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Appraising Endophyte–Plant Symbiosis for Improved Growth, Nodulation, Nitrogen Fixation and Abiotic Stress Tolerance: An Experimental Investigation with Chickpea (*Cicer arietinum* L.)

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Abstract: Chickpea is an important leguminous crop that improves soil fertility through atmospheric nitrogen fixation with the help of rhizobia present in nodules. Non-rhizobia endophytes are also capable of inducing nodulation and nitrogen fixation in leguminous crops. The aim of the current study was to isolate, characterize and identify the non-rhizobia endophytic bacterial strains from root nodules of chickpea. For this purpose, more than one hundred isolates were isolated from chickpea root nodules under aseptic conditions and were confirmed as endophytes through re-isolating them from root nodules of chickpea after their inoculation. Nineteen confirmed endophytic bacterial strains revealed significant production of indole acetic acid (IAA) both in presence and absence of L-tryptophan and showed their ability to grow under salt, pH and heavy metal stresses. These strains were evaluated for in vitro plant growth promoting (PGP) traits and results revealed that seven strains showed solubilization of P and colloidal chitin along with possessing catalase, oxidase, urease and chitinase activities. Seven P-solubilizing strains were further evaluated in a jar trial to explore their potential for promoting plant growth and induction of nodulation in chickpea roots. Two endophytic strains identified as *Paenibacillus polymyxa* ANM59 and *Paenibacillus* sp. ANM76 through partial sequencing of the 16S rRNA gene showed the maximum potential during in vitro PGP activities and improved plant growth and nodulation in chickpea under the jar trial. Use of these endophytic strains as a potential biofertilizer can help to reduce the dependence on chemical fertilizers while improving crop growth and soil health simultaneously.

Keywords: chickpea; *Paenibacillus* spp.; nodulation; nitrogen fixation; stress tolerance

1. Introduction

Chickpea (*Cicer arietinum* L.) is an important legume crop and is consumed all over the world as a source of protein, carbohydrate, fiber, oil, ash, minerals, vitamins, amino acids and unsaturated fatty acids [1]. Being a leguminous crop, chickpea forms a symbiotic relationship with rhizobia that support biological nitrogen fixation (BNF) in root nodules. Symbiotic N_2 fixation facilitates larger proportions of nitrogen (N) for food production and agricultural sustainability [2]. Leguminous plants in association with a diverse variety of bacteria can reduce atmospheric N_2 into a plant available form and as a result improve soil fertility and plant growth [3]. For decades, rhizobia were thought to be the only nitrogen-fixing inhabitants of legume nodules. However, non-rhizobial bacteria were detected within legume nodules revealing the existence of a phytomicrobiota where the interaction among the individuals is complex and affects the behaviour and fitness of the host plant [4]. These non-rhizobial bacteria are involved in BNF through inducing nitrogen fixing nodules on roots of host legume [5]. They benefit legume host by improving plant growth through producing phytohormones, fixing atmospheric N_2, and solubilizing mineral nutrients [6]. In the past, non-rhizobial bacteria remained largely ignored due to lack of knowledge on diversity of non-rhizobial bacteria which co-exist with rhizobia and involved in N_2 fixation [5]. Bacterial endophytes may took advantage over rhizobacteria as endophytes have more opportunity to be in contact with plant cells and exert direct beneficial effect on host plant [7,8] probably due to aggressive colonization patterns.

Plants are able to shape their rhizospheric and endophytic microbiome and can maintain environmental stress resistant bacterial communities having specific beneficial characteristics [9,10]. Bacterial endophytes have been found almost in every analysed plant species while, endophytic free plants are less able to cope with environmental stress conditions [11]. Previous reports described the diversity of bacterial endophytes in various plant species with agricultural interests. Diversity of endophytes found are comprised of various genera viz. *Acinetobacter*, *Agromyces*, *Azoarus*, *Bacillus*, *Brevibacillus*, *Brevundimonas*, *Burkholderia*, *Comamonas*, *Corynebacterium*, *Delftia*, *Dietzia*, *Enterobacter*, *Frigoribacterium*, *Kocuria*, *Lysinibacillus*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Serria*, *Sphingobacterium*, *Sphingomonas*, *Sporosarcina*, *Staphylococcus*, *Stenotrophomonas*, *Xanthomonas*, etc. [7,12–22]. All of these described bacterial endophytic genera are also common inhabitants of the rhizosphere [7,8]. Once symbiosis takes place, endophytes promote plant growth through the expression of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can sequester and cleave the ethylene precursor ACC to α-ketobutyrate and ammonia (NH_3) and decreases ethylene production in host plants [23,24]. They can alter the plant physiology including regulation in osmotic pressure, changes in stomatal responses, increase in root morphology and improved N accumulation and metabolism and uptake of minerals [7,23–25]. Bacterial endophytes can produce phytohormones as metabolites such indole-3-acetic acid (IAA), gibberellins and cytokinins [26]. These phytohormones are natural substances manufactured by plants to promote its growth and development however their supplementation through bacterial sources can be safeguarded from environmental stimuli [27]. Among phytohormones, IAA plays a vital role in several plant activities such as formation, initiation and development of plant leaf, root and fruit, and is involved in phototropism and geotropism, etc. It plays an important role in enhancement of root length, branches, hair and root lateral growth that increase uptake of nutrients from surrounding environments [28]. Its production is widespread among various class of endophytic bacterial genus such as *Acinetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas.* These endophytic bacterial genera were reported to produce IAA through indole-3-pyruvic acid, indole-3-acetamide and indole-3-acetonitrile biosynthesis pathways, which are part of tryptophan-dependent and -independent pathways [29]. The application of IAA producing endophytic strains viz. *Bacillus subtilis*, *Enterobacter ludwigii*, *Klebsiella* sp. Sal 1, and *Pseudomonas fluorescens* in onion, rice, tomato and radish crops has shown significant increase in plant growth and yield [30,31]. Bacterial endophytes are involved in biocontrol through producing enzymes (e.g., hydrolases, chitinases, laminarinases, glucanases etc.) as reported by Chernin and Chet [32]. In addition, endophytes have been reported to induce systemic resistance (ISR)-based plant growth promotion in host plants once exposed to environmental stresses [33,34]. Much of the research hitherto

has concentrated on rhizobia–legume interactions, whereas there is very limited work on exploring the role of non-rhizobial endophyte–legume symbiosis. Therefore, there is a need to investigate the role of non-rhizobial endophytes in improving crop growth, nodulation and nitrogen fixing ability of legumes. In addition, no information is available regarding abiotic stress tolerance capacity of non-rhizobial endophytes which in the present study constitutes the novelty.

Therefore, keeping in view the above facts, the current study was conducted for isolation of endophytic bacteria from chickpea nodules, the selection and identification of strains with better PGP abilities and analysis of the effect of these strains on chickpea plants compared with uninoculated control. The current study accomplished the potential utilization of PGP non-rhizobial endophytic bacteria (*Paenibacillus polymyxa* ANM59 and *Paenibacillus* sp. ANM76) to improve chickpea growth, nodulation and nitrogen fixation. Endophytic bacterial strains in this study were first isolated from chickpea nodules, screened for plant growth promotion and various types of stresses and identified as *Paenibacillus* spp. through 16S rRNA gene sequencing afterwards.

2. Materials and Methods

2.1. Isolation of Endophytic Strains

Plant samples along with roots were collected from chickpea grown in experimental fields of University College of Agriculture and Environmental Sciences (UCA and ES), the Islamia University of Bahawalpur (IUB), Pakistan. For isolation of endophytes, rhizosphere soil from chickpea roots was removed by washing with tap water. The healthy, unbroken and pink nodules were separated from roots by using sterile razor blade and placed in petri plates. These nodules were surface-disinfected with 95% ethanol for twenty seconds followed by immersing in 0.2% mercuric chloride ($HgCl_2$) solution for three minutes and five times washing with sterile-distilled water [35]. After that, nodules were aseptically crushed with a glass rod in a test tube containing distilled water, and a final suspension of 1 mL was prepared. The obtained suspension was spread on yeast extract mannitol agar (YEMA) and incubated at 28 ± 1 °C for bacterial growth. Morphologically distinct single colonies were purified and preserved in glycerol stock (50%) at −20 °C until further use.

2.2. Authentication of Endophytic Nature of Strains

To confirm isolates as true nodule endophytes, surface-disinfected chickpea seeds were inoculated with respective strains and aseptically sown in plastic jars filled with sand. Jars were kept in a growth room and watered daily with sterilized nitrogen free Hoagland solution. After 40 days of incubation, plants were harvested and analysed for induction of nodules in chickpea and presence of bacterial strains. Nineteen isolates were termed as true endophytic bacterial strains after detecting them in nodules of chickpea and promoters of nodulation. These strains were screened in vitro by Gram staining. The standard method of Vincent [36] was adopted for Gram staining. Various morphological features of endophytic strains such as colour, transparency, colony and cell shape, colony border, mucus production and colony diameter were examined on Congo red amended YEMA after an incubation period of 48 h at 28 ± 1 °C [36].

2.3. In Vitro Screening of Endophytes for their PGP Characteristics

Nineteen confirmed endophytic strains were selected for evaluation of their PGP characteristics. P-solubilization ability of strains was accessed qualitatively on Pikovskaya agar medium [37]. Appearance of a halo zone around the colonies was considered P-solubilization positive and the halo zone was measured to calculate P-solubilization index (PSI) through following Formula (1) as reported by Vazquez et al. [38] Premono et al. [39] and Singh et al. [40]. P-solubilization efficiency (PSE) was calculated using following Formula (2).

$$Solubilization\ Index\ (SI) = \frac{Colony\ diameter + Halo\ zone\ diameter}{Colony\ diameter}, \quad (1)$$

$$Solubilization\ Efficiency\ (SE) = \frac{Halo\ zone\ diameter}{Colony\ diameter} \times 100. \quad (2)$$

Qualitative zinc (Zn) solubilization test was performed on zinc oxide amended Tris-minimal salt agar (Tris-MSA) and halo zones around the colonies were observed [41,42]. For determination of ammonia production, strains were inoculated in 10 mL peptone broth in test tubes and incubated for 30 ± 1 °C at 72 h. After incubation, Nessler's reagent (1 mL) was added and a change in colour was observed [43]. For determination of acid production by strains, YEMA medium was amended with bromothymol blue and bacterial cells were inoculated. After 48 h of incubation at 28 ± 1 °C, change in colour from blue to yellow around bacterial cells was reported positive for acid producing endophytes [36].

The method of Ali and Hasnain [44] was modified to access the auxins production by strains. Dworkin and Foster (DF) minimal salt broth was amended both with and without 1.0 g L^{-1} L-tryptophan. Strains having 0.5 optical density measured at 600 nm (OD_{600}) by spectrophotometer (Agilent Technologies, mulgrave victoria, Melb, Australia) were inoculated and incubated at 28 ± 1 °C for 48 h. After incubation, cultures were centrifuged (10,000 rev/min for 10 min) and 1 mL Salkowski reagent was added in supernatant [45]. The absorbance of both with and without L-tryptophan amended media was observed through spectrophotometer at 600 nm and auxins production was calculated through drawing a standard curve for comparison by using indole acetic acid (Sigma-Aldrich, Steinheim, Germany) as a standard [42].

Selected strains were further screened for enzymatic activities, production of ammonia, triple sugar iron (TSI) test (related to glucose, sucrose, lactose) and hydrolysis of starch. Standard methods of Cappuccino and Welsh [43] were used for enzymatic activities viz. catalase, oxidase and urease and the TSI test. For chitinase activity, strains were grown on Dworkin and Foster (DF) minimal salt media modified with colloidal chitin (2% w/v). Formation of halo zones around the colonies was considered positive for chitinase activity [46]. Halo zone diameter of colloidal chitin solubilization was estimated and chitin solubilization efficiency (CSE) and chitin solubilization index (CSI) were calculated using Formulae 1,2. Starch agar was spot inoculated with overnight grown bacterial cells and incubated at 28 ± 1 °C. After 48 h, 0.1% iodine solution was spread in Petri plates and the result of starch hydrolysis was observed by adopting the method of Oliveira et al. [47]. P-solubilizing strains were selected for root colonization assay and the method of Mendis et al. [48] was followed to count bacterial colony forming units (CFUs) through serial dilution and pour plate techniques.

2.4. In Vitro Screening for Stress Tolerance

Nineteen selected endophytic strains were screened under heavy metals, temperature, pH and salt stress. Among heavy metals, mercury (Hg), Zn, copper (Cu) and chromium (Cr) tolerance was evaluated. The YEMA media was amended by adding $HgCl_2$ (20 μg mL^{-1}), $ZnSO_4$ (10 μg mL^{-1}), $CuSO_4$ (10 μg mL^{-1}), and $K_2Cr_2O_7$ (25 μg mL^{-1}) separately and autoclaved. Freshly grown strains were inoculated in all heavy metals amended media and incubated at 28 ± 1 °C for 72 h. After incubation, bacterial growth was observed as described by Carrasco et al. [49]. For temperature tolerance, strains were grown on YEMA media and incubated at 4, 28, 36 and 55 °C for 72 h to observe growth. Strains were also checked for their ability to grow under various pH levels. pH of yeast extract mannitol broth (YEMB) was maintained at 4.0, 5.0, 6.8, 8.0 and 9.0 by using 1 M HCl. Bacterial culture having 0.5 OD was inoculated and incubated at 28 ± 1 °C for 24 h. After incubation, OD was measured at 600 nm by spectrophotometer. Ability of strains to grow under salt stress was determined by growing strains in YEMB having salinity levels viz. 1.6 (normal), 4, 8 and 12 dS m^{-1} maintained by using NaCl. After 72 h of incubation at 28 ± 1 °C, optical density at 540 nm (OD_{540}) was measured through spectrophotometer.

2.5. Screening of Endophytes for Growth Promotion of Chickpea (Jar Trial)

Seven P-solubilizing endophytic strains were evaluated for their ability to promote growth of chickpea under natural climatic conditions. The YEMB media was cultured with overnight grown bacterial strains and incubated at 28 ± 1 °C for 48 h. Seeds of chickpea were surface-disinfected by dipping in 95% ethanol

for twenty seconds then 0.2% $HgCl_2$ solution for three minutes and rinsed five times with sterilized distilled water [35]. Three surface-disinfected seeds were inoculated with each of selected bacterial culture by dipping in the respective broth for 30 min. In the case of control, surface-disinfected seeds were treated with sterilized broth without bacteria. Inoculated seeds were sown in glass jars filled with sterilized sand. Modified, N-free sterilized Hoagland solution was watered to fulfil nutrients needs of seedlings. Jars were arranged in completely randomized design (CRD) in triplicate and placed in a wire house of the Department of Soil Science, IUB under natural climatic conditions. After two months of sowing, data regarding nodule formation and growth promotion were recorded.

2.6. Identification of Selected Endophytic Strains

Endophytic strains ANM59 and ANM76 were grown in YEMB media at 28 ± 1 °C overnight and slants and glycerol stocks were prepared. Commercial service of Macrogen, Seoul, Korea [50] was used for sequencing of bacterial strains. The 16S rRNA partial gene sequence through using universal primer 785F 5′ (GGA TTA GAT ACC CTG GTA) 3′ 907R 5′ (CCG TCA ATT CMT TTR AGT TT) 3′ was carried out. The resulting partial sequence of 16S rRNA were blasted using MEGABLAST on National Center for Biotechnology Information (NCBI) servers. Sequences of closely related fifteen published strains were retrieved from the database. A neighbour-joining phylogenetic tree was constructed using MEGA 7.0.14 software as described by Kumar et al. [51]. The tree topology of the phylogenetic tree was assessed by bootstrap resampling method of Tamura et al. [52] with 500 replications. The sequences were submitted to the NCBI gene bank to get accession numbers of the strains.

2.7. Statistical Assessment

Data were statistically analysed in software Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) and treatment means were compared using least significant difference (LSD) test at $p \leq 0.05$ [53].

3. Results

3.1. Morphological Characteristics of Endophytes

More than one hundred non-rhizobial endophytic isolates were isolated from nodules of chickpea and nineteen of these were authenticated as true endophytes by observing their presence in root nodules and induction of nodulation in chickpea. These endophytic isolates were selected and analysed for their colony morphological characteristics. All the colonies appeared as milky white with an entire margin. The majority of isolates showed mucoid growth and their colonies were translucent, round and small (Table S1).

3.2. Endophytic Strains Possess PGP Characteristics

All the tested strains showed IAA production both in the presence as well as absence of L-tryptophan (Table 1). Maximum IAA production with and without L-tryptophan was reported by the strain ANM79 followed by ANM78 and ANM76, while, ANM58 and ANM75 produced minimum IAA in presence as well as absence of L-tryptophan. Seven out of 19 strains performed solubilization of tri-calcium phosphate by showing halo zones around colonies. The diameter of P halo zones and their solubilization efficiency and solubilization index are given in Table 1, Figure 1. The strain ANM76 followed by ANM63 showed the maximum solubilization efficiency and solubilization index whereas, ANM73, ANM59 and ANM16 gave minimum values of P-solubilization efficiency and solubilization index. Eleven out of 19 strains were chitinase positive (Table 2) and showed halo zone formation around the colonies. Their colloidal chitin solubilization zone with solubilization efficiency and solubilization index are given in Table 1.

Table 1. Plant growth promoting characteristics in terms of production of indole-3-acetic acid (IAA), phosphate solubilization, colloidal chitin solubilization and root colonization ability by tested endophytic strains from chickpea.

Strains	Production of IAA (mg L^{-1}) [a]		Phosphate Solubilization [b]			Colloidal Chitin Solubilization [c]			Root Colonization [d]
	Without L-Tryptophan	With L-Tryptophan	P Halo Zone (mm)	PSE (%)	PSI	Colloidal Halo Zone (mm)	CSE (%)	CSI	
ANM9	4.78 ± 0.003	6.79 ± 0.005	ND [e]	ND	ND	9.67 ± 0.666	145± 3.316	2.45 ± 0.019	NS [f]
ANM12	3.67 ± 0.002	4.41 ± 0.003	ND	ND	ND	ND	ND	ND	NS
ANM16	4.21 ± 0.004	6.00 ± 0.001	18.3 ± 0.333	106 ± 2.976	2.06 ± 0.029	ND	ND	ND	4.23×10^5
ANM26	4.02 ± 0.002	4.91 ± 0.007	ND	ND	ND	ND	ND	ND	NS
ANM39	4.13 ± 0.005	4.98 ± 0.008	32.0 ± 0.523	128 ± 5.456	2.28 ± 0.055	16.66 ± 0.511	314 ± 1.852	4.14 ± 0.018	5.06×10^5
ANM58	3.56 ± 0.006	4.24 ± 0.002	ND	ND	ND	ND	ND	ND	NS
ANM59	4.27 ± 0.006	5.26 ± 0.001	21.0 ± 0.333	105 ± 2.253	2.05 ± 0.036	ND	ND	ND	4.44×10^5
ANM63	4.44 ± 0.003	6.39 ± 0.008	29.67 ± 0.856	203 ± 4.556	3.03 ± 0.033	13.33 ± 0.432	211 ± 1.201	3.11 ± 0.042	4.78×10^5
ANM66	3.78 ± 0.008	4.57 ± 0.011	ND	ND	ND	ND	ND	ND	NS
ANM69	4.38 ± 0.007	5.19 ± 0.012	33.00 ± 0.533	165 ± 8.335	2.65 ± 0.014	9.67 ± 0.333	147 ± 1.915	2.47 ± 0.072	4.12×10^5
ANM73	4.29 ± 0.010	5.15 ± 0.008	20.30 ± 0.765	105 ± 9.882	2.05 ± 0.018	14.33 ± 0.778	172 ± 4.167	2.72 ± 0.256	5.17×10^5
ANM75	3.57 ± 0.006	4.24 ± 0.002	ND	ND	ND	ND	ND	ND	NS
ANM76	5.24 ± 0.003	7.99 ± 0.004	38.33 ± 0.344	311± 3.316	4.11 ± 0.005	8.67 ± 0.667	144 ± 6.233	2.44 ± 0.0329	5.45×10^5
ANM77	3.68 ± 0.009	4.36 ± 0.009	ND	ND	ND	14.00 ± 0.856	193 ± 2.231	2.93 ± 0.015	NS
ANM78	5.31 ± 0.002	8.13 ± 0.005	ND	ND	ND	17.33 ± 0.333	308 ± 7.325	4.08 ± 0.045	NS
ANM79	5.33 ± 0.008	8.18 ± 0.001	ND	ND	ND	7.33 ± 0.667	122 ± 1.742	2.22 ± 0.082	NS
ANM81	3.97 ± 0.003	4.49 ± 0.007	ND	ND	ND	12.33 ± 0.556	186 ± 2.445	2.86 ± 0.051	NS
ANM82	5.22 ± 0.008	7.91 ± 0.005	ND	ND	ND	12.67 ± 0.856	126 ± 4.667	2.26 ± 0.027	NS
ANM100	3.87 ± 0.009	4.58 ± 0.006	ND	ND	ND	ND	ND	ND	NS

[a] Indole acetic acid (IAA) was estimated by observing optical density at 600 nm both in presence and absence of L-tryptophan; values are mean of three replicate ± standard error; [b] diameter of halo zones developed by endophytic strains was observed after 48 h of incubations and solubilisation efficiency and index were calculated; P = phosphorus, PSE = phosphorus solubilization efficiency, PSI = phosphorus solubilization index; means are average of three replications ± standard error; [c] yeast extract mannitol agar amended with colloidal chitin was centrally inoculated with respective endophytic strains and clearing zone was observed; CSE = chitin solubilization efficiency, CSI = chitin solubilization index; values are mean of three replicate ± standard error; [d] root colonization ability of endophytic strains was observed by colony forming unit (CFU) method from root of inoculated chickpea grown for one week; [e] ND stands for not detected; [f] NS stands for not studied.

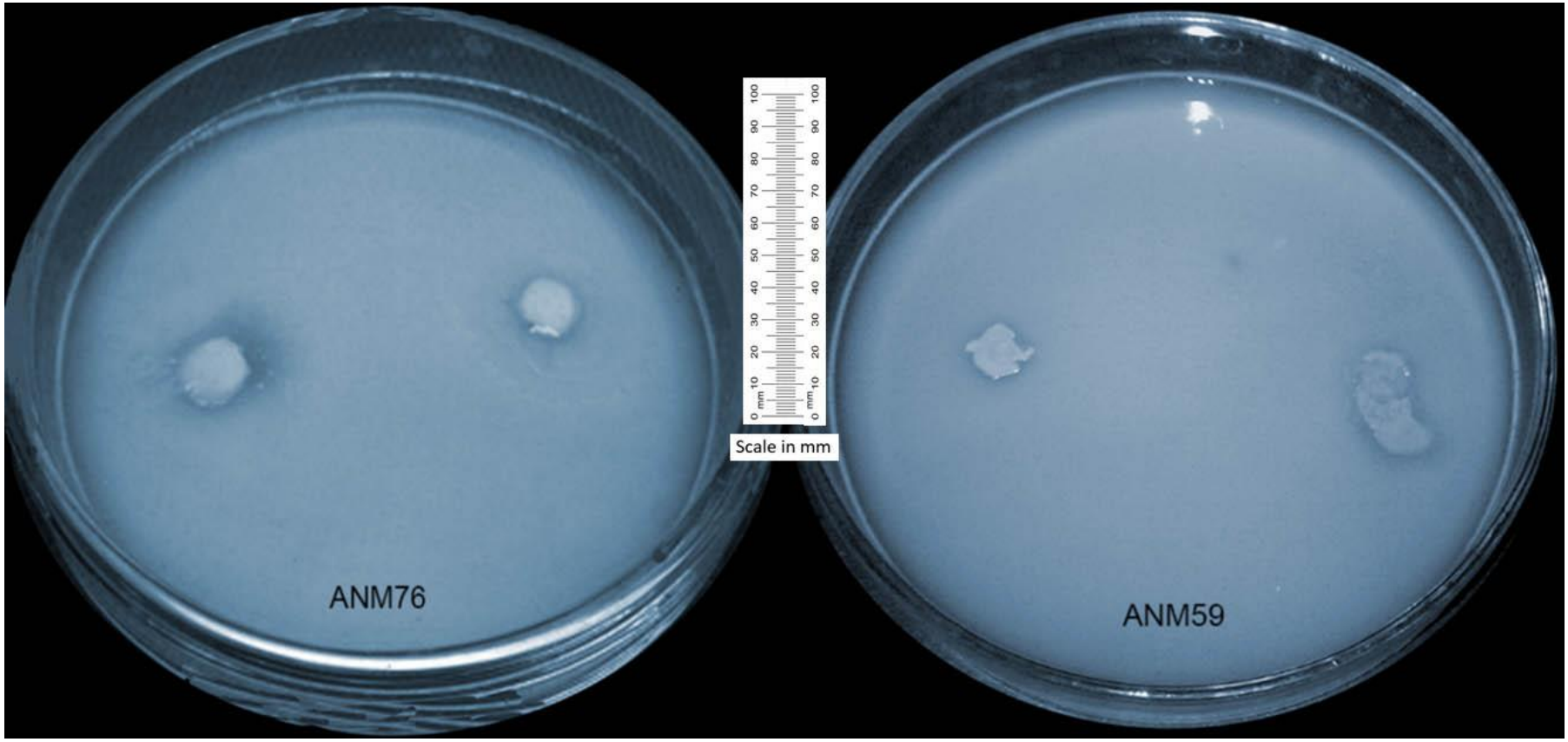

Figure 1. In vitro solubilization of inorganic phosphorus by endophytic strains ANM59 and ANM76 after 48 h of incubation at 28 ± 1 ºC. Halo zone around bacterial colonies indicated solubilization of phosphorus. Halo zone and bacterial colonies zones were calibrated through millimetre (mm) scale.

Table 2. Plant growth promoting characteristics of endophytic strains from chickpea.

Strains [a]	Solubilize Zn	Catalase Activity	Oxidase Activity	Urease Activity	Chitinase Activity	Starch Hydrolysis	NH_3 Prod.	Acid Prod.	Glucose Utilization	Sucrose Utilization	Produce CO_2	Produce H_2S	Gram Staining
ANM9	− [b]	+ [c]	+	+	+	+	+	+	+	+	−	−	−ve [d]
ANM12	−	+	−	−	−	+	−	−	+	+	−	−	−ve
ANM16	−	+	−	+	−	+	+	+	+	+	−	−	−ve
ANM26	−	+	+	+	−	+	+	+	+	+	−	−	−ve
ANM39	−	+	−	−	+	+	+	+	+	+	−	−	−ve
ANM58	−	+	+	+	−	+	+	−	+	+	−	−	−ve
ANM59	−	+	+	+	−	−	+	+	+	+	−	−	−ve
ANM63	−	+	−	−	+	+	+	+	−	−	−	−	−ve
ANM66	−	+	+	−	−	−	−	+	+	+	−	−	−ve
ANM69	−	+	−	−	+	−	+	+	+	+	−	−	−ve
ANM73	−	+	−	−	+	+	+	+	+	+	−	−	−ve
ANM75	−	+	+	−	−	+	+	+	+	+	−	−	−ve
ANM76	−	+	−	−	+	+	+	+	+	+	−	−	−ve
ANM77	−	+	−	−	+	+	+	+	−	−	−	−	−ve
ANM78	−	+	−	−	+	+	+	+	+	−	−	−	−ve
ANM79	−	+	−	−	+	+	+	+	+	+	−	−	−ve
ANM81	−	+	−	−	+	+	+	−	−	−	−	−	−ve
ANM82	−	+	−	−	+	+	+	+	−	−	−	−	−ve
ANM100	−	+	−	−	−	+	−	+	+	+	−	−	−ve

[a] Authenticated endophytic strains were screened for plant growth promoting characteristics and in vitro tests were repeated twice for confirmation of results with three replicates each time; [b] symbol − represents the absence of the traits; [c] symbol + represents the presence of the traits; [d] symbol −ve represents the Gram negative bacterial strains.

The strain ANM76 illustrated the highest P-solubilization zone, solubilization efficiency and solubilization index. The P-solubilizing strains were evaluated for root colonization and results indicated that all of these strains possessed strong root colonization ability. Maximum root colonization of 5.45×10^5 and 5.17×10^5 were shown by strains ANM76 and ANM73 (Table 1). Further, in vitro plant growth promoting characteristics of selected endophytic strains are described in Table 2. None of the tested endophytic strains were able to solubilize zinc oxide. All tested strains were catalase positive, production of CO_2 and H_2S negative and were Gram negative (–ve) bacteria. The majority of strains were able to hydrolyse starch, produced NH_3 and acid, and were able to utilize glucose and sucrose. Oxidase activity was performed by six strains out of 19 while five strains were urease positive.

3.3. Endophytic Strains were Salinity, pH and Heavy Metals Stress Tolerant

Nineteen selected strains were screened through various levels of salinity, pH, heavy metals and their results are given in Table 3. To assess salt tolerance by endophytic strains, these were grown in YEMB medium with 1% glucose as the sole carbon source at four salinity levels viz. 1.6, 4.0, 8.0 and 12.0 dS m^{-1} for determination of OD_{540}. Strains growth was decreased with increasing salinity and their tolerance ability was variable at highest salinity (12.0 dS m^{-1}). At this salinity level, the maximum OD was found in the case of ANM76 followed by ANM100 (Table 3). Yeast extract mannitol broth adjusted to starting pH values of 4.0, 5.0, 6.8, 9.0 and 10.0 was used to evaluate the effect of pH on growth of endophytic strains (their growth profiles are summarized in Table 3). All the strains showed optimum growth profiles at pH 6.8 but showed a drastic drop in growth with decrease in pH up to 4.0 as well as increase in pH up to 10.0. However, strains were still able to show their growth to some extent at acidic pH 4.0 as well as alkaline pH 10.0. To assess heavy metals tolerance, endophytic strains were grown on heavy metal (viz. Hg, Zn, Cu and Cr) amended YEMA plates and incubated for 48 h to observe their growth (growth is summarized in Table 3). Variable thirteen out of 19 strains were capable of growing on Hg and Zn amended media. Nine strains showed growth in Cu amended media while in Cr amended media only six strains showed their ability to grow.

3.4. Endophytic Strains Promoted the Growth and Nodulation in Chickpea (Jar Trial)

Seven P-solubilizing endophytic strains were selected to evaluate their ability to promote the growth and nodulation in chickpea by conducting a jar trial under natural environmental conditions and results are depicted in Table 4. Inoculation with endophytic strains significantly promoted the shoot and root growth (Figures 2 and 3), biomass and nodulation in chickpea. Increase in these attributes was non-significant between tested strains however, significantly different from un-inoculated control. Maximum shoot and root length with 87% and 100% increase was observed due to inoculation with ANM76 as compared to un-inoculated control. This strain also showed the highest shoot and root fresh biomass with 77% and 81% increase which was significantly higher than un-inoculated control. Highest shoot and root dry biomass were also reported by strain ANM76 by showing 84% and 85% increase over un-inoculated control. Inoculation with endophytic strains significantly promoted the number of nodules as well as their fresh and dry weight over un-inoculated control (Table 4). No nodule formation was observed in the case of un-inoculated control while inoculation with endophytic strains promoted nodulation in chickpea. Maximum number of nodules (7.3), nodules fresh weight (0.15 g) and nodule dry weight (0.10 g) of three chickpea plants in a jar were reported by strain ANM76 which was non-significant to other strains however significantly better compared to un-inoculated control.

Table 3. Growth of endophytic strains under various salinity and pH levels and heavy metals stress.

Strains	Salinity Tolerance [a]				pH [b]					Heavy Metals [c]			
	1.6 dS m^{-1} (Normal)	4.0 dS m^{-1}	8.0 dS m^{-1}	12.0 dS m^{-1}	4.0	5.0	6.8	9.0	10.0	Hg	Zn	Cu	Cr
ANM9	2.38 ± 0.013 [d]	2.15 ± 0.007	1.53 ± 0.009	1.46 ± 0.005	0.20 ± 0.005	0.29 ± 0.007	0.79 ± 0.001	0.21 ± 0.006	0.17 ± 0.003	++ [e]	++	− [g]	−
ANM12	2.01 ± 0.001	1.56 ± 0.002	1.58 ± 0.005	1.33 ± 0.007	0.21 ± 0.007	0.31 ± 0.004	0.69 ± 0.005	0.26 ± 0.005	0.20 ± 0.004	++	−	−	+
ANM16	1.95 ± 0.003	1.59 ± 0.005	1.84 ± 0.008	1.79 ± 0.010	0.37 ± 0.003	0.38 ± 0.005	0.78 ± 0.003	0.31 ± 0.008	0.18 ± 0.002	++	+ [f]	−	−
ANM26	1.51 ± 0.015	1.72 ± 0.006	1.69 ± 0.009	1.33 ± 0.005	0.22 ± 0.001	0.35 ± 0.015	0.66 ± 0.009	0.33 ± 0.009	0.21 ± 0.003	+	++	++	−
ANM39	1.84 ± 0.023	1.16 ± 0.002	1.23 ± 0.011	1.21 ± 0.006	0.16 ± 0.012	0.29 ± 0.003	0.65 ± 0.005	0.27 ± 0.002	0.15 ± 0.006	−	++	+	−
ANM58	1.77 ± 0.017	0.59 ± 0.001	0.89 ± 0.005	0.54 ± 0.008	0.13 ± 0.007	0.33 ± 0.001	0.73 ± 0.008	0.22 ± 0.007	0.11 ± 0.006	−	+	−	++
ANM59	2.37 ± 0.011	2.08 ± 0.002	1.59 ± 0.006	1.48 ± 0.007	0.18 ± 0.005	0.28 ± 0.006	0.72 ± 0.003	0.27 ± 0.004	0.14 ± 0.004	+	++	++	−
ANM63	2.53 ± 0.005	1.75 ± 0.026	1.79 ± 0.004	1.61 ± 0.013	0.14 ± 0.004	0.27 ± 0.003	0.64 ± 0.006	0.25 ± 0.011	0.11 ± 0.003	++	−	++	−
ANM66	2.39 ± 0.004	1.43 ± 0.002	1.11 ± 0.005	1.43 ± 0.008	0.14 ± 0.006	0.21 ± 0.007	0.77 ± 0.013	0.20 ± 0.007	0.11 ± 0.002	−	++	−	++
ANM69	1.67 ± 0.006	1.42 ± 0.003	1.30 ± 0.007	1.32 ± 0.002	0.19 ± 0.004	0.21 ± 0.004	0.69 ± 0.002	0.23 ± 0.003	0.18 ± 0.005	++	++	−	−
ANM73	2.04 ± 0.002	1.77 ± 0.001	1.46 ± 0.004	1.16 ± 0.007	0.17 ± 0.003	0.33 ± 0.008	0.70 ± 0.001	0.30 ± 0.006	0.15 ± 0.002	+	−	++	−
ANM75	2.13 ± 0.006	1.91 ± 0.004	1.34 ± 0.001	1.17 ± 0.005	0.17 ± 0.008	0.24 ± 0.003	0.65 ± 0.002	0.23 ± 0.001	0.16 ± 0.013	−	++	+	−
ANM76	2.43 ± 0.002	1.92 ± 0.002	1.93 ± 0.007	1.94 ± 0.005	0.19 ± 0.009	0.35 ± 0.007	0.68 ± 0.004	0.29 ± 0.008	0.18 ± 0.001	++	−	−	−
ANM77	1.97 ± 0.006	1.68 ± 0.003	1.37 ± 0.009	1.15 ± 0.006	0.14 ± 0.014	0.28 ± 0.001	0.71 ± 0.007	0.24 ± 0.006	0.12 ± 0.009	++	+	−	−
ANM78	1.65 ± 0.020	1.33 ± 0.005	1.22 ± 0.008	1.11 ± 0.009	0.20 ± 0.001	0.26 ± 0.003	0.66 ± 0.002	0.23 ± 0.002	0.25 ± 0.001	+	++	−	+
ANM79	1.83 ± 0.012	1.86 ± 0.009	1.54 ± 0.006	1.33 ± 0.010	0.10 ± 0.003	0.17 ± 0.010	0.64 ± 0.008	0.17 ± 0.007	0.11 ± 0.009	−	−	++	+
ANM81	2.09 ± 0.015	1.53 ± 0.006	1.39 ± 0.004	1.21 ± 0.007	0.16 ± 0.007	0.20 ± 0.005	0.63 ± 0.011	0.18 ± 0.008	0.15 ± 0.003	−	+	+	−
ANM82	2.15 ± 0.016	1.85 ± 0.011	1.93 ± 0.008	1.44 ± 0.012	0.14 ± 0.001	0.20 ± 0.007	0.71 ± 0.003	0.19 ± 0.014	0.13 ± 0.008	++	−	++	−
ANM100	2.53 ± 0.002	2.38 ± 0.005	0.84 ± 0.005	1.90 ± 0.003	0.16 ± 0.005	0.28 ± 0.003	0.73 ± 0.007	0.24 ± 0.001	0.15 ± 0.006	++	++	−	+

[a] Endophytic strains were inoculated in yeast extract mannitol broth amended with various salinity levels. Optical density (OD) was observed at 540 nm after 72 h of incubation; [b] pH levels were maintained through 1 M HCl solution in yeast extract mannitol broth and after 24 h of incubation optical density was observed at 600 nm; [c] target heavy metal was added in yeast extract mannitol agar and incubated for 72 h to observed bacterial growth; [d] values represent the average ± standard error of three replicates; [e] symbol ++ represent vigorous growth; [f] symbol + represent presence of growth; [g] symbol − represent absence of growth.

Table 4. Endophytic strains promoted growth and nodulation in chickpea under the jar trial.

Endophytic Strains *	Shoot Length (cm)	Root Length (cm)	Shoot Fresh Biomass (g)	Root Fresh Biomass (g)	Shoot Dry Biomass (g)	Root Dry Biomass (g)	Number of Nodules	Nodules Fresh Weight (g)	Nodules Dry Weight (g)
Control	13.83 ± 1.763 d	15.80 ± 0.878 d	1.11 ± 0.058 d	0.86 ± 0.002 b	0.27 ± 0.012 f	0.20 ± 0.005 b	0.00 ± 0.000 d	0.00 ± 0.000 f	0.00 ± 0.000 f
ANM16	23.83 ± 1.333 ab	25.43 ± 0.240 bc	1.39 ± 0.058 cd	1.19 ± 0.028 ab	0.35 ± 0.023 e	0.25 ± 0.023 ab	5.33 ± 0.853 abc	0.07 ± 0.009 cd	0.04 ± 0.008 cd
ANM39	18.27 ± 1.790 bcd	29.60 ± 0.416 ab	1.71 ± 0.115 abc	1.40 ± 0.114 a	0.43 ± 0.012 bc	0.34 ± 0.017 a	6.33 ± 0.667 ab	0.12 ± 0.006 b	0.08 ± 0.003 b
ANM59	25.50 ± 1.857 a	21.13 ± 0.862 c	1.59 ± 0.153 bc	1.29 ± 0.115 ab	0.37 ± 0.015 de	0.29 ± 0.012 ab	5.67 ± 0.882 abc	0.08 ± 0.005 c	0.05 ± 0.005 c
ANM63	21.67 ± 1.301 abc	28.60 ± 0.954 ab	1.55 ± 0.109 c	1.37 ± 0.173 ab	0.38 ± 0.029 cde	0.31 ± 0.014 b	4.67 ± 0.899 bc	0.07 ± 0.003 d	0.03 ± 0.003 de
ANM69	22.20 ± 1.595 abc	23.13 ± 0.960 c	1.65 ± 0.079 abc	1.22 ± 0.058 ab	0.41 ± 0.005 cd	0.27 ± 0.017 ab	3.67 ± 0.333 c	0.05 ± 0.003 e	0.02 ± 0.005 e
ANM73	17.50 ± 1.153 cd	30.37 ± 0.829 a	1.87 ± 0.051 ab	1.47 ± 0.017 a	0.48 ± 0.024 ab	0.35 ± 0.029 a	7.00 ± 0.577 ab	0.14 ± 0.005 a	0.09 ± 0.004 ab
ANM76	25.87 ± 1.718 a	31.60 ± 0.896 a	1.97 ± 0.155 a	1.56 ± 0.141 a	0.50 ± 0.021 a	0.37 ± 0.108 a	7.33 ± 0.882 a	0.15 ± 0.005 a	0.10 ± 0.005 a
LSD ($p \leq 0.05$) **	5.763	4.340	0.3146	0.5097	0.060	0.130	2.450	0.020	0.020

* Tested endophytic strains were selected on the basis of phosphate solubilization and evaluated for growth promotion and nodule induction in chickpea; values are mean of three plants in a single jar in triplicate ± standard error; means sharing same letter do not differ significantly; ** LSD = least significant difference at 5% level of significance.

Figure 2. Comparison of shoot growth of chickpea inoculated with endophytic strains grown under the jar trial.

Figure 3. Comparison of growth of selected chickpea roots inoculated with endophytic strains grown under the jar trial.

3.5. Identification of Selected Strains through 16S rRNA

The two endophytic strains viz. ANM76 and ANM59 were selected on the basis of their potential for nodulation, to increase plant growth and difference in their colony morphology. Partial sequences of 16S rRNA coding genes were determined and submitted to NCBI with accession numbers KX788870 and KX788871 for strains ANM59 and ANM76, respectively. Nucleotide BLAST search of NCBI was used to find homologous sequences to identify the strain ANM59 as *P. polymyxa* and ANM76 as *Paenibacillus* sp. (Table 5). The phylogenetic tree of *Paenibacillus polymyxa* ANM59 and *Paenibacillus* sp. ANM76 are given in Figures 4 and 5, respectively.

Table 5. Identification of endophytic strains ANM59 and ANM76 from chickpea by 16S rRNA partial gene sequencing.

Endophytic Strains	Identified Species	Accession Number	Closely Related Organism	BP	Similarity
ANM59	*Paenibacillus polymyxa*	KX788870	*Paenibacillus polymyxa*	719 bp	99%
ANM76	*Paenibacillus* sp.	KX788871	*Paenibacillus* sp.	629 bp	86.9%

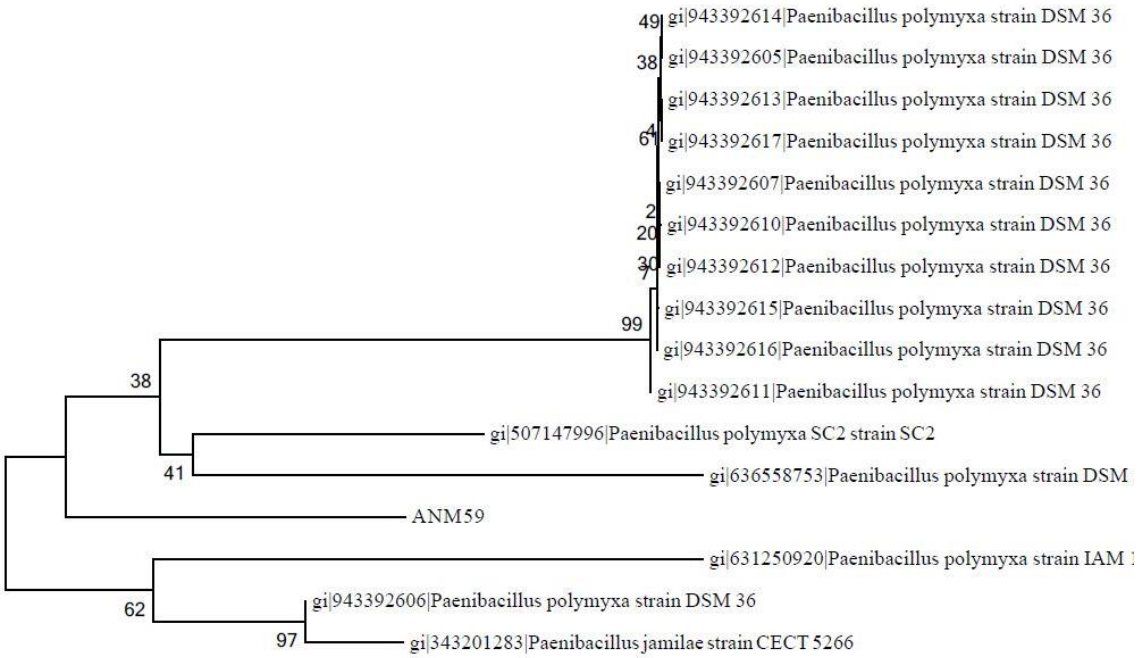

Figure 4. Neighbour-joining phylogenetic tree produced using multiple alignment of 16S rRNA gene sequence of *Paenibacillus polymyxa* ANM59 with those of other bacterial strains found in the GeneBank database.

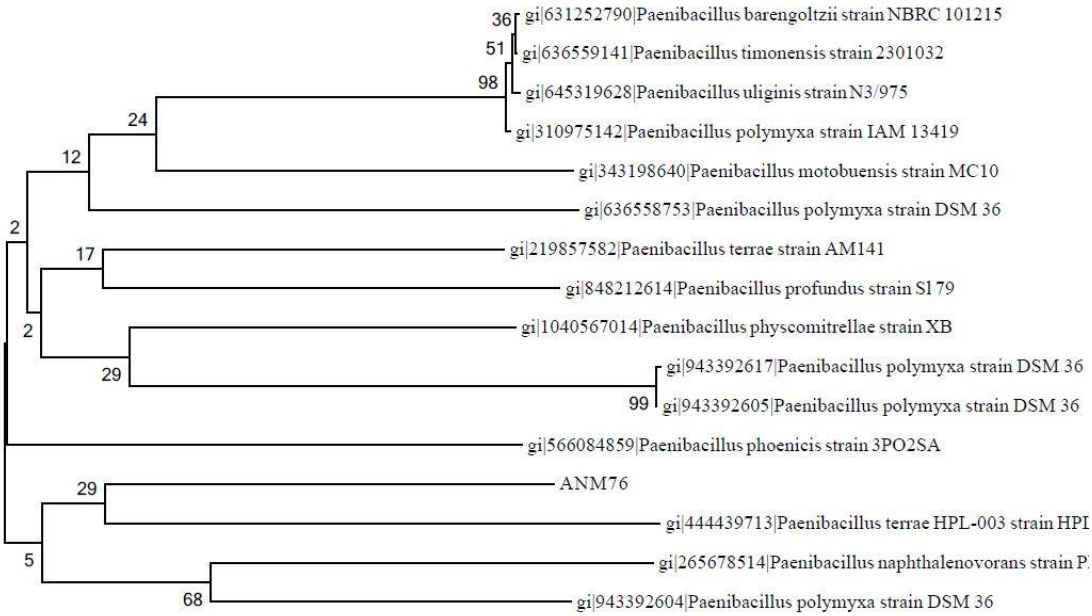

Figure 5. Neighbour-joining phylogenetic tree produced using multiple alignment of 16S rRNA gene sequence of *Paenibacillus* sp. ANM76 with those of other bacterial strains found in the GeneBank database.

4. Discussion

Legumes have a specific trait of nodule formation which is a supreme habitat for nitrogen fixing rhizobia as well as non-rhizobial endophytes. Many non-rhizobial endophytes from roots and nodules of legumes are involved in plant growth and nodulation by making a symbiotic relationship with the host [17]. It is identified that thirteen bacterial genera are able to nodulate different legumes which include ten genera from α-proteobacteria while three genera belong to β-proteobacteria [54,55]. Most of these bacteria in nodules originated from the rhizosphere and phyllosphere or were transmitted through seed. Mostly, endophytic bacteria reside in nodules as compared to roots which is due to

fact that nodules are much richer in nutrients. Isolation of both rhizobia as well as non-rhizobial endophytes from chickpea nodules need proper sterilization of nodules and every care taken to avoid surface contaminants [54]. In the current study, we isolated more than hundred non-rhizobial endophytic bacterial strains from the chickpea root nodules via the surface sterilization technique and authenticated them as endophytic in nature by re-isolation from chickpea root nodules after their inoculation under aseptic conditions. Similarly, Saini et al. [56] isolated endophytic *Bacillus subtilis* CNE215 and *Bacillus licheniformis* CRE1 from root or nodules of chickpea and authenticated them as true endophytic nature by re-isolating from chickpea nodules under sterilized conditions. In the current study, true endophytic *Paenibacillus* strains were characterized for PGP activities and selected strains able to promote chickpea growth and inducing nodulation were identified as *Paenibacillus polymyxa* ANM59 and *Paenibacillus* sp. ANM76 through 16S rRNA partial gene sequencing. Strain ANM76 was identified as *Paenibacillus* sp. with the possibility of having discovered a new species within the genus *Paenibacillus* in spite 86.9% similarity of the 16S rDNA, as this is low enough to speculate with this possibility. Moreover, in arid environments and soils submitted to several stresses such as drought, salinity, high temperatures, heavy metals, etc., new species of endophytes are being described as the phytomicrobiota of plants colonizing these areas.

Endophytic bacteria can promote plant growth through various direct and indirect mechanisms [7]. In the current study, endophytic bacterial strains were evaluated for plant growth promoting traits including IAA production, solubilization of P and colloidal chitin, enzymatic activity and production of ammonia. Various endophytic strains contributed in production of phytohormones and help to regulate developmental process in plants. It is now a matter-of-fact that a variety of endophytic bacteria can produce IAA [29,57,58]. Similarly, in the present study, all the tested endophytic strains showed production of IAA even without of precursor L-tryptophan however in presence of L-tryptophan there was significantly higher production of IAA (Table 1) which is according to the findings of Idris et al. [57] who revealed that IAA produced can be promoted by supplementing L-tryptophan. Khana et al. [58] also reported the production of IAA by endophytic *Bacillus subtilis* LK14, *Pseudomonas aeruginosa* LK17 and *Sphingomonas* LK18, while, application of *Bacillus subtilis* LK14 in *Solanum lycopersicum* showed significant increase in shoot and root biomass and chlorophyll contents. In vitro screening of endophytic bacteria to produce IAA could be a reliable trait for selection of effective plant growth promoting bacteria. The IAA production by bacteria is a prime trait for plant growth promotion as it is involved as a plant defence mechanism against stresses [59], may improve fitness of the plant–bacterium interaction [60], increases root exudation through loosening plant cell walls [61,62] and increases root surface area and length which provide plant access to soil nutrient and water uptake [63].

In the present study, seven strains were positive for P-solubilization and showed variation in halo zone formation (Table 1). These endophytic bacteria are able to convert insoluble P to soluble form possibly through producing low molecular weight organic acids which chelate the cation bound to phosphate through their OH and COOH groups or reduce the pH of medium by H^+ to release P [64–66]. *Paenibacillus* is reportedly one of the most significant P-solubilizing bacteria. In our study, *P. polymyxa* ANM59 and *Paenibacillus* sp. ANM76 showed strong solubilization of P which are handy in increasing plant nutrition through increased P-uptake. P-solubilizing endophytic bacteria were evaluated for their ability to colonize chickpea roots and it was found that all the tested strains were well-capable of colonizing the chickpea roots. Plants respond to bacterial as well as fungal infection by activating the plant-encoded synthesis of cell wall degrading enzymes including chitinase, protease and lipase which degrade plant cell-associated chitin, proteins and lipids, respectively [32,46,67–70]. In the present study, the majority of strains were positive for chitinase which could strengthen plant defence mechanisms as well as improve plant growth and yield. Endophytic bacterial strains genetically transformed with genes encoding these cell wall degrading enzymes to become more effective biocontrol agents [70]. Kowsari et al. [71] reported that *Trichoderma harzianum* can transform plant encoded chitinase genes tested with the inserted acetamidase gene *amds* used as a selectable marker on plasmid could help the enzyme to bind better to insoluble chitin and showed higher antifungal activity towards *Fusarium*

graminearum. The chitinase, peroxidases, lipase, protease are part of the pathogenesis-related proteins and their activation bring ISR in plants [7,72,73].

Colonies of endophytic strains in the current study appeared as mucoid milky white with an entire margin however there was a large variation in colony transparency, size and shape (Table 1). Other research also reported large variation in colony morphology of endophytic strains from nodules of *Glycine max* and *Lespedeza* sp. [74,75]. In our study, endophytic *Paenibacillus* strains were found Gram-negative which confirmed the findings of Dsouza et al. [75], Kittiwongwattana and Thawai [76], Valverde et al. [77] and Wang et al. [78] who reported several *Paenibacillus* strains including *P. prosopidis* PW21^T, *P. uliginis* DSM 21861^T, *P. purispatti* ES-MS17^T, *P. lactis* PSM15596^T and *P. lamnae* L7-75^T as Gram-negative or Gram-variable. Generally, *Paenibacillus* species showed a Gram-positive reaction however due to a progressive increase in the numbers, cells became sensitive to the decolorizing step of the Gram-stain and electron microscopy showed these cells to be Gram-negative. With time, the peptidoglycan layer of Gram-positive bacteria became thinner, fragile, un-visible and cells relied more and more on the outermost surface layer to maintain cell shape and showed Gram-negative reaction during staining [79].

Evaluation of bacterial endophytic efficacy is important as these infect roots nodules, improve nutrients availability and may facilitate rhizobia for nitrogen fixation [56,80]. Therefore, efficacy of seven non-rhizobial endophytic strains to promote chickpea plant growth were evaluated to develop better inoculants of endophytic bacteria having multiple PGP traits. The shoot and root fresh and dry chickpea biomass were significantly boosted by the application of these endophytic bacteria (Table 4). Increase in chickpea seedlings biomass could be due to production of IAA, cell wall degrading enzymes, ammonia, acids and increased nutrient uptake which were found positive in the current in vitro study. Egamberdieva et al. [81] reported positive correlation between the production of IAA, hydrogen cyanide, siderophore and cell wall degrading enzymes by *B. subtilis* NUU4 which indicated that these strains are able to promote chickpea growth. Park et al. [80] and Parray et al. [82] also reported the several mechanisms in arrears of plant beneficial effects including synthesis of plant growth regulators, antibiotics, antifungal compounds, cell wall degrading enzymes or modulation of physio-biochemical processes in plants. In the nodulation test, we re-inoculated these strains to their host chickpea to reconfirm their nodulating capacity and all the strains were found positive in forming nodules. Among these endophytic strains, *P. polymyxa* ANM59 and *Paenibacillus* sp. ANM76 showed the maximum nodule formation along with improvement in its fresh and dry nodule weights while no nodulation was observed in control chickpea plants. Previous studies reported the nitrogen-fixing ability in few *Paenibacillus* strains including *P. brasilensis*, *P. durus*, *P. sabinae*, *P. massiliensis*, *P. wynnii*, *P. peoriae* and *P. polymyxa* [83–85]. Silva et al. [85] studied the effects of different *Paenibacillus macerans*, *Paenibacillus durus*, *Paenibacillus polymyxa* and *Bacillus pumilus* strains on the symbiosis between *Bradyrhizobium* and cowpea, and showed that these strains stimulated nodulation and improved nitrogen fixation efficiency. Latif et al. [86] assessed *Paenibacillus* sp. (T.P2-4) along with *Rhizobia* and *Sinorhizobia* for nodule formation and reported for the first time a unique symbiotic association of *Paenibacillus* sp. (T.P2-4) nodulating *Trifolium pratense*. They also screened *nod D* genes in strains for genetics involved in nitrogen fixation by using nod primers deduced from *Sinorhizobium* sp. and reported maximum amplified band size of *nod D* gene in the case of *Paenibacillus* sp. (T.P2-4). However, in future further work on the mode of co-existence is needed to be investigated about the respective role of various rhizobia and non-rhizobial strains along with their associated nodulation genes.

5. Conclusions

In this study, endophytic *Paenibacillus* strains isolated from root nodules successfully formed a symbiotic association with chickpea, which was a novel finding. Our results suggest that endophytic *Paenibacillus polymyxa* ANM59 *and Paenibacillus* sp. ANM76 are well capable of colonizing chickpea roots and induced nodulation in chickpea. These strains possess multiple plant growth promoting traits as well as the ability to tolerate abiotic stresses. Plant growth promotion and biological nitrogen

fixation by *Paenibacillus* strains in addition to other PGP traits offer a potential candidature to these *Paenibacillus* strains as efficient plant growth promoting endophytes. In future, genetic characteristics of *Paenibacillus* strains involved in root nodulation and nitrogen fixation in combination with or without rhizobial strains should be evaluated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/10/621/s1. Table S1: Colony morphological characteristics of endophytic strains from chickpea grown on yeast extract mannitol agar.

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