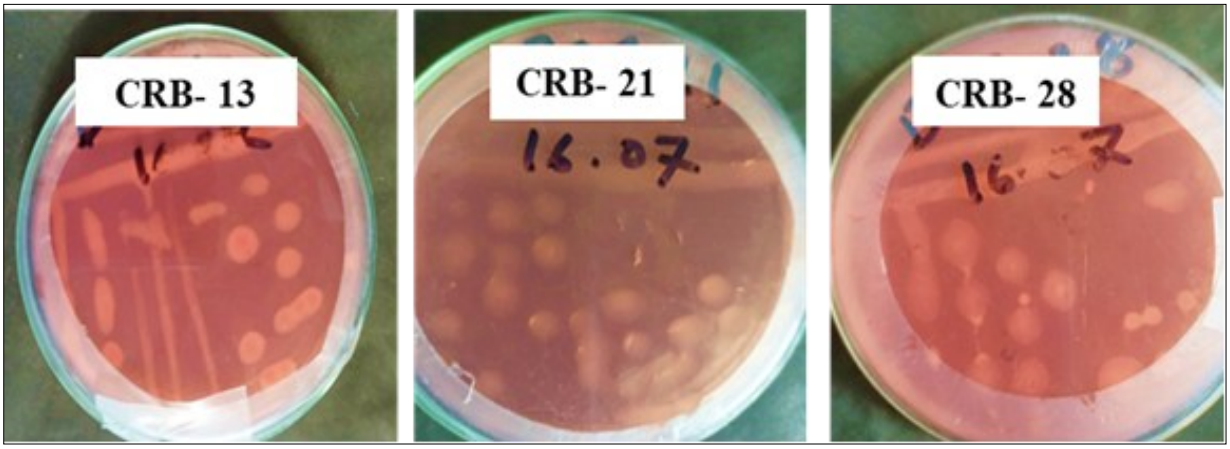

Fig. 1. Single colonies of isolated rhizobial strains on CRYEMA plate.

Table 1. General description and colony morphology of studied rhizobial strains

Strain identity	Collection area	Colony diameter (average) (mm)	Strain identity	Collection area	Colony diameter (average) (mm)
CRB-1	Station field, Chapainowabganj	1.17	CRB-36	Fukra, Gopalganj	4
CRB-3	Station field, Chapainowabganj	5	CRB-37	Fukra, Gopalganj	4.33
CRB-9	Station field, Chapainowabganj	5.33	CRB-39	Sub-station, Gopalganj	2.67
CRB-12	Sadipur, Bagharpara, Jessore	3.17	CRB-40	Sub-station, Gopalganj	8.33
CRB-13	Sadipur, Bagharpara, Jessore	8.33	CRB-43	RARS, BARI, Ishwardi, Pabna	3
CRB-16	Bunogati, Shalikha,Magura	6.33	CRB-45	RARS, BARI, Ishwardi, Pabna	4.33
CRB-17	Bunogati, Shalikha, Magura	2	CRB-46	RARS, BARI, Ishwardi, Pabna	4
CRB-18	Bunogati, Shalikha, Magura	2.83	CRB-47	Lalpur, Natore	6.67
CRB-19	Majhdia, Kaliganj, Jhenaidah	4.67	CRB-48	Lalpur, Natore	4.33
CRB-20	Majhdia, Kaliganj, Jhenaidah	4	CRB-51	Sub-station, Ishwardi, Pabna	3
CRB-21	Majhdia, Kaliganj, Jhenaidah	4.67	CRB-52	Sub-station, Ishwardi, Pabna	4
CRB-22	Majhdia, Kaliganj, Jhenaidah	5.33	CRB-54	Sub-station, Ishwardi, Pabna	5
CRB-23	Gobra, Jessore sadar	4	CRB-55	Bagha, Rajshahi	2
CRB-27	Rakudia, Babuganj, Barisal	4.33	CRB-56	Bagha, Rajshahi	1.33
CRB-28	Rakudia, Babuganj, Barisal	6	CRB-58	Bagha, Rajshahi	1.5
CRB-29	Rakudia, Babuganj, Barisal	4.33	CRB-61	Bagha, Rajshahi	2
CRB-30	Rakudia, Babuganj, Barisal	5.33	CRB-63	Bagha, Rajshahi	2.66
CRB-31	Sub-station, Barisal	5.33	CRB-64	Bagha, Rajshahi	2.33
CRB-32	Sub-station, Barisal	8.33	CRB-75	Bagha, Rajshahi	3.3
CRB-35	Fukra, Gopalganj	6	CRB-76	Bagha, Rajshahi	1.17

CRB= Chickpea rhizobia of Bangladesh[a].

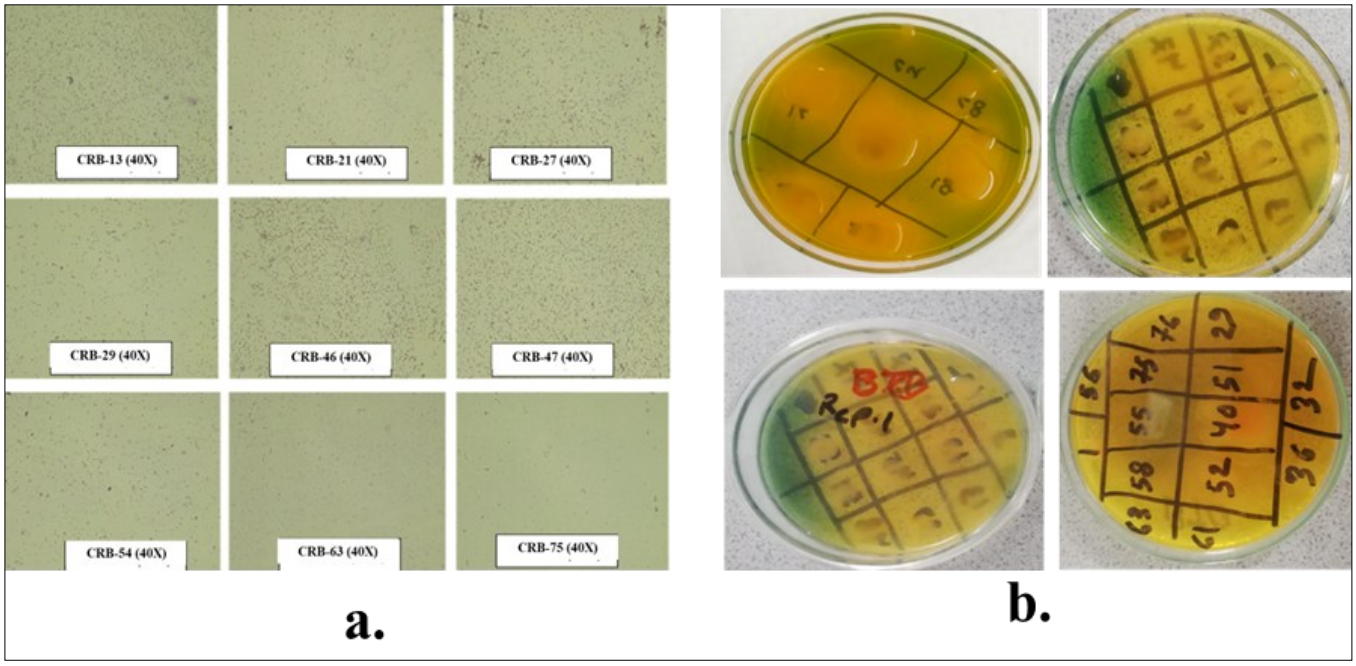

Fig. 2. (a) Microscopic view of the strains after Gram-staining and (b) Acid production at bromothymol blue medium.

Table 2. Effect of pH on growth of studied rhizobial strains

Strain name	NaCl concentrations		pH 4	pH 5	pH 9	pH 10	Strain name	NaCl concentrations		pH 4	pH 5	pH 9	pH 10
	4 %	5 %						4 %	5 %				
CRB-1	+	±	+	+	+	+	CRB-36	+	+	+	+	++	+
CRB-3	++	+	+	++	++	++	CRB-37	++	+	+	++	++	++
CRB-9	++	+	+	++	++	++	CRB-39	++	+	+	++	++	+
CRB-12	++	+	+	++	++	++	CRB-40	++	+	+	++	++	++
CRB-13	++	+	+	++	++	++	CRB-43	+	-	+	++	++	+
CRB-16	+	+	+	++	++	+	CRB-45	++	+	+	++	++	+
CRB-17	+	±	+	+	++	++	CRB-46	+	±	+	++	++	+
CRB-18	++	+	+	+	++	+	CRB-47	++	+	+	++	++	++
CRB-19	+	±	±	++	+	+	CRB-48	++	+	+	++	++	++
CRB-20	++	±	+	++	++	+	CRB-51	++	±	+	+	++	+
CRB-21	++	+	+	++	++	++	CRB-52	++	+	+	++	++	++
CRB-22	++	+	+	++	++	+	CRB-54	++	±	+	++	++	+
CRB-23	++	+	+	++	++	+	CRB-55	++	+	+	+	++	++
CRB-27	++	+	+	++	++	++	CRB-56	+	±	+	+	++	+
CRB-28	++	+	+	++	++	++	CRB-58	++	+	+	+	++	+
CRB-29	+	-	+	+	++	+	CRB-61	++	-	±	++	++	+
CRB-30	++	+	+	+	++	+	CRB-63	+	±	+	+	++	+
CRB-31	++	+	+	++	++	+	CRB-64	++	+	+	++	++	++
CRB-32	+	±	+	++	++	++	CRB-75	+	±	±	+	++	+
CRB-35	++	+	+	++	++	++	CRB-76	+	-	+	+	+	+

++ indicates vigorous growth, + indicates moderate growth, ± indicates sustain only and - indicates no growth[a].

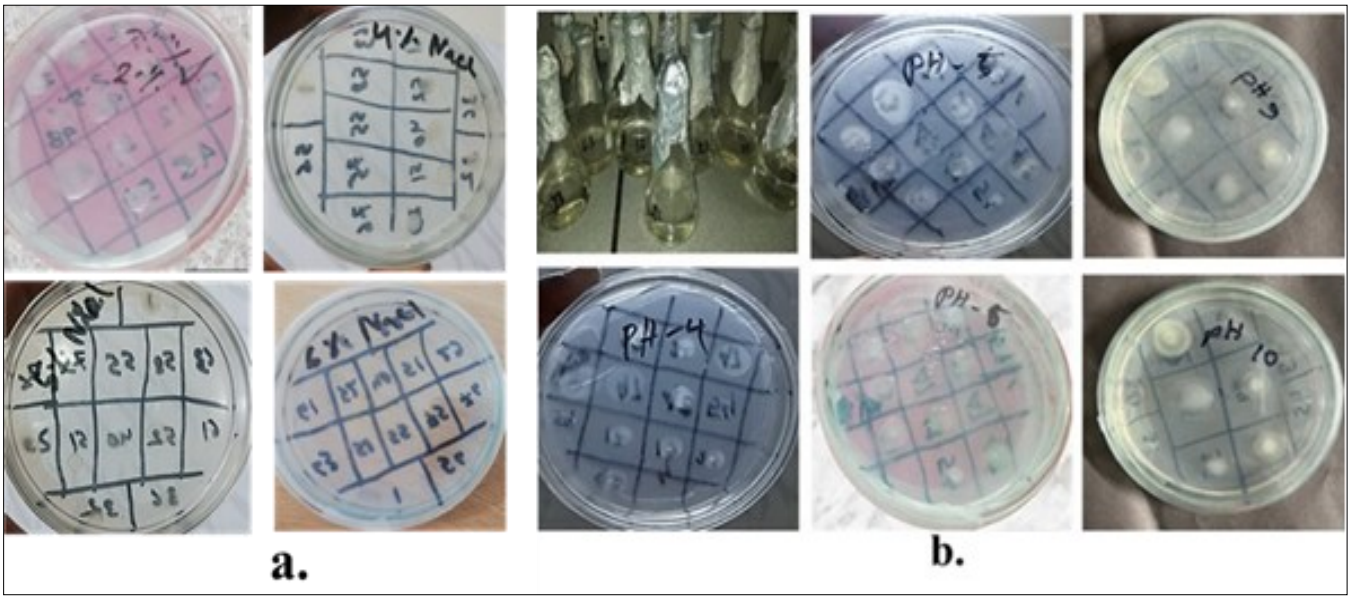

Fig. 3. (a) Pictorial view of salt and (b) pH tolerance test of studied rhizobia strains.

although 9 strains thrived. At 40 °C temperature, CRB-21, 31, 35, 37, 40 and 47 exhibited moderate growth, whereas CRB-32, 39, 43, 48 and 64 sustained only and the remaining 29 strains showed no growth. Among 40 isolates, only seven strains were highly capable of solubilizing $Ca_3(PO_4)_2$ on NBRIP agar medium, where PSI of all the isolates ranged from 1.00 to 3.08 (Fig. 4b and Table 3). Among the strains, CRB-55 (PSI= 3.08) was the best phosphate solubilizer, followed by CRB-31 (PSI=2.50), CRB -40 (PSI=2.18) and CRB-63 (PSI=2.13). Six isolates were incapable of solubilizing phosphate (PSI=1.00) whereas the remaining isolates had a low (PSI=1.1-1.5) to medium (PSI=1.5-2.0) phosphate solubilization potential. Out of 40 bacterial isolates, 21 were found to produce IAA and among them, 12 isolates (CRB-9, 18, 22, 23, 27, 29, 30, 37, 40, 47, 48 and 52) were better IAA producers and 9 strains produced little IAA. The remaining 19 strains could not produce IAA (Fig. 5 and Table 3).

Nodulation test

In the nodulation efficiency test, 32 among 40 could form nodules within 20-30 days and 8 could not (Table 4). The number of nodules in the inoculated chickpea plants varied from 3 to 40/plant in aseptic nitrogen-free conditions with CRB-37 having the highest number. The plant parameters such as branch number, fresh weight, dry weight and percent N were higher in inoculated plants than in control or nodulation-negative plants. Total N percentage in chickpea shoots range from 0.37 % (control) to 0.69 %. Higher percentages of N were found in the plants inoculated by the strains CRB-40 (0.69 %), CRB-37 (0.67 %), CRB-56 (0.66 %), CRB-47 (0.65 %), CRB-31 (0.64 %) and CRB-55 (0.64 %) (Table 4). The highest percent dry matter was in CRB-37 (23.05 %) whereas the lowest (12.32) was recorded in control. In total dry matter production, the best strains were CRB-37 (23.05 %), CRB-31 (22.97 %) and CRB-40 (22.9 %) (Fig. 6).

Amplification of *nifH* gene from isolated rhizobial strains

The strains in this study which demonstrated positive results in the nodulation tests, were also subjected to the confirmation of the presence of the *nifH* gene (Fig. 7). This confirmation was based on the observation of bands with the expected size of about 400 bp, as generated by the *nifH* primers (PolyF: 5-TGC GAY CCSAARGCBGACTC-3′and PolyR: 5-ATS GCC ATC ATY TCR CCGGA-3′) (31).

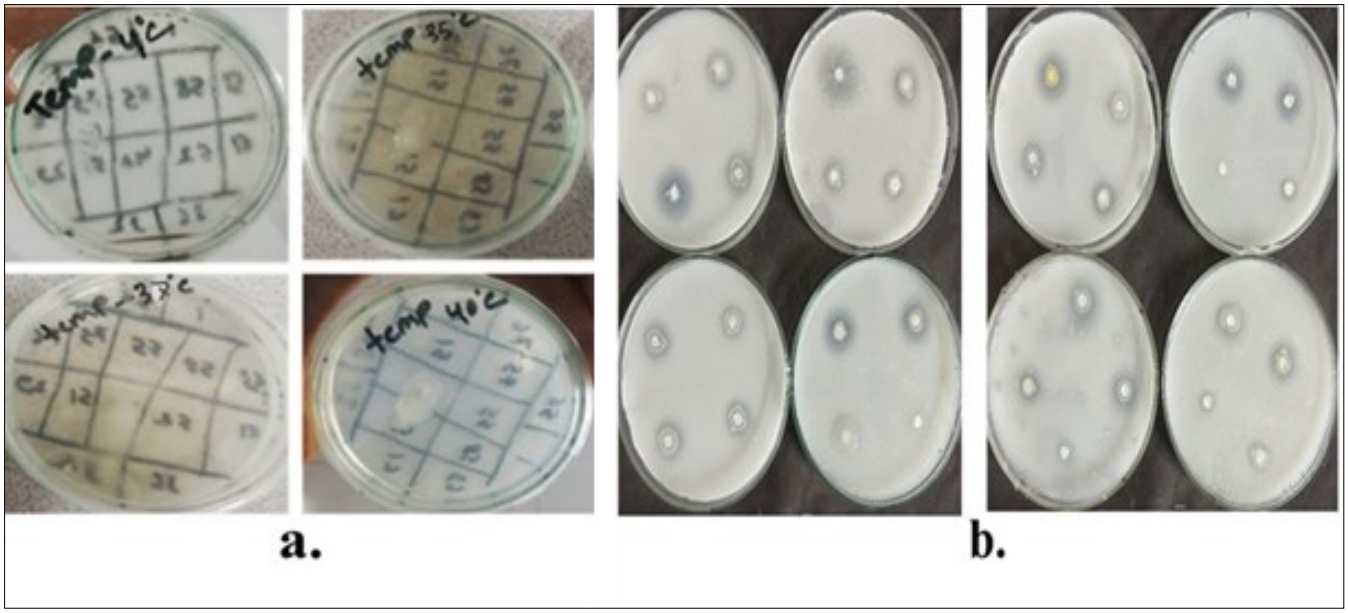

Fig. 4. (a) Temperature tolerance and (b) Phosphate solubilization by studied rhizobial strains.

Table 3. Phosphate solubilization and IAA production by studied rhizobia

Strain name	Temperature tolerance			PSI	IAA	Strain name	Temperature tolerance			PSI	IAA
	35 °C	37 °C	40 °C				35° C	37 °C	40 °C		
CRB-1	++	+	-	1.10	-	CRB-36	++	+	-	1.25	±
CRB-3	++	+	-	1.67	-	CRB-37	++	++	+	2.00	+
CRB-9	++	+	-	1.10	+	CRB-39	++	+	±	1.50	-
CRB-12	++	+	-	1.89	-	CRB-40	++	++	+	2.18	+
CRB-13	++	+	-	1.00	-	CRB-43	++	+	±	1.36	±
CRB-16	++	+	-	1.75	-	CRB-45	++	+	-	1.35	-
CRB-17	++	+	-	1.67	-	CRB-46	++	+	-	1.45	±
CRB-18	++	+	-	1.76	+	CRB-47	++	++	+	1.33	+
CRB-19	++	+	-	1.33	-	CRB-48	++	++	±	1.36	+
CRB-20	++	+	-	1.11	-	CRB-51	++	+	-	1.56	-
CRB-21	++	++	+	1.56	±	CRB-52	++	+	-	1.42	+
CRB-22	++	+	-	1.44	+	CRB-54	++	+	-	2.00	±
CRB-23	++	+	-	1.60	+	CRB-55	++	+	-	3.08	±
CRB-27	++	++	-	1.75	+	CRB-56	++	+	-	1.00	-
CRB-28	++	+	-	1.09	±	CRB-58	++	+	-	1.13	-
CRB-29	++	+	-	1.93	+	CRB-61	++	+	-	1.00	-
CRB-30	++	+	-	1.86	+	CRB-63	++	+	-	2.13	-
CRB-31	++	++	+	2.50	±	CRB-64	++	++	±	1.00	-
CRB-32	++	+	±	1.00	-	CRB-75	++	+	-	2.00	-
CRB-35	++	++	+	1.00	-	CRB-76	++	+	-	1.50	±

For temperature tolerance, ++ indicates vigorous growth, + indicates moderate growth, ± indicates sustain only and - indicates no growth[a]. For IAA production, + indicates well-developed color, ± indicates a little development of color and - indicates no color development[b].

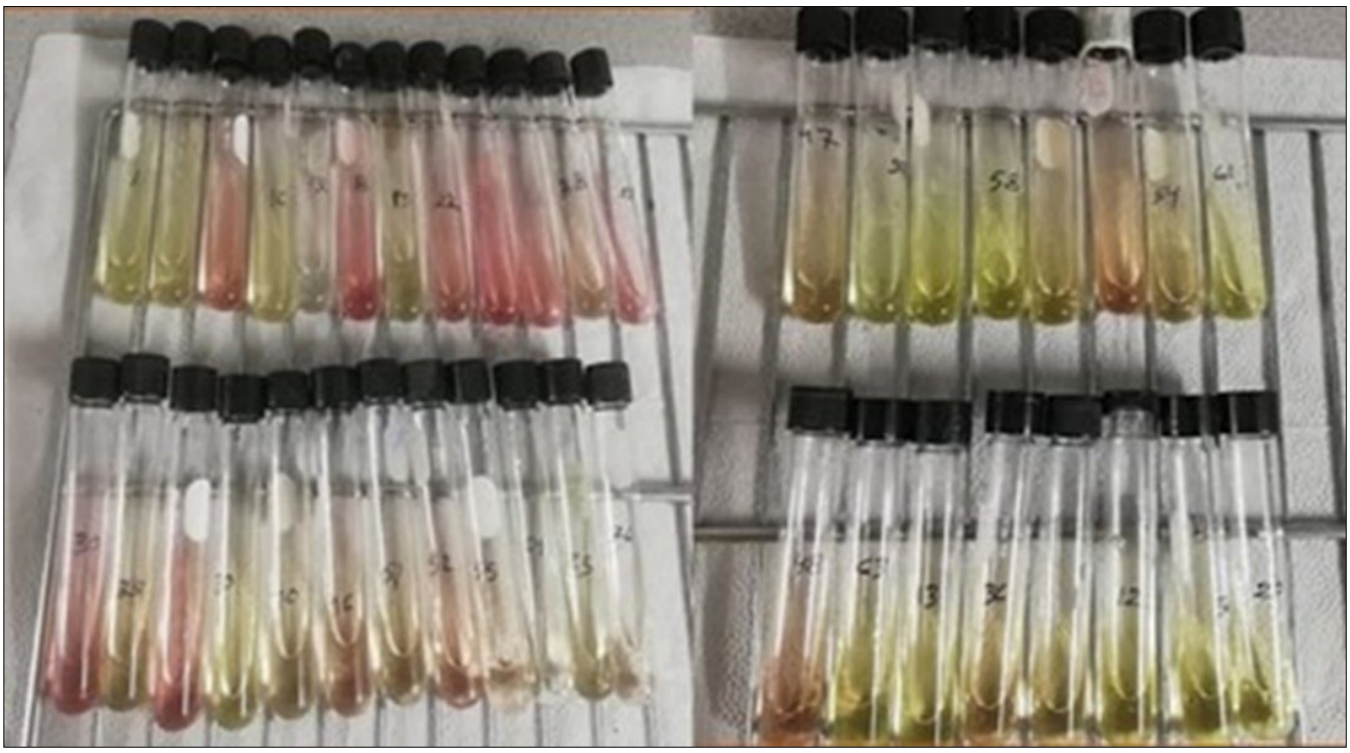

Fig. 5. Indole acetic acid production by studied rhizobial strains.

Table 4. Nodulation test of studied rhizobial strains under *in vitro* (aseptic) condition

Strains	Nodulation	Different plant parameters influenced by rhizobial strain inoculation			
		Branch no.	Nodule number	Dry weight of nodule (mg/ plant)	% N in shoot at nitrogen free media
Control	-	1±0 e	0	0	0.37±0.02^{j}
BCR-1	+	4±1^{abc}	3±0^{lmnop}	4.9±0.6^{efghi}	0.42±0.06^{hij}
BCR-3	+	3.5±0.5^{abcd}	3.5±0.5^{klmnop}	15±0^{bcdef}	0.45±0.01^{efghij}
BCR-9	+	3.5±0.5^{abcd}	11±1^{efg}	8.55±1.55^{defghi}	0.5±0.03^{bcdefghij}
BCR-12	+	3.5±0.5^{abcd}	26.5±0.5^{b}	18±1^{bcd}	0.49±0.02^{cdefghij}
BCR-13	+	5±0^{a}	10.5±0.5^{fgh}	21±1^{b}	0.51±0.02^{bcdefghij}
BCR-16	-	1±0^{e}	0	0	0.41±0.07^{hij}
BCR-17	-	1±0^{e}	0	0	0.46±0.15^{efghij}
BCR-18	-	2±0^{cde}	0	0	0.5±0.01^{bcdefghij}
BCR-19	+	2.5± 0.5^{bcde}	7±0^{ghijk}	3.55±0.45^{fghi}	0.41±0^{ij}
BCR-20	+	2±0^{cde}	2.5±0.5^{mnop}	1±0^{hi}	0.61±0.02^{abcdef}
BCR-21	+	3.5±0.5^{abcd}	9±1^{ghij}	5.8±3.4^{efghi}	0.48±0.02^{defghij}
BCR-22	+	2.5±0.5^{bcde}	15±1de	15.5±0.5^{bcde}	0.5±0.03^{bcdefghij}
BCR-23	-	2±0^{cde}	0	0	0.45±0.02^{fghij}
BCR-27	+	3.5±0.5^{abcd}	10±2^{ghi}	7.1±4.9^{defghi}	0.5±0.02^{bcdefghij}
BCR-28	+	3±0^{abcde}	6±3.74 g^{hij}	9±5.61^{bcdefg}	0.53±0.02^{abcdefghij}
BCR-29	-	2±0^{cde}	0	0	0.51±0.05^{bcdefghij}
BCR-30	+	2±0^{cde}	6±1^{ijklm}	4.15±0.35^{efghi}	0.49±0.02 ^{cdefghij}
BCR-31	+	3.5±0.5^{abcd}	17±1^{d}	20.5±1.5^{bc}	0.64±0.02^{abcd}
BCR-32	+	2±0^{cde}	6.5±0.5^{hijklm}	1.5±0.5^{hi}	0.62±0.03^{abcde}
BCR-35	+	2±0^{cde}	7.5±0.5^{ghijk}	4.55±0.55^{efghi}	0.53±0.03^{abcdefghij}
BCR-36	+	2.5±0.5^{bcde}	21.5±1.5^{c}	6.3±0.7^{efghi}	0.6±0.03 ^{abcdef}
BCR-37	+	3.5±0.5^{abcd}	40±1^{a}	59.5±9.5^{a}	0.67±0^{a}
BCR-39	+	4.5±0.5^{ab}	14.5±0.5^{def}	13.8±2.8^{bcdef}	0.59±0.07^{abcdefg}
BCR-40	+	3±0^{abcde}	4.5±0.5^{klmno}	4.65±0.35^{efghi}	0.69±0.09^{a}
BCR-43	+	3±0^{abcde}	3.5±0.5^{klmnop}	9±1^{cdefghi}	0.6±0.07^{abcdef}
BCR-45	-	1.5±0.5^{de}	0	0	0.5±0.05^{bcdefghij}
BCR-46	-	2.5±0.5^{bcde}	0	0	0.45±0.03^{fghij}
BCR-47	+	2.5±0.5^{bcde}	5±0^{jklmno}	11.5±1.5^{bcdefghi}	0.65±0.06^{abc}
BCR-48	+	2.5±0.5^{bcde}	2.5±0.5^{mnop}	12±2^{bcdefgh}	0.56±0.01^{abcdefghi}
BCR-51	+	2±0^{cde}	14.5±1.5^{def}	6.5±1.5^{defghi}	0.62±0.05^{abcd}
BCR-52	+	2±0^{cde}	2.5±0.5^{mnop}	1±0^{hi}	0.56±0.1^{abcdefghi}
BCR-54	+	3.5±0.5^{abcd}	9±0^{ghij}	7.3±0.7^{defghi}	0.57±0.09^{abcdefghi}
BCR-55	+	2±0^{cde}	4±0^{klmnop}	1.15±0.15^{hi}	0.64±0.19^{abcd}
BCR-56	+	3.5±0.5^{abcd}	2±1^{nop}	1.4±0.1^{hi}	0.66±0.06^{b}
BCR-58	+	2.5±0.5^{bcde}	10±1^{ghi}	5.5±0.5^{efghi}	0.6±0.06^{abcdef}
BCR-61	+	2±0^{cde}	1.5±0.5^{op}	2.2±0.1^{ghi}	0.43±0.07^{ghij}
BCR-63	+	3±0^{abcde}	1±0^{op}	0.9±0^{hi}	0.58±0.03^{abcdefgh}
BCR-64	-	1±0 ^{e}	0	0	0.44±0.02^{ghij}
BCR-75	+	2.5±0.5^{bcde}	4±0^{klmnop}	4.95±0.05^{efghi}	0.43±0.02^{hij}
BCR-76	+	2.5±0.5^{bcde}	6±0^{ijklmn}	11.5±0.5^{bcdefghi}	0.44±0.02^{ghij}
CV%		20.73	14.14	37.14	15.4
LOS		***	***	***	***

Here, CV (%) = coefficient of variation, LOS = levels of significance. Figures in a column having common letters do not differ significantly at 5 % level of probability (*** = p<0.001. ** = p<0.01, * = p<0.05)[a].

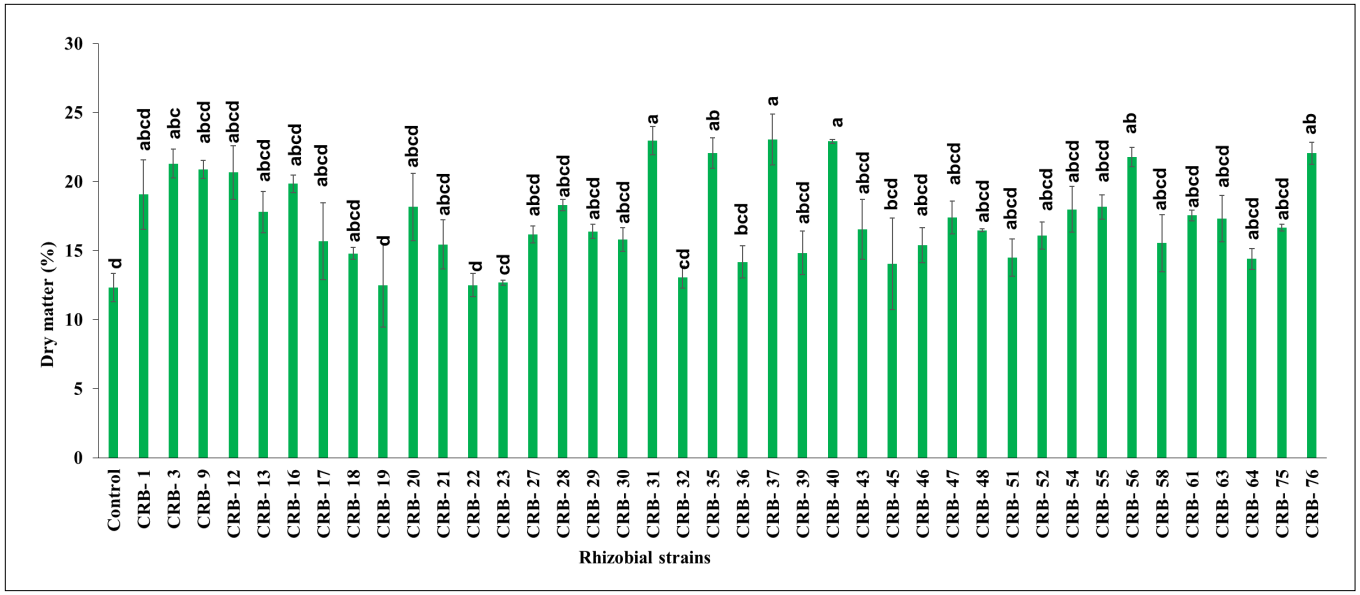

Fig. 6. Effect of inoculation of rhizobial strains on total dry matter (%) of chickpea plants.

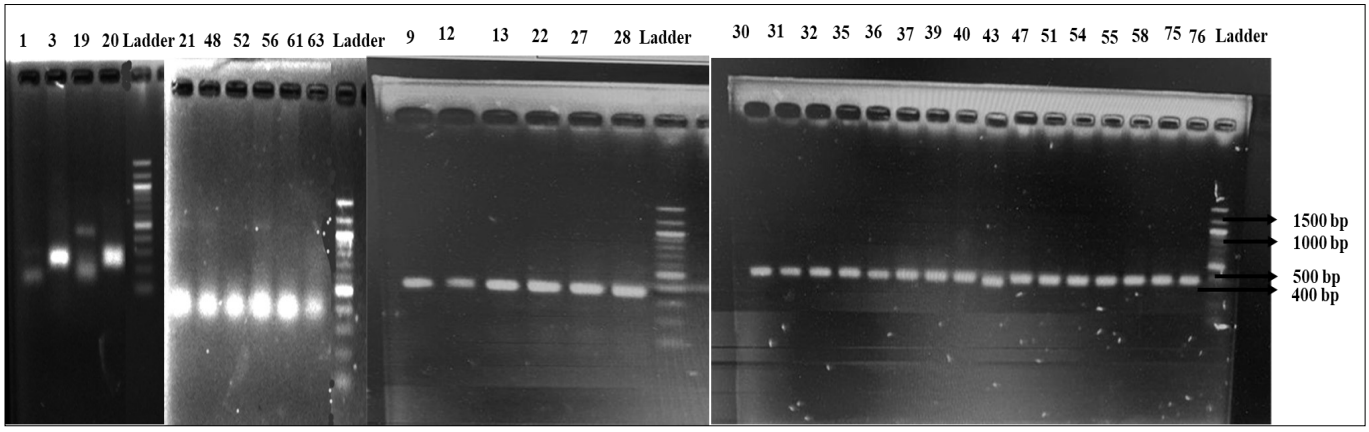

Fig. 7. Molecular detection of the *nifH* gene through PCR.

DNA fingerprinting

Genomic DNA from nodule-forming bacterial strains was subjected to ERIC-PCR-based fingerprinting (Fig. 8). The cluster analyses based on high-resolution ERIC-PCR fingerprints revealed different, well-defined banding patterns of different strains. Studied 32 strains formed two major clusters (A and B) with different subclusters (Fig. 9). In total, 31 different DNA profiles were found where CRB-63 and CRB-76 were 100 % similar in their DNA profile. Cluster A was the larger and contained more diverse strains. It was divided into two sub-clusters (A1 and A2). In A2, there were more separate sub-clusters (A2a and A2b) which may indicate substantial genomic variation. Sub-cluster A1 comprised strains CRB-3, 21, 36, 43, 58, sub-cluster A2a included strains CRB-1, 12, 13, 19, 27, 30, 31, 32, 39, 40, 55, 56, 61, 63, 75, 76 and subcluster A2b exhibited strains CRB-9, 35, 37, 47, 51. The strain CRB-28 was the most diverse strain in cluster A. In contrast, cluster B represented a smaller group with higher genetic similarity among its members and consisted of strains CRB-20, 22, 48, 52 and 54. Within this cluster, a single subcluster, B1, was observed and despite this overall similarity, strain CRB-20 stood out as the most genetically divergent strain in Cluster B.

Discussion

The fast-growing rhizobia are found in peas, beans, clover, alfalfa, chickpea and *Leucaena* and slow-growing rhizobia in cowpea and soybean plants (32). In this experiment, rhizobial strains formed creamy colonies (1.17-8.33 mm) as like previous experiments (33). All strains were gram-negative fast growers and acid producers which is essential for pH regulation in alkaline soil. A researcher also found the same result on rhizobial strains in similar conditions (34).

Salinity reduces chickpea growth, nitrogen fixation, nodule numbers and nitrogen content at field conditions (35). Interestingly, rhizobia isolated from chickpea root nodules are more salt-tolerant than the host (36). In this study, rhizobia showed diverse responses to higher salt concentrations with 62.5 % of strains exhibiting tolerance at 5 % NaCl. A similar response was found in another study (37). This finding is significant for saline regions, indicating these strains could enhance chickpea yields when used as inoculants. Rhizobia have a wide pH tolerance range, which is necessary for symbiosis with leguminous plants and adaptation to varied conditions, hence improving nitrogen fixation in agriculture. In present study, the strains had tolerance to extremes pH like pH 9 and 10 and some showed resistance to acidic environments (pH 4 and 5). In other studies, various responses were also observed of chickpea nodulating rhizobia in different pH conditions (37).

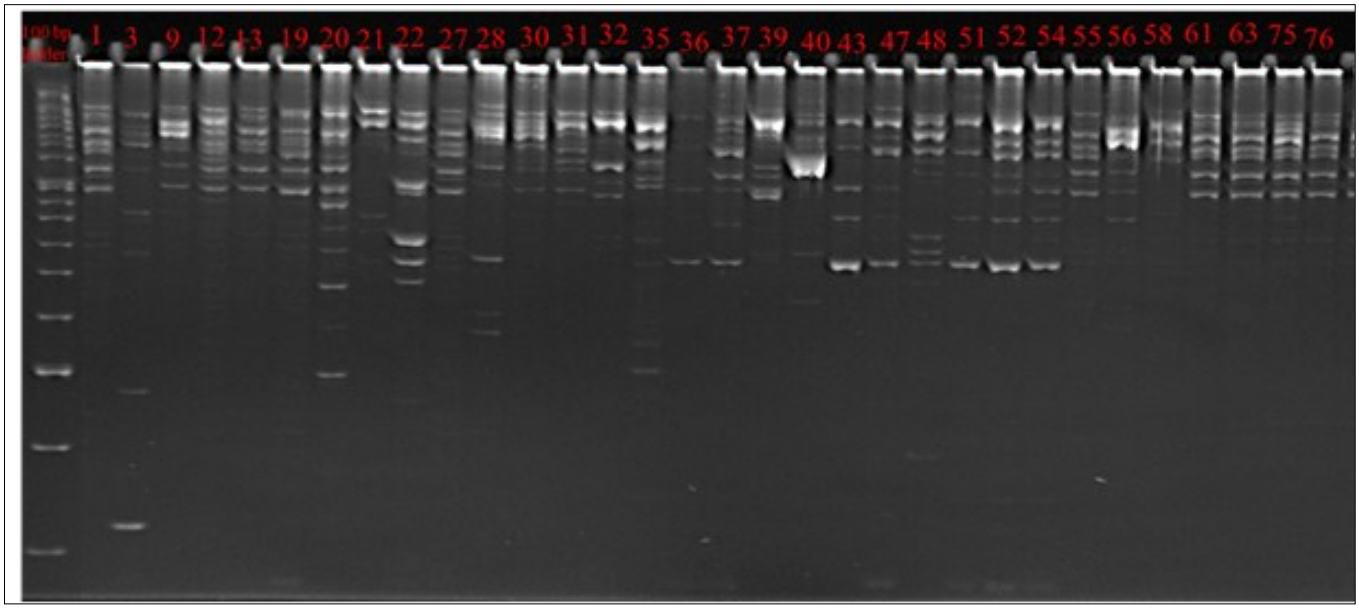

Fig. 8. High-resolution ERIC-PCR fingerprint for genes of rhizobial isolates.

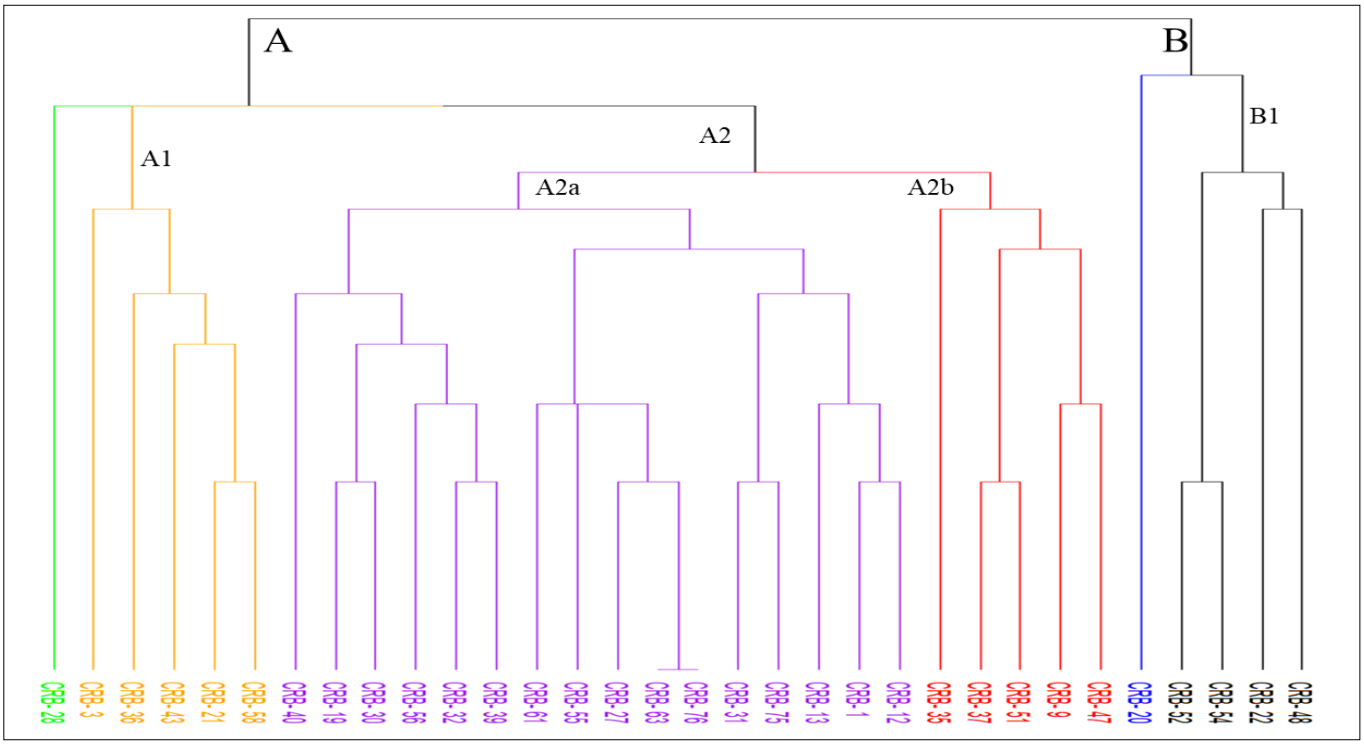

Fig. 9. The UPGMA dendrogram generated from ERIC-PCR banding pattern of bacterial genes.

Temperature tolerance tests are important to find out rhizobial effectiveness in different climates. The isolated strains exhibited optimal growth at 28-35 °C, which is in line with the typical temperature range in Bangladesh. A 4 °C test was done to observe recent growth, as all strains were stored at this temperature. Strains showed vigorous growth up to 37 °C and at 40 °C, but their responses varied, which reflected their adaptability to warm conditions. As reported in another study, similar patterns were found, with optimal growth at 28 °C and limited growth at 5 °C and 40 °C (6). Other researchers also observed that rhizobial strain survival varied with temperature: best growth occurred at 23 °C, was reduced at 15 °C and 37 °C and nearly all strains died at 45 °C (38).

Rhizobia-mediated phosphate solubilization promotes plant growth, root development and nitrogenase activity, which is necessary for nodulation and N_2 fixation (39). Phosphorus is the second limiting nutrient after nitrogen and is essential for plant growth. Rhizobacteria capable of phosphate solubilization are important in improving phosphorus availability and productivity (40). In current study, 17.5 % of isolated rhizobial strains showed significant phosphate solubilization capacity, with the strain CRB-55 having the highest solubilization index (3.08). Researchers found that around 41.66 % of isolates had phosphate solubilization capacity in their study, with solubilization index ranging from 2.15 to 3.33 (41). Another study observed that 34.4 % of the 61 rhizobial strains were able to solubilize phosphorus (42). These strains improve phosphorus availability in chickpea cultivation, lowering the need for chemical fertilizers.

Indole acetic acid, a naturally occurring auxin produced by microorganisms, promotes root growth, increases surface area and improves plant nutrient absorption (43). In present experiment, 52.5 % of isolates were able to produce IAA. This can be compared to a previous study, where 13 out of 17 chickpea rhizobial strains were capable of producing IAA (44). In another investigation, only 27 out of 118 isolates showed a pink or pink-red color with Salkowski's reagent, which indicates a positive result for IAA production based on the qualitative determination (45).

In nodulation test under *in vitro* conditions, maximum strains successfully formed symbiotic relationships with chickpea plants. This symbiosis is essential for nitrogen fixation and enhancing crop yield. The fact that eight strains could not induce nodulation might be attributed to various factors such as strain specificity, proper root development and growth chamber environments or they may be different bacteria but enter in the nodules within the cracks produced by rhizobia. These results align with a previous study, who found that the majority of isolates formed nodules on chickpea roots and some could not nodules (33). According to a separate study, 42 out of 53 rhizobial isolates were able to form nodules on chickpea roots (46). Nodulation-positive plants produce higher dry matter percentage than nodulation-negative or uninoculated plants because nodulation-positive plants fix nitrogen through nodules from atmosphere and produce more biomass. Besides, nitrogen concentration of inoculated plants was also higher than control in aseptic conditions. legume-rhizobia symbiosis meets approximately 45 % of agricultural nitrogen requirements and improves ecological resilience under a variety of environmental conditions (47).

The *nifH* gene is the most used biomarker in studies of the ecology and evolution of nitrogen-fixing bacteria. In this experiment, the *nifH* gene was successfully detected in all the nodulation-positive strains, which aligns with the prior study, who amplified the *nifH* gene with band sizes of 360-400 bp, affirming the presence of nitrogen-fixing genes in those bacterial strains (40). Other researchers also detected the *nifH* gene, as evidenced by the presence of DNA fragments of approximately 300 bp in gel electrophoresis, indicating that these bacterial isolates possess the genetic potential for

nitrogen fixation (45). This finding suggested that all nodule formation strains were positive for nitrogen fixation, an important character for rhizobia.

ERIC sequences are commonly found in the genome of Gram-negative soil bacteria. In ERIC-PCR, the amplified ERIC-like elements are useful for fingerprinting genera, species and strains of bacteria. This method is considered an effective tool in bacterial taxonomy and can aid in determining phylogenetic relationships (27, 31). In this study, fingerprint patterns helped to distinguish between different isolates and revealed diversity among the strains of different regions. Researchers observed a significant level of genetic diversity and variation in both fast and slow-growing peanut nodulating rhizobial strains using ERIC-PCR (48). Similarly, in another experiment, the genetic diversity of 50 pea rhizobial isolates was examined based on their ERIC profiles, revealing 7 to 10 distinct band patterns (49). In additional research ERIC-PCR analysis of 22 bacteria from *G. monspessulana* nodules showed 2-15 bands, which revealed high heterogeneity and an abundance of repetitive sequences (50). An investigation conducted with chickpea rhizobia reported that ERIC-PCR was the most effective fingerprinting method for determining genomic diversity among *Rhizobium leguminosarum* subsp. *ciceri* strains (18).

Conclusion

Rhizobia are vital microbes for ecosystems because of their ability to fix nitrogen in symbiosis with legume plants. The decline in chickpea production in Bangladesh could be due to inadequate native soil rhizobial populations and their ineffective nitrogen fixation along with other factors. Inoculating chickpea plants with effective nitrogen-fixing and growth-promoting rhizobia could enhance various growth and yield parameters and nutrient uptake. This study demonstrated that bacteria isolated from the root nodules of *C. arietinum* shows considerable genetic and phenotypic diversity. The phenotypic analysis helped identify strains capable of growing under various stress conditions. The presence of the *nifH* gene confirmed that these rhizobial isolates can successfully establish symbiosis with chickpeas. Based on ERIC-PCR fingerprinting, strains appear to be substantially diverse among the nodulating rhizobia, indicating a range of effective indigenous strains. Some of these highly effective strains could be used to produce inoculants, offering an alternative to costly chemical fertilizers. Further research is needed to assess the symbiotic potential of these microsymbionts fully at field conditions and their species declination.

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Authors' contributions

TSH, MHR and MAH[1] conceived the study and developed the methodology. NJM, AUR and KK carried out the experimentation and formal analysis. NJM and AUR performed the data analysis. NJM and TSH prepared the original draft. NJM edited the tables and figures. NJM and MHR contributed to writing, reviewing and editing. MHR and TSH supervised the work. MHR and MAH[2] administered the project. All authors read and approved the final version of the manuscript. (MAH[1] stands for M Anamul Hoque and MAH[2] stands for Mohammad Anwar Hossain)

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues : None

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