Characterization of Root-Endophytic Actinobacteria From Cactus (*Opuntia Ficus-Indica*) for Plant Growth Promoting Traits

V Govindasamy (govindasamy@iari.res.in)
ICAR-Indian Agricultural Research Institute https://orcid.org/0000-0003-3083-8093
Priya George
National Institute of Abiotic Stress Management
S V Ramesh
Central Plantation Crops Research Institute
P Sureshkumar
National Research Centre on Banana
Jagadish Rane
National Institute of Abiotic Stress Management
PS Minhas
National Institute of Abiotic Stress Management

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Abstract

The present study is the first report of isolation and characterization of endophytic actinobacteria from cactus (*Opuntia ficus-indic*a). A total of 179 morphologically distinct endophytic actinobacterial isolates were purified from the roots of two different genetic accessions of cactus. All these isolates were screened for their plant growth promotion traits namely growth on N-free medium, P-solubilization, siderophore production, ACC deaminase activity and IAA production. A majority of the endophytic actinobacterial isolates (85%) exhibited the potential for plant growth promotion under *in vitro* conditions. Ten among the isolates were selected based on their multi-PGP traits and were identified as *Streptomyces* sp. based on the 16S rRNA gene sequencing and phylogenetic analysis. Plant growth promoting potential of these selected endophytic *Streptomyces* was studied in wheat seedlings. All these selected isolates significantly enhanced the growth parameters like seedling length and rootlets number compared to the uninoculated control. The wheat seeds inoculated with *Streptomyces tuirus* VL-70-IX exhibited maximum number of rootlets (6.33) compared to uninoculated control (3.67). The inoculation of endophytic actinobacteria *Streptomyces pseudogriseolus* VL-70-XII caused maximum seedling length (20.53 cm) and root length (8.26 cm) while the inoculation of *S. radiopugnans* HV-VIII resulted in highest shoot length (12.33 cm). These endophytic actinobacteria isolated from the roots of cactus accessions showed potential PGP traits. This work lays foundation for characterization and selection of endophytic actinobacteria from the under-exploited, drought tolerant species such as cactus with potential cross-compatibility for the improvement of plant growth of field crops especially under abiotic stress conditions..

Introduction

Understanding and managing the plant-microbial interactions will accord considerable benefits especially in improving the crop production under stressed environments. Among these plant-microbe interactions, endophytes are the microbes inhabiting inner tissues of a plant and confer neutral, positive or negative effects to the hosts. Endophytic microbes that live within plant tissues without causing any visible damage to the host and promote plant growth directly or indirectly through a combination of mechanisms are considered as plant-beneficial endophytes [1, 2]. The ecological role of beneficial endophytes is more conspicuous due to the enhanced nutrient use efficiency, biotic or abiotic stress tolerance of plants. All the three domains of life *viz*., *Bacteria*, *Archea* and *Eukarya* are reported to form endophytic association with various plant parts under different climatic conditions [3, 4]. The structural composition of endophytic bacterial communities depends on the host genotype, plant tissue and its vegetation stage. Also, the microbial species composition is significantly influenced by the plant stress and soil types [5–7].

The *Actinobacteria*, one of the dominant bacterial phyla found widely distributed in terrestrial (in soil) and aquatic ecosystems play a significant role in decomposition process and humus formation. They are commonly referred as “actinomycetes” and are Gram-positive having high G + C content in their genome. This phylum comprises wide array of bacterial diversity such as those residing in the soil (*Streptomyces*), N-fixing symbiont of non-leguminous plants (*Frankia*), an important plant pathogen (*Streptomyces scabies*) among others [8]. Diverse group of endophytic actinobacterial species such as *Streptomyces* spp., *Microbispora*, *Micromonospora*, *Nocardioides*, *Streptosporangium, Actinoplanes, Aeromicrobium, Arthrobacter, Brevibacterium, Corynebacterium, Microbacterium* and new genera including *Jatrophihabitans, Herbiconiux, Jishengella, Koreibacter, Phytohabitans, Phytomonospora, Flindersiella, Actinophytocola* and *Allonocardiopsis* etc. were isolated and characterised from various plant species conferring various ecological advantages [4, 9–11]. *Actinobacteria* are known to exhibit diverse physiological and biochemical properties, such as production of extracellular enzymes and formation of a wide variety of secondary metabolites. In recent years, research pertaining to endophytic actinobacteria has gained immense attention attributed mainly to their plant growth promoting (PGP) properties. Endophytic actinobacteria were reported from various plants including wheat, lucerne, tomato, jatropha etc. across the world [9, 12–15]. Endophytic actinobacteria are known to confer ecological advantages to the crop plants grown under abiotic stresses, even though the plant-microbe interactions under these adverse environmental conditions are still inadequately understood [9–11].

Cactus (*Opuntia ficus-indica*), commonly known as prickly pear, belongs to the plant family *Cactaceae*. It uses the Crassulacean acid metabolism (CAM) for photosynthesis. It is highly water-use efficient and adapted to arid and semiarid environments [16, 17]. Cacti develop an association with niche soil microbes which could also potentially contribute to overcome these stress conditions [18]. Endophytes of cacti are comparatively less explored and actinobacterial inhabitants of cactus endosphere in particular are yet to be characterized for their PGP activities. Hence, the present study was planned to isolate root-endophytic actinobacteria from the cactus plants using different enrichment media combinations and to characterize them for various PGP traits.

Materials And Methods

Collection of Cactus- root samples and isolation of endophytic actinobacteria

Two genetic accessions (Acc. No. 1280 and 1287) of cactus (*Opuntiaficus-indica*) were explored for the isolation of root-endophytic actinobacteria. These accessions are obtained from the cactus germplasm collection of ICAR-Central Soil Salinity Research Institute, Karnal, India. Cactus Acc. No. 1280 is a thorn less type bearing yellow fruits and Cactus Acc. No. 1287 is a thorny type with pink fruits. Root samples were collected from a depth of 15-30 cm of the cactus plants which were grown in murrum soil of the research farm at ICAR-National Institute of Abiotic Stress Management, Baramati, Maharashtra, India. Collected roots were washed in running tap water for 5 minutes to remove the soil debris. The air dried root samples were then surface sterilized following standard procedures as below: 1 min. initial wash in 90% ethanol; 4-5 min. in 4% (v/v) NaOCl; 30 sec in 90% ethanol; Samples were washed twice in the sterile water followed by 5 min. wash in 5% $Na_2S_2O_3$ and the final rinse in sterile water for 5 times. The surface sterilization procedure was further validated by examining the final washed solution for no bacterial growth on the tryptone soy agar (TSA) medium [6]. The surface sterilized roots were air dried and cut it in to small fragments (0.5-1 cm) under aseptic conditions.

The dry root bits from the cactus plants were separately placed on Petri plates containing 5 different actinobacterial-specific isolation media namely Humic acid vitamin-B (HV) agar [19], Tap water yeast extract (TWYE) agar [20], Mannitol soya (MS) agar, VL-70agar [21] and VL-70+Cactus extract (VLCE) agar (this study). Briefly, cactus extract was prepared by grinding the fresh cactus roots followed by filtration of the extract through a muslin cloth. Ten mL of the filter sterilized cactus extract was added in to 1L of sterile VL-70 agar and used as VL-70+Cactus extract (VLCE) agar medium. The chemical composition of VLCE agar medium developed and used in this study is provided in Supplementary Table 1. Each sterile medium was supplemented with benomyl (50 mg L^{-1}) to inhibit the fungal growth. The wax/parafilm-sealed plates were incubated for 3 months at 28 °C and 37 °C in a plastic boxes closed with a lid. Plates were observed regularly for actinobacterial colonies and the emerging colonies were regularly picked and purified on the half strength potato dextrose agar (HPDA) plates [12]. Morphological features/cultural characteristics of these isolates were documented.

Screening of endophytic actinobacterial isolates for plant growth promotion traits

Qualitative determination of PGP traits

All the isolates were screened for PGP traits like N-fixation, phosphate solubilization [22] and siderophore production [23]. N-fixation was determined by streak inoculation of individual cultures on N-free medium (Jensen's N-free medium, HiMedia, India) and incubation at 28 °C for 5-6 days (15). The presence of mucoid and slimy growth of actinobacterial isolates on N-free culture plates was considered as putative N-fixers and the cultures were further subjected to confirmatory analysis through acetylene reduction activity. Solubilization of phosphate was determined by spot inoculation of the actinobacterial isolates on Pikovskaya's agar (HiMedia) followed by incubation at 28°C for 6 days. Actinobacterial isolates exhibiting clear zones were considered to possess P-solubilization trait [22]. Bacterial isolates were assayed for their ability to produce siderophores on Chrome Azurol S (CAS) agar medium [23] following spot inoculation of individual actinobacterial isolates and incubation at 28 °C for 6 days. Development of a yellow–orange halo zone around the bacterial growth was construed as a potential for siderophore production.

Quantitative estimation of nitrogenase activity by acetylene reduction assay

All the endophytic actinobacterial isolates showing growth on the N-free Jensen medium were streaked onto N-free Jensen medium slants in glass tubes and were incubated at 28°C for 7 days. In the total headspace, 10 percent volume was exchanged with an equal amount of acetylene and were sealed with stoppers, the culture tubes were further incubated for 24 h. Reduction of acetylene to ethylene by the nitrogenase enzyme was measured with a gas chromatograph (Agilent Technologies 7890A) using a flame ionization detector. Non-streaked slants injected with acetylene served as a negative control, and *Azotobacter chroococcum* isolate (Ac-EPS-1) was used as positive control. The experiment was conducted twice and each time in triplicates [24, 25].

Quantitative estimation of Indole 3-acetic acid (IAA) production

IAA production was quantitatively determined following the method suggested by Gordon and Weber [26] and Bric et al [27]. Actinobacterial isolates were grown in International *Streptomyces* Project-2 (ISP-2) medium [13] supplemented with L-tryptophan (100 mg mL^{-1})-a precursor/inducer of IAA synthesis. Cultures grown for five days were centrifuged at 8000 rpm at room temperature (25 °C) for 10 min and the supernatant obtained was mixed with Salkowski's reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution) in the ratio of 2:1 and kept in dark for 30 min. The pink colour developed was measured at 530 nm using spectrophotometer (Shimadzu, Japan). The concentration of IAA produced by the individual bacterial isolates was determined from a standard curve prepared using known concentrations of IAA (Hi-media, India).

Quantitative estimation of 1-Aminocyclopropane-1-carboxylate (ACC) consumption

ACC deaminase activity of actinobacteria was indirectly estimated by measuring the consumption of ACC-provided as a sole N-source in the medium [28]. Briefly, actinobacteria were inoculated in ISP-2 broth and incubated in a refrigerated incubator shaker (180 rpm) at 28°C for 5 days. The fully grown cultures were centrifuged at 8000 rpm at room temperature for 10 min and actinobacterial cell pellets were washed thrice with sterile DF medium. Cell pellets were re-suspended in DF medium supplemented with ACC (3 mmol L^{-1}) and incubated at 30 °C in incubator shaker at 200 rpm for 48 h. From each of these cultures, 1 mL of culture fluid was centrifuged at 8000 rpm at room temperature (25 °C) for 10 min and 100 µL of supernatant was diluted to 1 mL with DF medium. To this, 2 mL of ninhydrin reagent was mixed in the test tubes and kept in boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min, and absorbance was measured spectrophotometrically at 570 nm. Leftover ACC in the bacterial grown DF liquid medium was quantitatively estimated by developing a standard curve for ACC (Sigma-Aldrich, USA). The amount of ACC consumption (mmol L^{-1}) by the individual actinobacterial isolates was calculated from the initial ACC concentration (3.0 mmol L^{-1}) of DF medium.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted from the selected isolates following the standard methods [29, 30] with slight modifications [31]. Quantity and purity of isolated genomic DNA was ascertained by gel electrophoresis. The 16S rRNA genes from the genomic DNA of the actinobacterial isolates were PCR amplified. The universal bacterial primers 8F (50-AGAGTTTGATCCTTGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) were used for the amplification of 16S rRNA genes [32]. The resulting PCR products were analyzed by performing electrophoresis in 1.2% agarose gel followed by observation in a UV trans-illuminator. The PCR products were sequenced at Sci-Genome Pvt. Ltd. Kochin, India. The Seq-Man software version 4.1 (DNASTAR.) was used to compile the 16S rRNA gene sequences and individual isolates were identified based on a BLAST search. The 16S rRNA gene sequences were submitted to NCBI GenBank repository and the accession numbers were assigned. For the phylogenetic analysis, 16S rRNA gene sequences derived from the WGS information of type species of *Streptomyces* in the Bacterial 16S Ribosomal RNA RefSeq Targeted Loci Project (Bacteria FTP: ftp://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/Bacteria/) and previously described actinobacterial strains (type species or type strains), available in the NCBI database were used. The sequences were aligned in ClustalW using CLC

Genomics Workbench 20.0 software (https://digitalinsights.qiagen.com) and a Maximum-Likelihood (ML) phylogenetic tree with a bootstrap value of 1000 replicates was generated.

PCR-based detection of *nif*H gene

The genomic DNA isolated from the actinobacterial isolates was used as a template to ascertain the amplification of *nif*H gene using the primers IGK3/DVV and PCR conditions as enumerated in Ando et al [33] Gaby and Buckley [34].

Wheat seedling growth assay

Wheat seeds (cultivar - Nethravati) obtained from Wheat Crop Improvement Project, Mahatma Pule Krishi Vidyapeeth, Rahuri, Maharashtra, India were used for the seedling vigour assays. Wheat seeds were surface sterilized by soaking in ethanol (70%) for 30 seconds followed by 2-3 min in 4 % (v/v) NaOCl and eventually performing multiple washes of seeds in the sterile water. Selected endophytic actinobacterial spores were collected by growing respective cultures on mannitol soy agar (MS agar) for 5-7 days following the method of spore preparation suggested by Conn and Franco [35]. Surface sterilized seeds were immersed in respective actinobacterial spore suspension (~ 10^8 cells mL^{-1}) for 4 h. The spore coated seeds were air dried and transferred in to Petri plates containing two sheets of sterile filer papers moistened with 10 mL of sterile distilled water. Seeds added with sterile water served as the control and all the Petri plates were incubated in a plant growth chamber (25°C, 60% RH). After 10 days of incubation without external supply of water, number of rootlets, root length, shoot length and total seedling length were measured.

Statistical analysis

Statistical analysis was carried out using the SPSS statistical software package version 16.0 (IBM SPSS, USA). Data regarding plant growth measurements on wheat seedlings were analyzed by performing analysis of variance (ANOVA) and the treatment means were subjected to the least significant difference (LSD) followed by Duncan's Multiple-Range Test (DMRT) post-hoc analysis. All the hypotheses were tested at the 95% confidence interval ($\alpha = 0.05$).

Results

Isolation of endophytic Actinobacteria

Endophytic actinobacterial colonies growing around the root bits placed in actinobacterial isolation media were carefully transferred to purification medium (Supplementary Figure 1). A total of 179 phenotypically distinct endophytic actinobacterial isolates were purified on HPDA medium. The number of isolates obtained from each of the actinobacterial isolation medium (incubated at two different temperatures) is given in Table 1. HV agar medium was found to be more effective in yielding more morphologically diverse endophytic actinobacteria (62 nos.) from both the cactus accessions. Incubation temperature also affected the number of actinobacterial isolates obtained from the specific isolation media as well from the two different accessions of cactus. Results showed that 122 isolates (68 %) (71 and 51 isolates from the cactus accessions 1280 and 1287, respectively) were purified from different isolation media while incubating at 37°C. The selected endophytic actinobacterial isolates purified from the surface sterilized roots of cactus accessions exhibiting different colony morphology on HPDA medium are presented in Fig. 1. The endophytic actinobacterial isolates were named according to the media and cactus accessions that yielded them. Isolates named after isolation medium followed by Roman numerals indicate their origin from the cactus accession 1280, while isolates appended with Arabic numerals indicate their origin from the cactus accession 1287.

Screening of endophytic actinobacteria for PGP traits

Most of the isolates of endophytic actinobacteria showed at least one of the PGP characters studied; however 26 isolates did not show any of the PGP traits tested. Of the endophytic actinobacterial isolates from the cactus accessions 1280 and

1287, 73 % and 92 % of them exhibited the putative N-fixing ability based on their growth in the N-free Jensen medium, respectively (Supplementary Figure 2). Whereas, 68% and 65% of the endophytic actinobacterial isolates from the cactus accession 1287 showed siderophore (Supplementary Figure 3) and IAA production ability, respectively. All the PGP traits, except ACC deaminases activity, was found high in the endophytic actinobacterial isolates purified from the Cactus accession 1287 compared to the isolates derived from the accession 1280 (Fig. 2).

Experiment to quantify putative nitrogenase activity of the selected root-endophytic actinobacterial isolates by performing the acetylene reduction assay did not confirm nitrogen fixation trait since no detectable nitrogenase activity was observed except *A. chroococcum* isolate (Ac-EPS-1), which served as a positive control (1032.28 nmol C_2H_5. h^{-1} mg $protein^{-1}$) (Supplementary Figure 4). In addition, none of selected actinobacterial DNA samples yielded a positive PCR amplification of *nif*H gene with the IGK3/DVV primers (Supplementary Figure 5). The non-specific PCR bands amplified in some of the endophytic actinobacterial isolates were gel eluted and sequenced, but does not show any sequence similarity with *nif*H gene (data not shown). The IAA production capability of the isolates in the presence of precursor L-tryptophan varied from 10 to 200 µg/mL of the ISP-2 broth. The maximum IAA production was exhibited by the isolate VL-70-PIII (200.82 µg/mL) followed by the isolate HV-18 (170.80 µg/mL) (Fig. 3a). These two isolates were obtained from the roots of cactus accessions 1280 and 1287 using VL-70+Cactus extract agar (VLCEA) and Humic acid Vitamin-B agar (HVA) medium, respectively. Quantitative measurement of IAA production by the selected root-endophytic actinobacterial isolates having multi-PGP traits is shown in Fig. 3a. Endophytic actinobacterial isolates from the cactus accession 1280 showed a relatively high ACC consumption, than the isolates obtained from the accession 1287. ACC consumption ranged from 0.018 to 2.3 mmol L^{-1}. The endophytic actinobacterial isolate HV-VIII exhibited a maximum consumption of 2.3 m mol L^{-1} of ACC after 48 h of incubation (Fig. 3b) which was followed by the isolate VL-70-PIII (1.754 mmol L^{-1}). These two isolates originated from the roots of cactus accession 1280 in HVA and VLCEA medium, respectively. Quantitative measurement of ACC consumption by the selected root-endophytic actinobacterial isolates having multi-PGP traits is shown in Fig. 3b.

Identification of endophytic actinobacteria and their phylogenetic analysis

Based on the above PGPR properties, ten superior endophytic actinobacterial isolates, having multiple PGP traits among others, were selected for further studies (Table 2). Morphological features of the selected root-endophytic actinobacteria in the purification medium are given in Table 2. These selected endophytic actinobacterial isolates were identified using 16S rRNA gene sequence and sequence-based phylogenetic analysis. BLAST analysis of sequences revealed that all the ten isolates belong to different species of the genus *Streptomyces.* The closest phylogenetic identity and 16S rRNA gene sequence features of each endophytic actinobacteria are given in the Table 3. The phylogenetic tree of the endophytic actinobacterial isolates was rooted to *Bradyrhizobium diazoefficiens* strain USDA-110 as an out-group taxon and evolutionary history was inferred based on the Maximum-Likelihood (ML) method (Fig. 4). The molecular phylogeny revealed that all the ten isolates reported in this study formed the components of the distinct clades of *Streptomyces* spp. quite away from the actinobacterial genera such as *Nocardia* sp., *Kocuria* sp., *Arthrobacter* sp., *Microbacterium* sp. and *Micromonospora* sp. (Fig. 4). Molecular evolutionary lineage analysis discloses that the isolate HV-I reported in this study exhibited closest phylogenetic relationship with *Streptomyces radiopugnans* HV-VIII and *S. mutabilis* HVA-18 isolates forming a distinct sub-clade of the main Clade I suggesting their monophyletic origin. The endophytic actinobacterial isolates of *S. tuirus* VL-70-IX*, S. pseudogriseolus* VL-70-XII*, S. collinus* HV-6 and *S. tricolor* VL-70-PIII though formed a distinct sub-clades were found in the same Clade I. Nevertheless, the isolate of *Streptomyces* sp. MS-10 which was identified as *S. hygroscopicus* based on sequence homology search did not cluster with a sub-clade containing *S. hygroscopicus* type strain (NBRC-13472) in the main Clade II suggesting the isolate could be a distinct one sharing a close phylogeny with *S. scabei* or *S. ipomoea* strains (Fig. 4). Similarly, *Streptomyces* sp. HV-19 which could not be identified in homology based search clustered with the *S. scabei* or *S. ipomoea* containing sub-clade implying close evolutionary lineage among the strains (Clade II). Analyzing the phylogenetic relationship among the endophytic actinobacterial isolates in the context of multi-PGP traits disclose that the Clade I comprises isolates [namely *Streptomyces mutabilis* HV-I, *S.*

radiopugnans HV-VIII, *S. mutabilis* HVA-18, *S. collinus* HV-6, *S. tricolor* VL-70-PIII, *S. pseudogriseolus* VL-70-XII] which exhibited all the positive PGP traits. Quite interestingly, the isolate *S. tuirus* VL-70-IX with little P-solubilization trait share closest phylogenetic relationship with the isolates of Clade I however, the isolate *Streptomyces* sp. HV-19 sharing PGP features with *S. tuirus* VL-70-IX did not form a part of the Clade I and present in the Clade II along with *Streptomyces* sp. MS-10 and *S. pseudovenezuelae* VL-70-XIII (Fig. 4).

Effect of endophytic actinobacteria on the growth of wheat seedlings

Significant differences were observed in the growth parameters like root length and rootlet numbers of wheat seedlings following root inoculation of these endophytic actinobacteria compared to the un-inoculated control (Fig. 5a). The highest number of rootlets (6.33) was recorded in the seedlings coated with *Streptomyces tuirus* VL-70-IX. Root length was maximum (8.26 cm) in wheat seedlings inoculated with *S. pseudogriseolus* VL-70-XII followed by the inoculation with *S. tuirus* VL-70-IX (8.14 cm). The inoculation of wheat seeds with *S. radiopugnans* HV-VIII resulted in maximum shoot length of 12.33 cm after 10 days of incubation (Fig.5b) followed by the inoculation with *S. tuirus* VL-70-IX that increased the shoot length to 12.667 cm. The total seedling length was increased by 67 % (20.53 cm) with the inoculation of *S. tuirus* VL-70-IX over the uninoculated control (12.27 cm). All the wheat seedlings treated with endophytic actinobacteria maintained green and healthy growth even after 10 days of incubation without the supply of external moisture compared to the control seedlings (Fig. 6).

Discussion

Cactus (*Opuntia ficus-indica*) is one of the most drought tolerant plants growing in arid environments [16, 17]. Although cactus species are adapted to desert conditions, diverse endophytic bacterial groups are found to inhabit their roots [18, 36]. Prominent among them are actinobacteria belonging to gram positive bacteria with high DNA G + C content exhibiting filamentous growth and formation of spores. Furthermore, members of the phylum *Actinobacteria* are the largest ecological resource for secondary metabolites (plant hormones, antibiotics and other bioactive compounds), with potential biotechnological applications in agriculture, industry and medicine [4, 9]. Actinobacteria could withstand extreme desiccation conditions and hence they are ecologically significant in imparting abiotic stress tolerance among the crop plants [37, 38]. In this context, this is the first report on the isolation and characterization of endophytic actinobacteria from the roots of cactus (*Opuntia ficus-indic*a) plants. Herein, 179 actinobacteria species were isolated from the surface sterilized roots of two cactus accessions using various growth media and incubation temperature combinations. Congruent with the findings of Zhao et al [39], humic acid-vitamin agar (HV agar) medium, having soil humic acid as a sole carbon and nitrogen source, supported the maximum number of endophytes (35 % of the total isolates). This media was developed for the selective isolation of soil actinomycetes supporting the growth of largest number of actinobacteria such as *Streptomyces, Micromonospora, Microbispora*, *Nocardia*, etc. [19].

Actinobacteria have been reported to possess PGP traits in addition to their ability to produce other secondary metabolites. Also their endophytic nature confers them with relative efficiency in promoting the plant growth and crop yield [40]. Endophytic actinobacteria adapt a wide range of mechanisms including nutrient acquisition, phytohormone production, removal of contaminants, and direct suppression of pathogens *via* antibiosis or competition, and induction of plant defence responses to promote the plant growth. Biological N-fixation (BNF) is one of the most common plant beneficial mechanisms shown by endophytic PGPRs [6, 41] which ensure the supply of considerable quantum of N for the diverse agronomically important crops [42]. A majority of the endophytic actinobacteria of cactus root origin (85 % of the total) exhibited growth on an N-free Jenson medium. Similarly, N-fixing ability of culturable endophytic actinobacteria associated with *Jatropha curcas L*. grown in Panxi dry-hot valley soil based on its growth in N-free medium was reported by Qin et al [15]. However, the acetylene reduction assay and non-amplification of *nif*H gene product confirmed their inability to fix atmospheric nitrogen (with no detectable nitrogenase activity) (Supplementary Fig. 4 &5). N-fixing ability based on growth in N-free medium and nitrogenase activity based acetylene reduction assay of an endophytic *Streptomyces chartreusis*

strain WZS021 isolated from the sugarcane shown enhanced the crop biomass [43]. Nonetheless, N-fixation ability of various other endophytic actinobacteria such as *Arthrobacter*, *Mycobacterium*, *Propionibacteria* and many other genera isolated from the root nodules of leguminous and actinorhizal plants are reported [44, 45]. *Streptomyces* improve the plant growth promotion either by improving the nutrition acquisition or production of phytohormones or through the suppression of plant diseases [46]. Here, the cactus root-derived endophytic actinobacteria exhibited other PGP traits like siderophore production (59 % of the total), phytohormone (IAA) production (54 % of the total), P-solubilization (53 % of the total) and ACC deaminase activity (28 % of the total). The ability to produce siderophores, the second most predominant plant growth promotional trait, by the endophytic actinobacteria could have helped the cactus plants to extract various micronutrients such as Fe, Zn, and Cu [47]. It appears that the rhizospheric relationship with these siderophore producing endophytes bestows the cactus with the ability to grow on any micro-nutrient deficient environments [15, 47].

Another important PGP trait of the cactus-origin endophytic actinobacteria is the production of phytohormone, auxins. Indole 3- acetic acid (IAA) is one of the most physiologically important auxins, having pivotal functions in the lateral, adventitious root formation and in root elongation. Rhizo-microbial auxin synthesis contributes to the enhanced total plant auxin pool thereby influencing the overall root growth and plant development [48]. Similarly, root endophytes assist the plants in the uptake of soil mineral nutrients. In this study, almost half of the cactus root-actinobacterial endophytes exhibited *in vitro* P-solubilization activity. It suggests that the cactus accessions depend on these endophytes for their phosphorus requirement as the available P is very low in nutrient poor soils like murrum [6]. Exploration of ACC deaminase activity of the actinobacterial endophytes [28, 49] revealed that all the isolates exhibited ACC deaminase activity. ACC deaminase activity is considered a very potent PGP trait as it enhances the plant growth by overcoming the deleterious effects of ethylene-induced abiotic stress responses. ACC deaminase producing PGP rhizobacteria have been shown to mitigate the adverse effects of drought in plants suggesting the possibility of isolates reported herein to confer abiotic stress tolerance [50]. In this study, the cactus accessions were grown in native murrum soil characterized with relatively low nutrient content [6] and devoid of external supply of nutrients in the form of fertilizers. Consequently, it is rational to assume that these adverse plant growth conditions caused the cactus plants to accommodate/recruit as many PGP endophytic microbes as possible in its exo- and endo-rhizosphere which helps to promote its growth in this nutrient deficient soil.

The 16S rRNA gene sequence-based identification of endophytic actinobacterial isolates revealed the predominance of the genus *Streptomyces*. Similar preponderance of *Streptomyces* spp. among the actinobacterial endophytes in many other crop plants ecosystem was also reported [11, 13, 15, 40]. Phylogenetic studies of the selected isolates based on 16S rRNA gene sequences also reiterated that they belonged to the genus *Streptomyces.* In the phylogenetic tree, the endophytic actinobacterial isolates formed distinct clades. Also, the correlation of phylogenetic relationship among the isolates with their multi-PGP traits divulged similar characters among the isolates of monophyletic origin with a notable exception of *S. tuirus* strain VL-70-IX. However, given the little analysis of 16S rRNA gene sequences employed in this study, the evolutionary or phylogenetic lineage of endophytic actinobacterial isolates requires further corroboration by performing multiple locus sequences analysis (MLSA) with many other conserved marker genes among the groups of actinobacteria [9, 15]. Furthermore, the drawback of 16S rRNA gene sequences in separating the prokaryotes at finer taxonomic levels suggests the utilization of additional nearly universal marker genes in resolving the phylogeny of closely related species or strains of same species [51, 52]. Quite interestingly, all of these selected endophytic actinobacterial isolates reported herein were identified as *Streptomyces* sp. devoid of actual N-fixation ability. Nevertheless, the observed growth of some of these isolates on N-free agar medium could be attributed to the ability of these to utilize the traces of combined nitrogen from agar medium and also scavenge residual ammonia from the atmosphere [53]. In this context, BNF capability of free-living *Streptomyces* was supported by *nif*H gene product amplification and through radio-isotope studies [54]. Nevertheless, there are no recent reports of *Streptomyces* sp. exhibiting nitrogen fixing ability including *S. thermoautotrophicus* [55].

Although endophytic actinobacteria are ubiquitous, their utilization as biofertilizer or PGPR is rather restricted. Hence, PGP traits and their effect/potential on the promotion of wheat seedling growth were evaluated. All the ten selected endophytic actinobacterial isolates significantly improved the seedling growth parameters over the uninoculated control. The isolates

Streptomyces tuirus VL-70-IX and *S. pseudogriseolus* VL-70-XII significantly improved the root number (73 % over control) and length (77 % over control) of wheat seedlings upon 10 days of incubation without the supply of external moisture/water. The inoculation of wheat seeds with *S. radiopugnans* HV-VIII resulted in 61 % increase in shoot length over the uninoculated control. These isolates also possess multiple PGP traits like siderophore production, N-fixation, IAA production and ACC deaminase activity significantly contributing to the improved growth of wheat seedlings. Similarly, harnessing of PGP effects of *Streptomyces* spp. isolated from the different plants species were reported [56–58]. These endophytic actinobacteria reported herein were found to be promising and further investigations are required to explore their secondary metabolites production potential which influences the biotic and abiotic stress tolerance in the crop plants. However, this work provides the basis for characterization and selection of potential endophytic actinobacteria from the under-exploited, drought tolerant species such as cactus. Further it would add to the current state of knowledge regarding the development of an endophytic actinobacterial consortium from cactus plants with potential cross-compatibility for the improvement of plant growth of field crops especially under abiotic stress conditions.

Declarations

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Conflict of interest The authors declare that they do not have any conflict of interest.

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Tables

Table 1
Number of root-endophytic actinobacterial isolates obtained from different actinobacteria isolation medium and incubation temperature combinations

Isolation Media	**No. of isolates purified (Cactus Acc.No.1280)**		**No. of isolates purified (Cactus Acc.No.1287)**	
	Incubation Temperature		**Incubation Temperature**	
	28°C	**37°C**	**28°C**	**37°C**
Humic acid vitamin-B agar (HVA)	6	12	21	23
Mannitol soya agar (MSA)	5	5	10	8
Tap water yeast extract agar (TWYEA)	4	11	2	25
VL-70 agar (VLA)	5	13	0	6
VL-70 + Cactus extract agar (VLCEA)	3	10	1	9
Total	**23**	**51**	**34**	**71**
	74		**105**	

Table 2
Morphological features and Plant growth promoting traits exhibited by selected root-endophytic actinobacteria of cactus plants

Endophytic Actinobacterial Identity	Morphological features on growth purification medium		Plant growth promoting traits (Qualitative screening)				
	Substrate mycelium	Aerial mycelium & spores	Growth in N-free Jensen medium	P-solubilization	Siderophore production	IAA production	ACC deaminase
Streptomyces mutabilis strain HV-I	Brown	Grey	+	+	+	+	+
Streptomyces radiopugnans strain HV-VIII	Light brown	Grey with white spots	+	+	+	+	+
S. mutabilis strain HV-18	Brown centre and encircled by off white coloured ring	Off white cottony	+	+	+	+	+
S. pseudovenezuelae strain VL-70-XIII	Red/pink with diffusible pigment	Off white with outer rings	+	-	+	+	+
S. collinus strain HV-6	Light brown-feathery appearance	Off white cottony and sunken at the centre	+	+	+	+	+
Streptomyces sp. HV-19	Light brown centre and encircled by off white coloured ring	Light grey cottony	+	-	+	+	+
Streptomyces sp. MS-10	Cream coloured	Light black plus white	+	+	+	+	+
S. tuirus strain VL-70-IX	Yellowish white	Off white cottony	+	-	+	+	+
S. pseudogriseolus stain VL-70-XII	Red/pink with diffusible pigment	Off white with outer rings	+	+	+	+	+

Note: +, indicates the presence of PGP trait and -, indicates absence of PGP trait

Endophytic Actinobacterial Identity	Morphological features on growth purification medium		Plant growth promoting traits (Qualitative screening)				
	Substrate mycelium	Aerial mycelium & spores	Growth in N-free Jensen medium	P-solubilization	Siderophore production	IAA production	ACC deaminase
S. tricolor strain VL-70-PIII	Brown diffusible pigment with yellowish outer border	Grey with white spots	+	+	+	+	+
Note: +, indicates the presence of PGP trait and -, indicates absence of PGP trait							

Table 3
Molecular identity of selected root-endophytic actinobacterial isolates having multi-PGP traits screened from roots of cactus accessions

Isolate No.	Source and incubation temperature	Closest type strain (NCBI-GenBank accession)	Sequence similarity (%)	Sequence length (bp)	NCBI-GenBank accession
HV-I	Acc.No.1280 28°C	*Streptomyces mutabilis* strain SAIG321 (MT355865)	99.78	1344	KU550044.2
HV-VIII	Acc.No.1280 37°C	*Streptomyces radiopugnans* strain HBUM174084 (FJ486341)	99.89	1429	KU550045.2
HV-18	Acc.No.1287 28°C	*Streptomyces mutabilis* strain HBUM174166 (FJ532445)	99.86	1418	KU550046.1
VL-70-XIII	Acc.No.1280 28°C	*Streptomyces pseudovenezuelae* strain B201 (DQ462662)	99.07	1402	KU550047.2
HV-6	Acc.No.1287 28°C	*Streptomyces collinus* strain Str-6 (JX050226)	99.76	1405	KU885910.2
HV-19	Acc.No.1287 28°C	*Streptomyces* sp. 6R001 (LC497905)	99.75	1411	KU885911.2
MS-10	Acc.No.1287 28°C	*Streptomyces hygroscopicus* strain SA57 (MH265973)	99.66	1399	KU885912.2
VL-70-IX	Acc.No.1280 28°C	*Streptomyces tuirus* strain PAS9 (KR296715)	100	1420	KU885913.2
VL-70-XII	Acc.No.1280 28°C	*Streptomyces pseudogriseolus* strain A22 (KM978827)	99.88	1397	KU885914.2
VL-70-PIII	Acc.No.1280 28°C	*Streptomyces tricolor* strain AS4.1867 (AY999880)	99.76	1381	KU885916.2

Figures

Figure 1

Representative images showing the colony morphology of endophytic actinobacterial isolates purified from the surface sterilized roots of Cactus on HPDA medium.

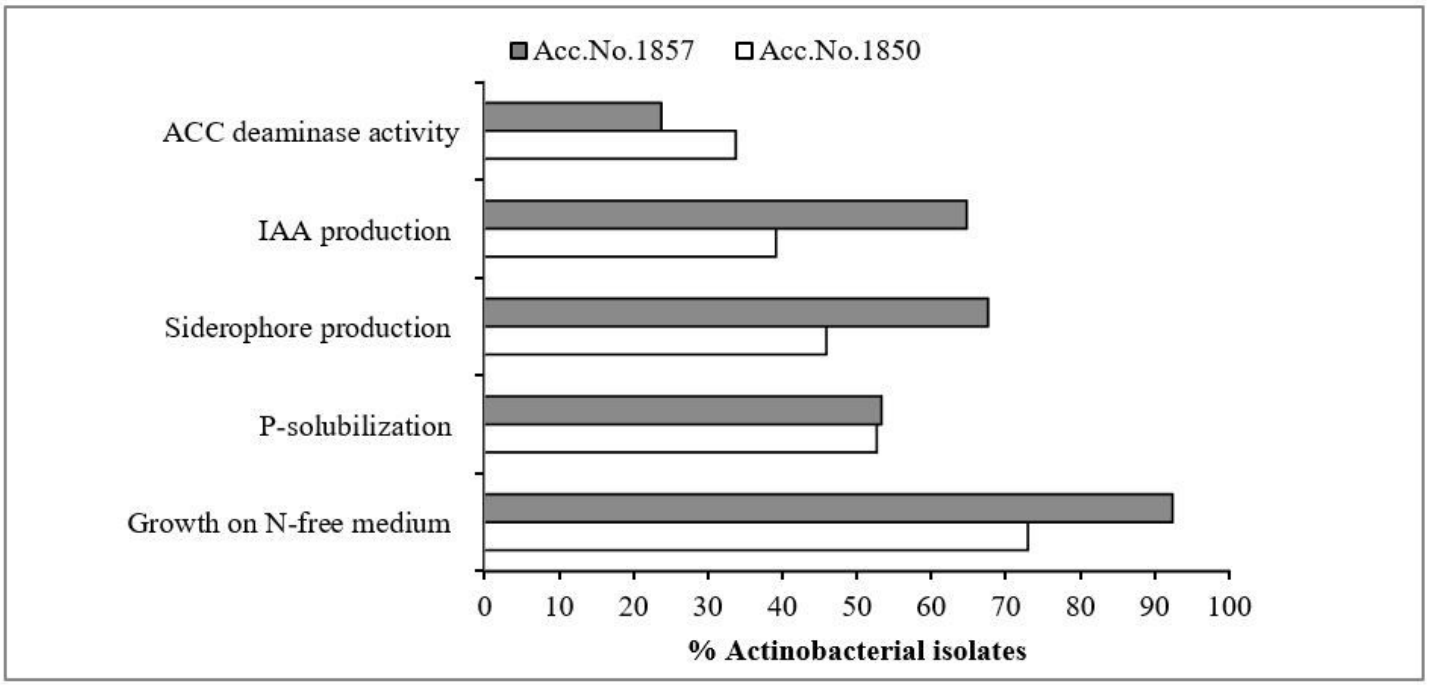

Figure 2

Percentage of root-endophytic actinobacterial isolates purified from two Cactus accessions exhibiting various plant growth promoting traits based on qualitative screening.

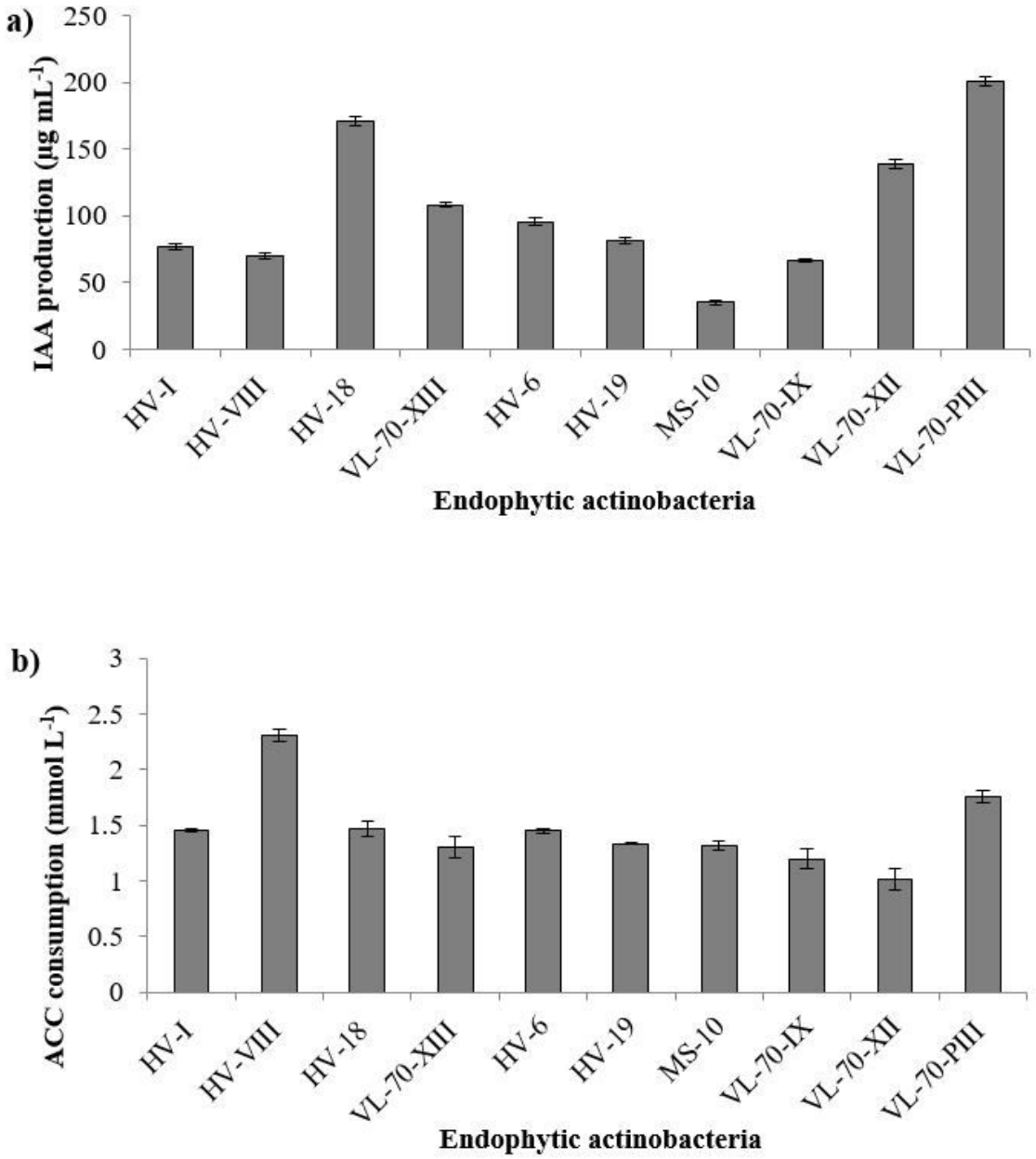

Figure 3

Quantitative estimation of IAA production (a) and ACC consumption (b) traits of the selected root-endophytic actinobacteria of Cactus. Isolates named with Roman and Arabic numerals are obtained from the cactus accessions 1280 and 1287, respectively. Values are the mean of three replications ± standard error.

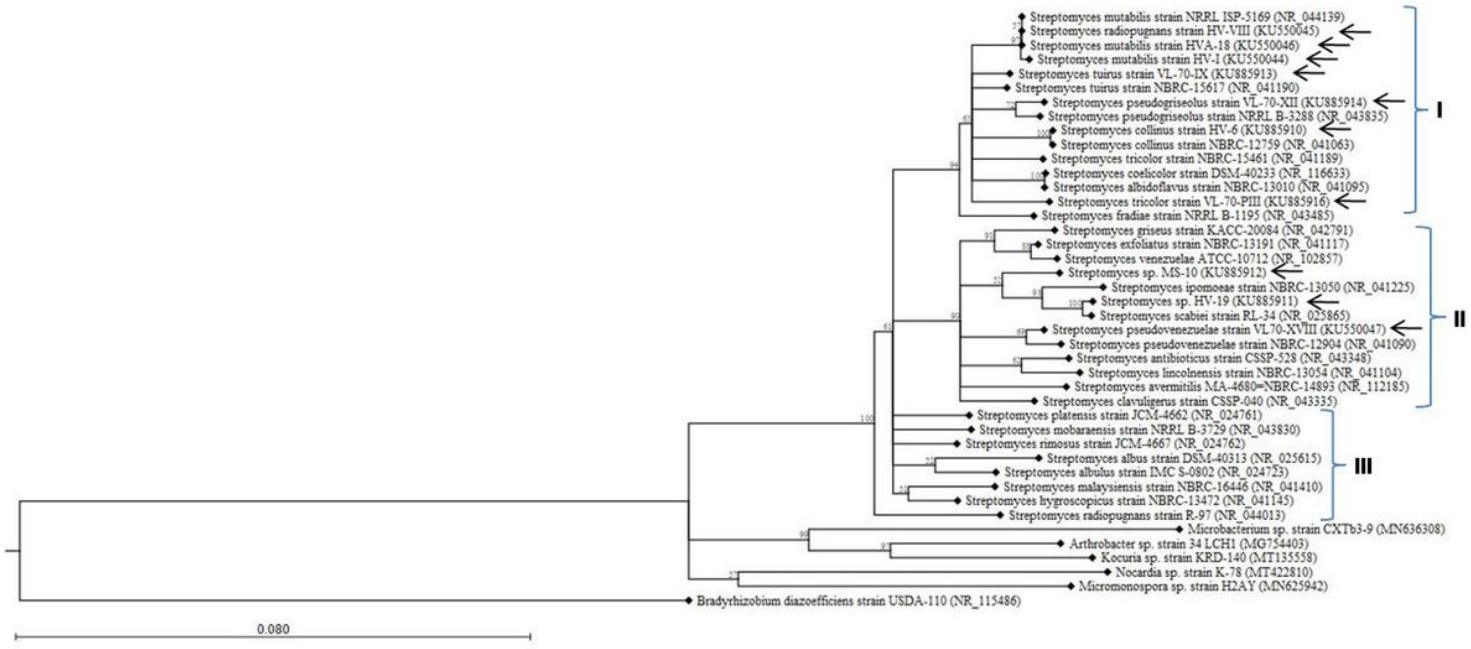

Figure 4

Maximum- likelihood (ML) phylogenetic tree based on the 16S rRNA gene sequences of selected Cactus-root endophytic actinobacteria possessing multiple PGP traits and previously described actinobacterial strains (type species or type strains whose 16S rRNA gene sequences were derived from the WGS information available at Bacterial 16S Ribosomal RNA RefSeq Targeted Loci Project, NCBI database). Bradyrhizobium diazoefficiens strain USDA 110 was used as an out group taxon. Cactus-root endophytic actinobacteria reported in this work are highlighted with arrows. The numbers at the nodes indicate the percentage of bootstrap support, based on the analysis of 1000 replicated datasets.

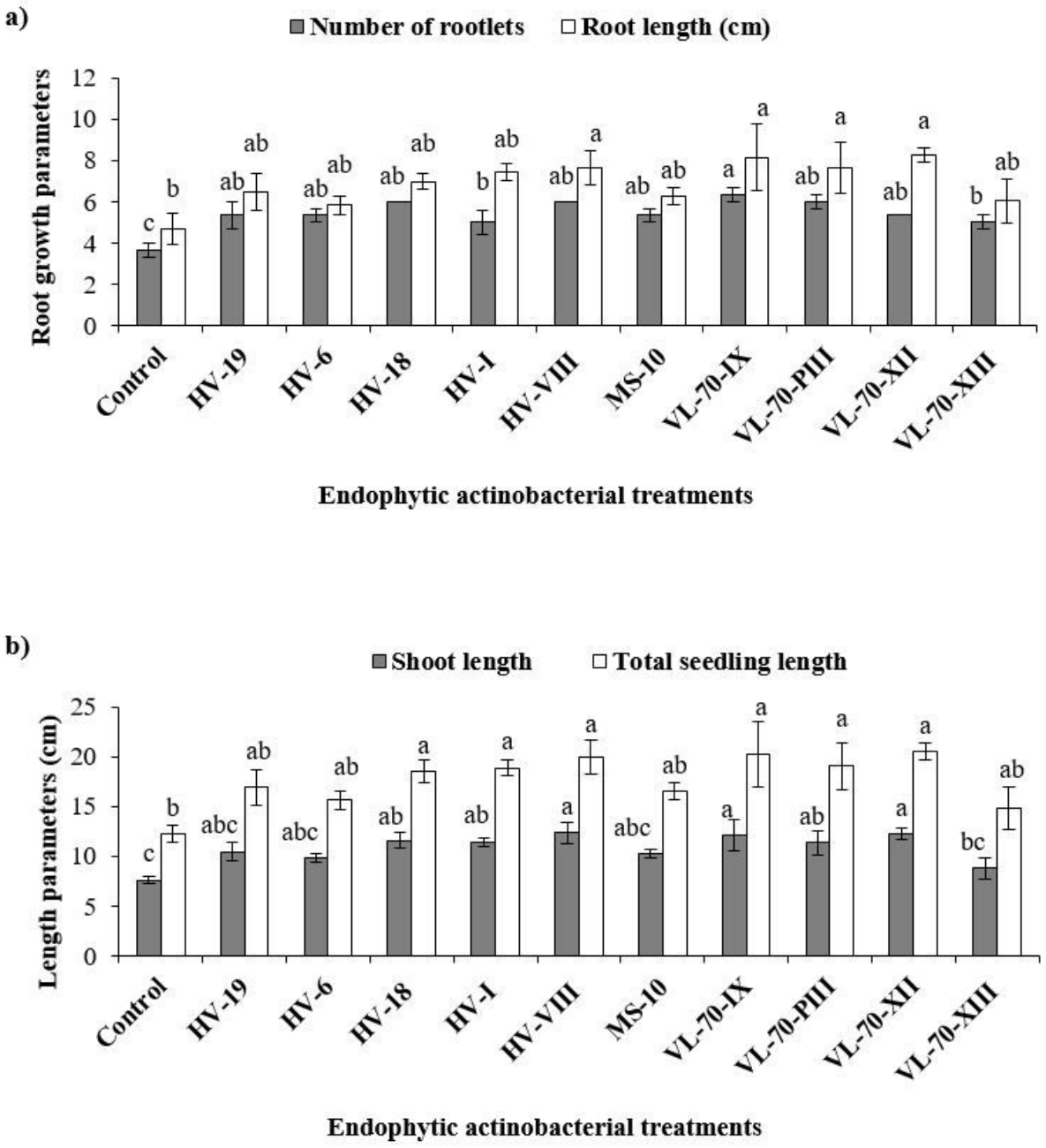

Figure 5

Growth promoting effects of selected root-endophytic actinobacteria on wheat seedlings: (a) root and (b) length parameters. Isolates named with Roman and Arabic numerals originate from the cactus accessions 1280 and 1287, respectively. Values are the mean of three replications ± standard error. The bars in graph denoted by the same alphabet indicate non-significance at $P \geq 0.05$ based on Duncan's Multiple-Range Test (DMRT).

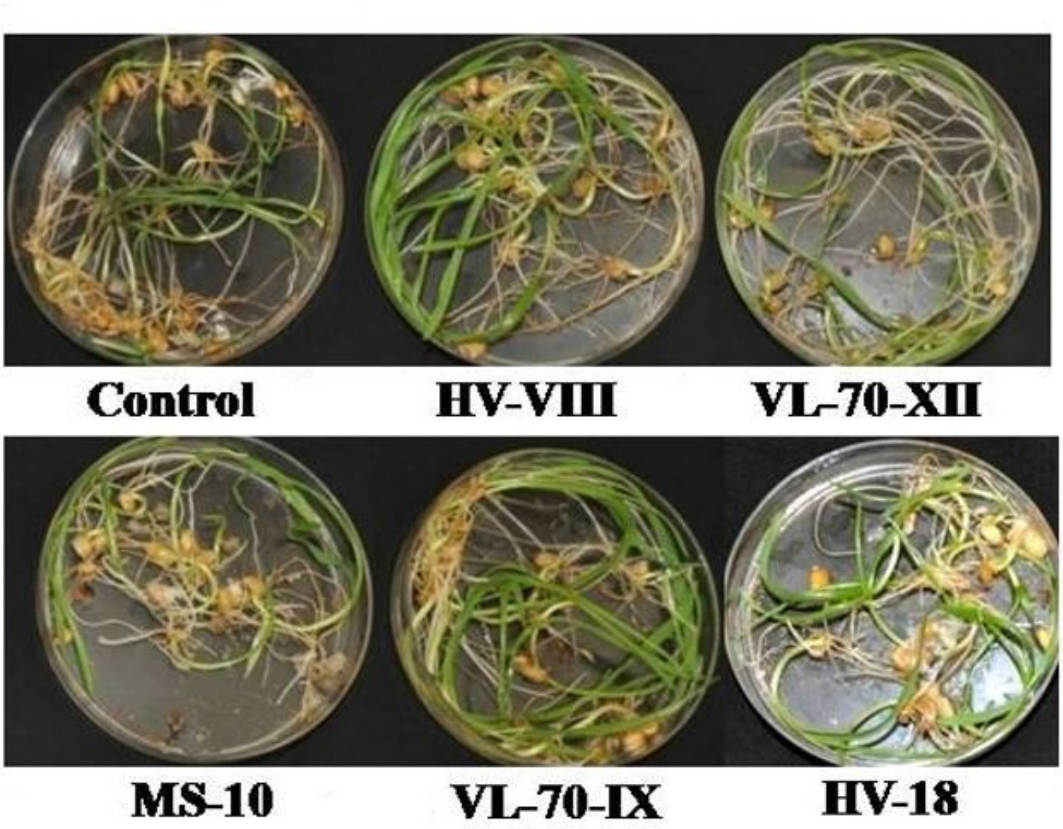

Figure 6

Growth of the wheat seedlings under moisture deficit conditions, following the inoculation of the selected root-endophytic actinobacterial isolates obtained from the Cactus plants.

Supplementary Files

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