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Identification of Actinobacteria from Rice Endo-Rhizosphere for Salt Stress Tolerance and Plant Growth Promotion

T. Kavya[1], Venkadasamy Govindasamy[1*], Gerard Abraham[2] and Archna Suman[1]

[1]Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi -110 012, India
[2]Centre for Conservation and Utilization of Blue Green Algae (CCUBGA), ICAR-Indian Agricultural Research Institute, New Delhi -110 012, India

**Corresponding author*

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A B S T R A C T

The endo-rhizospheric microbiome plays a vital role in improving plant tolerance to abiotic stresses such as salinity. This study focuses on the morphological characterization and molecular identification of actinobacteria from the endo-rhizosphere of rice (*Oryza sativa*) of different doses of N applied soils. A total of 15 Actinobacterial isolates were obtained and evaluated for salt stress tolerance at varying NaCl concentrations (up to 15%). All the studied isolates showed tolerance up to 7.5% NaCl concentration, only 3 isolates showed growth up to 12.5% and none of the isolates showed growth at 15 % NaCl concentration. Molecular identification using 16S rRNA gene sequencing revealed the predominance of genera such as *Streptomyces* spp. The N- fixation through acetylene reduction assay was in the range 0.22-0.87 µmoles ethylene/hr, all the isolates showed the ammonia and siderophore production, two isolate showed phosphorus solubilization, eight and ten isolates showed HCN (0.09-0.44), and exopolysaccharide production respectively. The IAA production 1.03-60.88 µg/ml and 1.41-32.45 µg/ml in presence and absence of tryptophan respectively. There was no significance difference was observed among the endophytes in ACC deaminase activity. These findings suggest that endo-rhizospheric actinobacteria possess dual functionality as salt stress alleviators and biofertilizers, emphasizing their potential contribution to sustainable agriculture in saline-affected areas.

Introduction

Soil salinity is one of the most important environment stresses which play major role in decreasing agriculture productivity. It leads to the loss of around 2,000 hectares of agricultural land daily, contributing to a 1–2% annual decline in agricultural soils worldwide. This results in crop yield reductions of up to 70%, particularly in arid and semi-arid regions, causing an estimated economic loss of US $27.3 billion per year (Paul and Lade, 2014;

Zaman *et al.,* 2018). It is important to combat soil salinity to feed the world's growing population. In this regard, many researches developed transgenic plants which can overcome salt stress (Kumar *et al.,* 2017; Chantre Nongpiur *et al.,* 2016), but these approaches are time-consuming and costly due to the impressive charges required to validate the consumption or cultivation of genetically modified plants (Bianco and Defez, 2009). Exploring the potential of microbial associates and their biomolecules could significantly contribute to stress mitigation and plant growth promotion, presenting strategic opportunities for large-scale application in crops cultivated in saline areas.

Endophytic bacteria are widespread in nature, residing within plant tissues across most ecosystems and completing their life cycle, often in facultative or obligate symbiotic relationships. These beneficial microorganisms play a important role in promoting eco-friendly and sustainable agricultural practices by enhancing plant growth and protecting crops from microbial diseases, thereby improving crop yields (Nagrale *et al.,* 2023; Rashid *et al.,* 2012). In this regard, recently the endophytic actinobacteria gaining importance as a potential bioinoculant under stressful environment (Kavya *et al.,* 2024). These species are prominent producers of secondary metabolites and bioactive compounds (Sánchez-Suárez *et al.,* 2020). Most of the actinobacteria isolated from soil as well as from various environments such as seawater, fresh water, desert, vermicompost rhizosphere and plant tissues (Sharma *et al.,* 2009; Sreevidya *et al.,* 2016; González *et al.,* 2023). The relevant literature contains endophytic actinobacteria of the representative genera *viz*., *Streptomyces*, *Nocardiodies, Actinopolyspora, Micromonospora*, *Saccharopolyspora, Dactylosporangium*, *Corynebacterium*, *Frankia*, *Rhodococcus* and *Mycobacterium* (Tian *et al.,* 2007; Gangwar *et al.,* 2012) in plant growth promotion. Reflecting their enormous diversity in different niches and ecosystems, many reports indicate that actinobacteria residing in the endosphere of plants have potential character to augment plant growth directly through production of plant growth regulators or phytohormones (auxins, cytokinins and gibberellins), production of HCN siderophores to scavenge ferric iron from the environment, solubilization of inorganic phosphate, fixing nitrogen and suppression of stress ethylene in plant by production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Sadeghi *et al.,* 2012; Saikia and Bora, 2021; Kumar *et al.,* 2015; Govindasamy *et al.,* 2014; 2018). They also employ various strategies to combat abiotic stress, including the synthesis of osmolytes, plant hormones, and enzymes, as well as the regulation of osmotic balance (Narsing Rao *et al.,* 2022; Kavya *et al.,* 2024). Many reports also evident for the role of endophytes in improving plant growth through stress tolerance in different plants like wheat (Yandigeri *et al.,* 2012), rice (Sukpanoa *et al.,* 2024), maize (Chukwuneme *et al.,* 2020), Soyabean (Dubey *et al.,* 2021), tomato (Xiong *et al.,* 2019). This versatility of endophytic actinobacteria, encouraged us to explore plant growth-promoting potential of native endospheric actinobacterial strains of rice under salt stress condition.

Materials and Methods

Source of Actinobacterial isolates

The actinobacterial strains in the laboratory collection which were originally isolated from four distinct plant-associated niches: rhizosphere, rhizoplane, root-endosphere, phyllosphere, and leaf-endosphere (phyllo-endosphere) of two rice varieties, BPT-5204 and Varadhan. The media used for actinobacteria isolation included Humic Acid Vitamin Agar (HVA), Tap Water Yeast Extract Agar (TWYA), Tryptone Soy Agar (TSA), and Nitrate Mineral Salts Agar (NMSA). The actinobacterial isolates were purified by selecting the individual colonies from these media and streaking them to Half Potato Dextrose Agar (HPDA), after which they were differentiated based on their morphological features.

Cultural characterization of actinobacterial isolates

The cultural characteristics of all actinobacterial isolates were assessed by culturing them on Half Potato Dextrose Agar (HPDA) and five different International Streptomyces Project (ISP) media such as Yeast Malt Agar (ISP-2), Oatmeal Agar (ISP-3), Inorganic Salt Starch Agar (ISP-4), Glycerol Asparagine Agar (ISP-5), and Tyrosine Agar (ISP-7). Morpho-cultural characteristics were recorded after incubating the streaked Petri plates with actinobacterial isolates for 7 days at 28 ± 2º C in a BOD incubator. The growth of actinobacterial colonies was examined for various cultural traits, including colony shape, surface texture, the color of both aerial and substrate mycelium, spore presence and coloration, and the production of soluble pigments on the respective media.

Screening of actinobacterial isolates for salt stress tolerance

The ability of the purified actinobacterial isolates to grow at various sodium chloride concentrations (5%, 7.5%, 10%, 12.5%, and 15% w/v) was tested on ISP-2 medium supplemented with the respective NaCl concentration. The osmotic potential of ISP-2 medium supplemented with varying concentrations of NaCl was determined using a psychrometer (PSψPRO, WESCOR INC). Fresh cultures of actinobacteria were streaked onto ISP-2 agar plates containing different levels of NaCl and incubated at 28 ± 2°C for seven days in a BOD incubator. The ability of the isolates to tolerate salt stress was evaluated based on their growth under these conditions.

Plant Growth-Promoting Properties of Actinobacterial Isolates

Ammonia Production

Ammonia production by the actinobacterial isolates was evaluated using the method described by Islam *et al.,* (2009). Pure culture of each isolate was inoculated in 5 ml of peptone water medium and incubated at 28 ± 2°C for 7 days. Following incubation, 0.5 ml of Nessler's reagent was added to each test tube, mixed thoroughly, and left at room temperature for 30 minutes. The appearance of a yellow to orange or deep pinkish-red color indicated positive ammonia production.

Nitrogen Fixation

The nitrogen-fixing ability of the actinobacterial isolates was qualitatively assessed by growing them on nitrogen-free Jenson medium. Newly cultured isolates were inoculated onto medium-containing Petri plates and incubated at 28 ± 2°C for 7 days. Isolates showing mucoid and slimy growth were considered potential nitrogen fixers. The isolates were subsequently evaluated for quantitative nitrogen fixation through acetylene reduction activity (ARA) assays using gas chromatography (Hardy *et al.,* 1968; Dahal *et al.,* 2017). For the ARA assay, actinobacterial isolates were streaked on slants containing nitrogen-free Jenson medium, covered with cotton plugs, and incubated at 28 ± 2°C for 7 days. Afterward, 10% of the air in each tube was substituted with acetylene, followed by a 24-hour incubation. After incubation, 1 ml of gas was withdrawn using a syringe, and ethylene production was analyzed using a gas chromatograph (GC-2014, SHIMADZU) equipped with a flame ionization detector and a capillary column. Non-inoculated slants injected with acetylene were used as negative controls.

$$\text{ARA (µmoles of ethylene/hr)} = \frac{\text{Total ethylene in ppm} \times \text{volume of the container}}{\text{Molecular mass of ethylene} \times 24}$$

Mineral phosphate solubilization

Phosphate solubilization activity of the actinobacterial isolates was tested following the method by Sylvester-Bradley *et al.,* (1982), as cited in Beneduzi *et al.,* (2008). Actinobacterial isolates were inoculated by streaking onto glucose yeast (GY) medium plates and incubated at 28 ± 2°C for 7 days. The formation of clear halo zones surrounding the colonies was taken as evidence of mineral phosphate solubilization. Isolates that exhibited positive activity were subjected to quantitative analysis by inoculating them into 5 ml of GY broth (pH 7.2 ± 0.2) and incubating at 28 ± 2°C for 7 days. The pH of the broth was measured after incubation, with greater pH reduction indicating higher production of organic acids, correlating with enhanced phosphate solubilization.

Indole 3-acetic acid (IAA) production

Indole acetic acid (IAA) production by actinobacterial isolates was quantified by inoculating cultures into two sets of tubes: one containing 5 ml of ISP-2 broth supplemented with 10 mg/ml of L-tryptophan, and the other containing ISP-2 broth without L-tryptophan. Both sets of tubes were incubated in shaking condition at 28 ± 2°C for 7 days. Further, the cultures were transferred to sterile Eppendorf tubes and centrifuged at 5000 g (3K30, Sigma) for 15 minutes. After centrifugation, 2 ml of the supernatant was transferred into 15 ml test tubes. Subsequently, 2–3 drops of orthophosphoric acid and 4 ml of Salkowski's reagent were added. The mixtures were incubated in the dark at room temperature for 20–30 minutes. Following incubation, absorbance was recorded at 530 nm using a spectrophotometer (HALO DB-20S, Dynamica), in accordance with the procedure outlined by Ndeddy Aka and Babalola (2016).

Siderophore production

Siderophore production by the actinobacterial isolates was assessed using the universal CAS agar method,

which utilizes blue chrome azurol S (CAS) plates. The molten medium was dispensed into sterile Petri dishes and left to solidify. After 24 hours, actinobacterial isolates were streaked onto the solidified CAS agar and incubated at 28 ± 2°C for seven days. The production of siderophores was detected by the formation of yellowish-orange halo zones around the colonies, caused by the decolorization of the blue ferric dye complex, as described by Schwyn and Neilands (1987).

HCN production by spectrophotometry

The hydrogen cyanide production by the actinobacterial isolates was analysed following the protocol of Bakker and Schippers (1987). Actinobacterial isolates were streaked on Petri plates containing King's B medium supplemented with 4.4 g/L of glycine. A filter paper soaked in picric acid solution (0.5% picric acid in 2% sodium carbonate) was placed inside the lid of each Petri dish. A filter paper soaked in picric acid solution (0.5% picric acid in 2% sodium carbonate) was placed inside the lid of each Petri dish. The plates were sealed with parafilm and incubated at 28 ± 2°C for 5 to 7 days. A change in filter paper from yellow to orange-brown indicated positive HCN production. Subsequently, the filter paper was cut into small fragments and immersed in 5 ml of sterile distilled water. These samples were kept in a shaker at 28 ± 2°C for 3-5 days, and the spectrophotometric absorbance of the supernatant was measured at 625 nm using a HALO DB-20S spectrophotometer.

ACC deaminase activity by ACC-Ninhydrin assay

ACC deaminase enzyme activity of the actinobacterial isolates was indirectly estimated by measuring the consumption of ACC as the sole nitrogen source in the liquid DF medium (Li *et al.,* 2011). The actinobacterial isolates were cultured in ISP-2 broth and incubated in a shaker at 180 rpm and 28 ± 2°C for 5 days. The cultures were subsequently centrifuged at 8000 rpm for 10 minutes at room temperature using a 3K30 Sigma centrifuge. The resulting cell pellets were washed three times with sterile DF medium and then resuspended in DF medium supplemented with 3 mmol/L ACC solution. The suspensions were incubated at 30°C for 48 hours in a shaker set to 200 rpm. After incubation, 1 ml of the culture was centrifuged again at 8000 rpm for 10 minutes. From the supernatant, 100 µl was taken and diluted to 1 ml with DF medium. Then, 2 ml of ninhydrin reagent was added to the mixture, and the tubes were heated in a boiling water bath for 15 minutes. Once cooled to room temperature, the absorbance was recorded at 570 nm. ACC utilization by the isolates was determined by comparing the absorbance values against a standard ACC calibration curve.

Exopolysaccharide production by spectrophotometry

To assess exopolysaccharide production, actinobacterial cultures were grown in ISP-2 broth to an optical density of OD600 = 2.0. The cultures were centrifuged at 8000 rpm for 2 minutes to pellet the cells, followed by washing with sterile distilled water. The cell concentration was then adjusted to an OD600 of 0.2 using the same medium. A 150 µl aliquot of the bacterial suspension was added to individual wells of a 96-well polyvinyl chloride (PVC) plate and incubated at 28°C for 48 hours. After incubation, the medium was discarded, the wells were rinsed with sterile distilled water, and 150 µl of 0.001% crystal violet solution was added for 15 minutes. Excess dye was removed, and the wells were washed again with sterile distilled water. The bound dye was then solubilized by adding 150 µl of 95% ethanol. The dye concentration was quantified using a plate reader at 570 nm, as described by Verhoef *et al.,* (2003).

Molecular identification and phylogenetic analysis of the selected actinobacterial isolates by 16S rDNA sequencing

Extraction of genomic DNA and PCR amplification of 16S rRNA gene

Genomic DNA from the selected actinobacterial isolates was isolated following the method outlined by Coombs and Franco (2003). Actinobacteria were grown on ISP-2 medium overlaid with cellophane in Petri dishes for 7 days, and 2-3 loopfuls of actinobacterial mycelium were transferred to 1.5 ml Eppendorf tubes containing 500 µl of Tris-EDTA (TE) buffer for DNA extraction. The extracted DNA was confirmed by electrophoresis on a 0.8% agarose gel and visualized using a UV transilluminator. DNA yield and purity were determined using a NanoDrop spectrophotometer. Amplification of the 16S rRNA gene was carried out using the universal bacterial primers 27F (5' AGAGTTTGATCMTGG CTCAG 3') and 1492R (5' TACGGYTACCTTGTTAC

GACTT 3') (Lane, 1991). The PCR reaction was prepared in a final volume of 25 µl, containing 1 µl of DNA template, 5 µl of 5X Taq buffer, 2.5 µl of 1mM dNTP mixture, 0.5 µl of each primer, 2.5 µl of 25 mM MgCl2, 0.2 µl of Taq DNA polymerase (2 U), and 12.2 µl of nuclease-free water.

PCR amplification was performed for 35 cycles in a thermal cycler (Himedia), starting with an initial denaturation at 95°C for 5 minutes. This was followed by denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute and 30 seconds, and a final extension at 72°C for 7 minutes.

16S rRNA gene sequencing and BLAST analysis

16S rRNA gene-PCR amplified products were purified and sequenced by outsourcing with Barcode Biosciences India Pvt. Ltd. The 16S rRNA gene sequences obtained from the selected actinobacterial isolates were analyzed using the BLASTn tool against known sequences available in the NCBI database (www.ncbi.nlm.nih.gov), and sequences showing more than 97% similarity were selected for species-level identification based on the closest matches to known type strains.

Results and Discussion

Morphological characterization of actinobacterial isolates

Thirteen actinobacterial isolates were purified from the endo-rhizosphere of rice plants grown in soils with three different nitrogen regimes (0%, 50%, and 100% of applied N) at the research farm of ICAR-Indian Institute of Rice Research (IIRR), Rajendra Nagar, Hyderabad (Table1).

The isolates morphological characterization was performed based on their different cultural characters such as shape, the surface texture of colonies, the colour of colonies aerial and substrate mycelium, presence or absence of spores, its colour and soluble pigment formation using a 6 different media such as HPDA, ISP2, ISP3, ISP4, ISP5 and ISP7. The cultural characteristics of all actinobacterial isolates on various media are presented in Table 2. The majority of isolates from the endo-rhizosphere of rice plants exhibited slow growth and had a rough texture. The shapes were varied from round, irregular, minute sunken, irregularly raised and encircled. In addition, the surface texture varied from rough to smooth, powdery, and slimy. The colour of substrate mycelium observed were brown, dull white, blackish green, light orange to whitish yellow. Likely, the colour of aerial mycelium was like dull white, grey to light yellow. And also, three isolates produced soluble pigment viz., Tap3c (Brownish), Tap3d (Light pink) and Tap5 (Beige) in HPDA medium. Two isolates Tap1 and Tap2 Vo produced light brown and brown soluble pigment in ISP-2 medium. Whereas, only one isolate produced soluble pigment in ISP3 (Tap5) and ISP5 (Tap3c) medium. No soluble pigment production was detected in ISP4 and ISP7 medium for any of the actinobacterial isolates.

Screening of rice endo-rhizosphere actinobacterial isolates for salt stress tolerance

All the 13 endo-rhizosphere actinobacterial isolates were evaluated for their salt tolerance by assessing their growth in ISP-2 medium supplemented with varying NaCl concentrations of 5%, 7.2%, 10%, 12.5%, and 15%. Among the 13 endo-rhizosphere actinobacterial isolates all the isolates were able to show growth at 5% and 7.5% NaCl. Under 10% NaCl concentration 7 isolates were able to show growth, at 12.5 % NaCl concentration 3 isolates (Tap1, Tap2 Vo and Tap3b) and at 15% NaCl concentration no growth was observed for any of the isolates (Table 1).

In-vitro Screening of actinobacteria for their qualitative and quantitative plant growth promoting traits

Ammonia Production

All 13 endo-rhizospheric actinobacterial isolates showed the ability to produce ammonia under salt stress conditions at 5% NaCl concentration. Among them Ha12b isolate showed highest ammonia production compared to other (Table 2).

P-solubilization

All the actinobacterial isolates were screened for their mineral phosphate-solubilization potential using GY medium. Among the 13 endo-rhizosphere isolates, only one isolate Tap6, was able to solubilize the phosphorous by forming a clear zone around the colonies on GY media plates (Table 2).

Siderophore and EPS production

Siderophore producing ability of all the actinobacterial isolates was ascertained in solid CAS agar medium under *in vitro* conditions. All the 13 endo-rhizosphere isolates were able to produce siderophores as shown by formation of yellow to orange colour hallow around the colony. Exopolysaccharide production ability of all the endo-rhizosphere isolates was evaluated. Among the 13 endo-rhizosphere isolates, 10 isolates were able to produce exopolysaccharide (Table 2, Fig.1A). Two isolates were highest producer with OD value of 0.251±0.02 and 0.224±0.04 followed by Ha1 and Tap7, while Tap1 recorded lowest exopolysaccharide production.

Acetylene reduction assay for quantitative screening of actinobacterial isolates

In this study, initially all the 13 endo-rhizosphere actinobacterial isolates were screened for their ability to fix nitrogen by using N-free Jensen medium in presence of 5% NaCl salt stress. 10 isolates were showing growth on N-free Jensen medium among them Tap3c showed profuse growth (Table 2) These 10 isolates were quantified for nitrogen fixing ability through acetylene reduction assay using gas chromatography. Among the isolates the strain Tap1 reached high levels of nitrogenase activity of 0.87±0.07µmoles of ethylene/hr followed by isolate Tap3c (0.65±0.02µmoles of ethylene/hr) and Tap3d (0.57±0.01µmoles of ethylene/hr) (Fig.1B). Ihe isolates Tap3b and Tap5 showed the same value of nitrogenase activity with 0.53±0.02µmoles of ethylene/hr. And also, Tap6 and Tap2V100 were statistically on par in their nitrogenase activity. While isolates Ha12b showed the significantly lowest ARA activity of 0.22±0.01µmoles of ethylene/hr.

HCN production

Among the 13 endo-rhizosphere actinobacterial isolates 8 tested positive for HCN production under 5% NaCl-induced salt stress (Table 2). The studied isolates exhibited notable variation in HCN production, with OD value at @625nm ranging from 0.41±0.33 to 0.10±0.12**.** The significantly highest HCN production was observed by the two isolate Ha6 (0.41±0.03) and Tap2 V100 (0.44±0.02) followed by Tap1 with 0.32±0.02 (Fig. 1C). The significantly lowest HCN production was observed by the 2 endo-rhizosphere actinobacterial isolates Tap2Vo (0.15±0.02).and Tap7 (0.13±0.01).

IAA Production

The endo-rhizosphere actinobacterial isolates are quantitatively analysed for IAA production both in the presence and absence of precursor metabolite, L-tryptophan under 5 % NaCl salt stress condition. Five isolates were able to produce IAA in absence of L-Tryptophan (Ha6, Tap3c, Tap7, Ha1 and Ha12a) (Table 2, Fig. 2A) and presence of L-tryptophan (Ha6, Tap7, Ha1, Ha12a and Tap2 V100) (Table 2, Fig. 2B).. IAA production by the tested endo-rhizosphere actinobacterial isolates varied from 11.65±0.36 to 60.88±4.57 µg/ml and 10.70±0.04 to 32.45±0.02 µg/ml in presence and absence of L-tryptophan respectively. Ha6 produced maximum IAA both in the presence and absence of L-tryptophan followed by Ha1 (27.49±1.11 µg/ml), Tap2 V100 (26.28±0.99 µg/ml) in presence of L-tryptophan, whereas in the absence of L-tryptophan Tap3c showed 18.77±0.29 µg/ml followed by Ha1 and Ha12a. Significantly lowest IAA production in was observed by Tap7 with production of 11.65±0.36 µg/ml and 10.70±0.04 µg/ml in the presence of L-tryptophan and absence of L-tryptophan respectively

ACC deaminase activity

All the endo-rhizosphere isolates are screened for ACC deaminase activity, seven out of 13 isolates showed ACC deaminase activity (Table 2). And there was no significant variation in the utilization of ACC as a sole nitrogen source was observed in all the tested endo-rhizosphere actinobacterial isolates (Fig. 2C). The amount of ACC consumed by these isolates was ranged from 2.66±0.09 to 2.79±0.04 mmol ACC. Highest amount of ACC consumption was observed for the actinobacterial isolate Ha12b followed by the isolates Tap7 and Tap3c with 2.78±0.06 mmol ACC and 2.77±0.13 mmol ACC, respectively. Isolate Ha12b consumed lowest ACC of about 2.66±0.09mmol ACC.

Taxonomic identification and BLAST analysis of actinobacterial isolates

Among the 13 endo-rhizosphere actinobactetial isolates, 6 isolates DNA was isolated and confirmed using gel electrophoresis. The 16S rRNA gene of all six isolates was successfully PCR-amplified, yielding a single band of approximately 1500 bp in length using specific primers (Table 2). Further these isolates were identified using 16S rRNA gene sequencing and BLASTn analysis,

results inferred that the isolates belong to the group of GC-rich Gram positive actinobacteria. Based on molecular analyses of 16S rRNA sequence data, the 6 actinobacterial isolates among the 13 endo-rhizosphere isolates were confirmed as *Streptomyces* spp. Phylogenetic analysis revealed that the isolate Tap3c shared a 99.89% similarity with *Streptomyces mutabilis* strain HBUM174166 and 99.79% similarity with *Streptomyces radiopugnans* strain HBUM174084. Isolate Tap7 shared a 99.93% homology with *Streptomyces tendae* strain ATCC 19812 and *Streptomyces tendae* strain MCCB 209. Isolate Tap2 V100 showed a highest similarity to the closest phylogenetic type strain *Streptomyces mediolani* strain cfcc3065 and *Streptomyces* sp. strain QJSt8 with 99.58% homology.

Isolate Ha6 showed a 99.86% similarity with *Streptomyces champavatii* strain NBRC 15392 and *Streptomyces* sp. strain BC1004, isolate Tap2_V0 showed 100% similarity with *Streptomyces griseus* strain S59 and *Streptomyces griseus* DGS3. Whereas, isolate Tap1 showed 99% similarity with *Streptomyces* sp. MPWIWI03_BRO3 and 100% similarity with *Streptomyces* sp. strain huaiR 18. Isolate Tap10 showed highest similarity to the closest phylogenetic type strain of *Streptomyces celluloflavus* strain D4-17 99.79%, isolate Met9 showed highest similarity to its phylogenetic type strain *Streptomyces parvus* strain LSA-14 with 99.93%, isolate Ha1b to *Streptomyces hydrogenans* strain Kris3 with 98.69% and Tap10 to *Streptomyces* sp. strain AUR2 (KX427130.1) with 99.85% similarity.

Further, sequences were deposited in GenBank and accession numbers respective isolates were given in the Table.3. The 16S rRNA gene sequences of the actinobacterial isolates were deposited in GenBank. The respective accession numbers for each isolate are provided in Table 3. Among the 13 actinobacterial isolates, three endo- rhizosphere actinobacterial *Streptomyces mutabilis* strain Tap3c, *Streptomyces tendae* strain Tap7 and *Streptomyces mediolani* strain Tap2 V100 were selected as a potential based on their NaCl tolerance and also on the results of qualitative and quantitative PGPR traits.

In the present scenario, salinity is one of the most brutal environmental factors that has limited the crop productivity worldwide. Salt stress in plants cause various changes and can be noted at different growth stages of plants (Kaleem *et al.,* 2018). The effect may be caused due to osmotic and ionic stress developed due to accumulation Na (Munns, 2002). Microbes inhabit rhizosphere and endosphere forms a complex ecological community that influences plant growth and productivity through its metabolic activities (Yandigeri *et al.,* 2012; Zahra *et al.,* 2023; Kavya *et al.,* 2024). In the present study, we screened 13 actinobacterial isolates obtained from endo-rhizosphere of rice plant for PGP traits under salt stress.

The Morphological characterization of 13 endo-rhizosphere actinobacterial isolates on different media (HPDA, ISP-2, ISP-3, ISP-4, ISP-5, and ISP-7) resulted in 13 different groups of actinobacteria. Lee *et al.,* (2008) studied 3 strains of actinobacteria isolated from roots of Chinese cabbage and characterized them based on their growth, colour of aerial and substrate mycelium on six different media viz., ISP-2, ISP-3, ISP-4, ISP-5, ISP-6 and ISP-7.

All the 13 endo-rhizosphere actinobacterial isolates were able to grow under salt stress of 7.5 % NaCl. In the previous report the actinobacteria *Streptomyces* sp. strain KLBMP5084 obtained from the root of halophyte *Limonium sinense* tolerated 7% w/v NaCl (Gong *et al.,* 2020) and four *Streptomyces* strain (VAI-7, VAI-40, SAI-13 and SAI-29) isolated from herbal vermicompost tolerated NaCl concentration of 8% (Sreevidya *et al.,* 2016). And in another report out of the 39 actinobacteria isolated from rhizosphere of halotolerant cash crop *Salicornia bigelovii*, 22 actinobacteria were tolerant to high salinity up to 8% w/v NaCl concentration (Mathew *et al.,* 2020).

In this study among the 13 endophytic isolates were screened for different PGP traits, the highest frequency of PGP trait possessed by rice endo-rhizosphere isolates was observed for ammonia production (13 isolates) and siderophore production (13 isolates), followed by nitrogen fixation (10 isolates), exopolysaccharide production (10 isolates), HCN production (8 isolates). ACC consumption (7 isolates), IAA production (6 isolates) and P-solubilization (1 isolate).

In the earlier study by Yandigeri *et al.,* (2012), the endophytic actinobacteria *Streptomyces coelicolor* DE07, *Streptomyces olivaceus* DE10, *Streptomyces geysiriensis* DE27 isolated from roots of 5 cultivated plant species collected from arid and drought affected fields of Bikaner and Jaisalmer, Rajasthan exhibited many PGP traits like ammonia production.

Table.1 Screening of rice endo-rhizosphere actinobacterial isolates for salt stress tolerance using ISP-2 with different concentration of NaCl

Isolate	0%NaCl Ψ_S = -0.122	5%NaCl Ψ_S = -2.667	7.5%NaCl Ψ_S = -2.832	10%NaCl Ψ_S = -3.449	12.5%NaCl Ψ_S = -3.598	15%NaCl Ψ_S = -4.568
Ha6	+	+	+	+	-	-
Tap1	+	+	+	+	++	-
Tap2 Vo	+	+	+	+	++	-
Tap3b	+	+	+	+	+	-
Tap3c	+	+	+	+	-	-
Tap3d	+	+	+	+	-	-
Tap5	+	+	++	-	-	-
Tap6	+	+	+	-	-	-
Tap7	+	+	+	+	-	-
Ha1	+	+	++	-	-	-
Ha12a	+	+	++	-	-	-
Ha12b	+	+	++	-	-	-
Tap2 V100	+	+	++	-	-	-

Note: (Low growth), ++ (High growth), - (No growth)

Figure.1 Quantitative estimation of PGP traits of selected salt stress tolerant rice endo-rhizosphere actinobacterial isolates (A) Exopolysaccharide production (B) N-fixation by ARA and (C) HCN production. Values are the mean ± standard error of repeated experiments with 3 replications. The bars in graph denoted by the same alphabet indicate non-significance at P≥0.05 based on Duncan's Multiple-Range Test (DMRT).

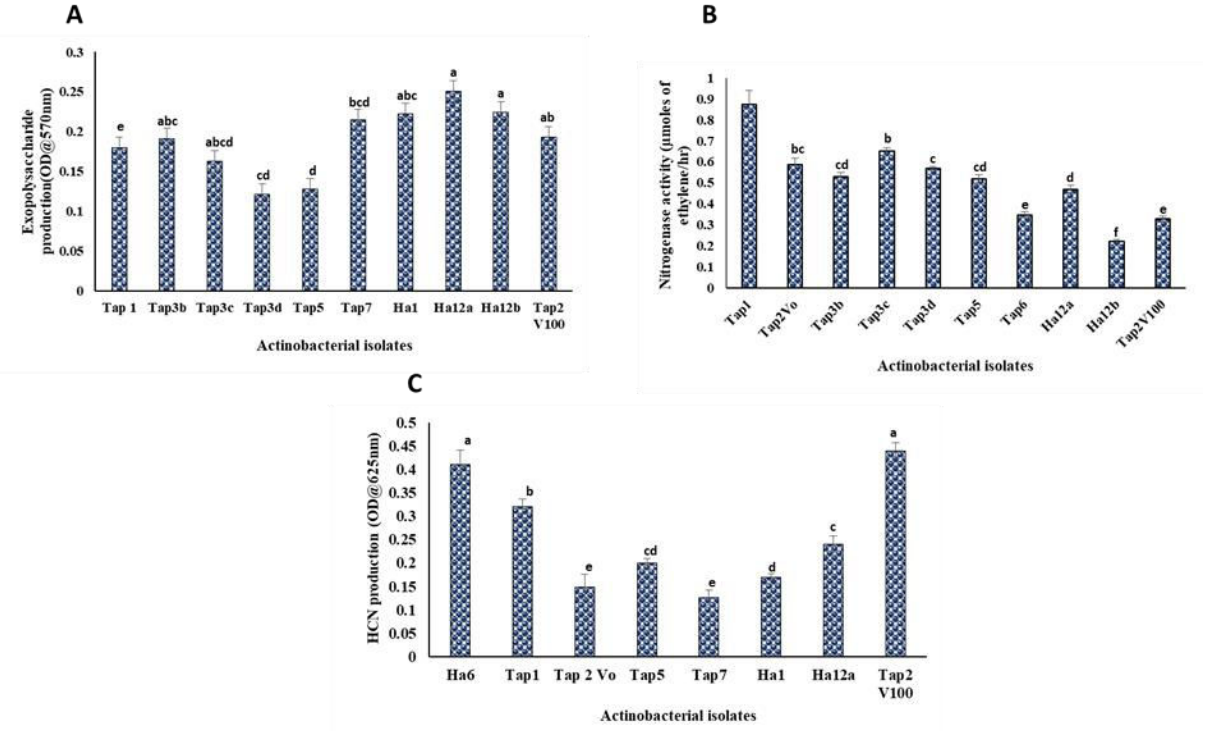


Table.2 Qualitative PGPR traits of rice endo-rhizosphere actinobacterial isolates

Isolate	Ammonia production	Growth on N-free media	P-solubilization	Siderophore production	HCN production	IAA production		ACC deaminase activity	Exopolysaccharide production
						Trp +	Trp -		
Ha6	+	-	-	+	+	+++	+++	-	-
Tap1	+	+	-	+	+	-	-	++	+
Tap 2 Vo	+	+	-	+	+	-	-	-	-
Tap3b	+	+	-	+	-	-	-	-	+
Tap3c	+	++	-	+	-	-	+	++	+
Tap3d	+	+	-	+	-	-	-	+++	+
Tap5	+	+	-	+	+	-	-	-	+
Tap6	+	+	++	+	-	-	-	-	-
Tap7	+	-	-	+	+	+	+	++	++
Ha1	+	-	-	+	++	++	+	-	++
Ha12a	+	+	-	+	+	+	+	+	++
Ha12b	++	+	-	+	-	-	-	+++	++
Tap2 V100	+	+	-	+	+	++	-	++	+

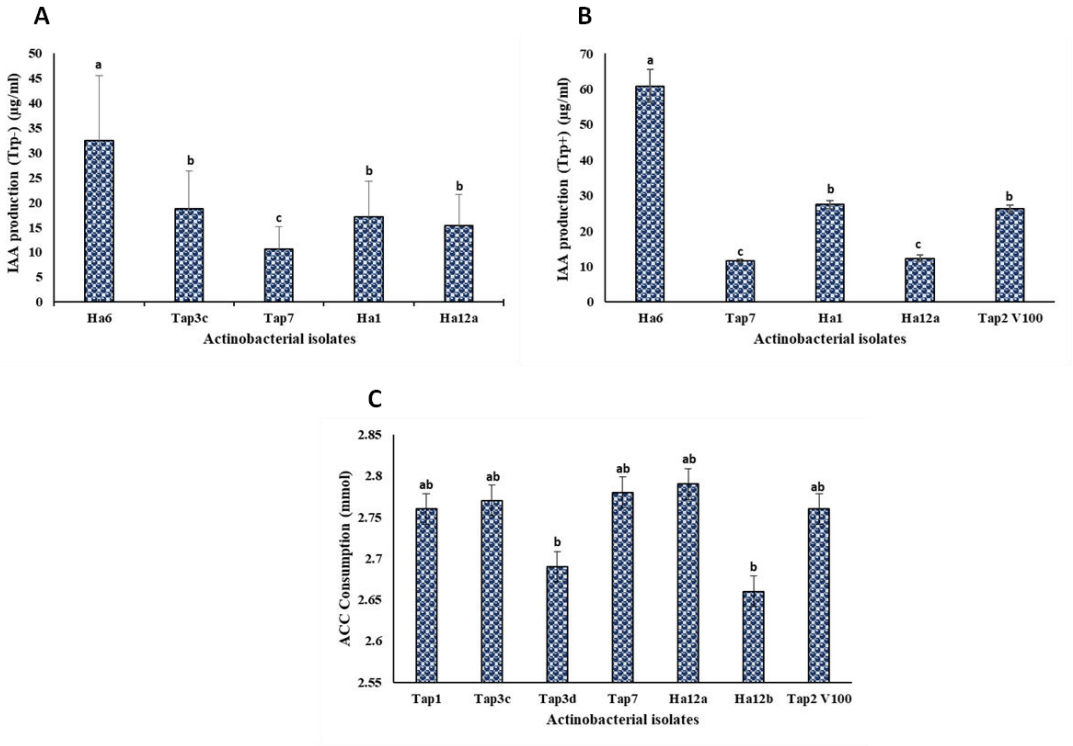
Table.3 BLAST analysis of 16S rRNA gene and taxonomic identification of salt stress tolerant rice endo-rhizosphere actinobacterial isolates

Isolates	Closest Phylogenetic type strain (NCBI-GenBank accession)	Scientific Name	Sequence similarity (%)	Sequence length (bp)	Accession number
Tap3c	*Streptomyces mutabilis* strain HBUM174166 (FJ532445.1)	*Streptomyces mutabilis*	99.89	1416	OK284534
	Streptomyces radiopugnans strain HBUM174084 (FJ486341.1)	*Streptomyces radiopugnans*	99.79		
Tap7	*Streptomyces tendae* strain ATCC 19812 (NR_025871.2)	*Streptomyces tendae*	99.93	1379	OK284535
	Streptomyces tendae strain MCCB 209 (KF454844.1)	*Streptomyces tendae*	99.93		
Tap2 V100	*Streptomyces mediolani* strain cfcc3065 (FJ792545.1)	*Streptomyces mediolani*	99.58	1421	OK284536
	Streptomyces sp. strain QJSt8 (KX950907.1)	*Streptomyces* sp.	99.58		
Ha6	*Streptomyces champavatii* strain NBRC 15392 (NR_112451.1)	*Streptomyces champavatii*	99.86%	1406	PQ479222
	Streptomyces sp. strain BC1004 (MK134629.1)	*Streptomyces* sp.	99.86%		
Tap2_V0	*Streptomyces griseus* strain S59 (JX007982.1)	*Streptomyces griseus*	100.00%	1375	PQ479226
	Streptomyces griseus DGS3 (KF704237.1)	*Streptomyces griseus*	100.00%		
Tap 1	*Streptomyces* sp. MPWIWI03_BRO3 (EF414027.1)	*Streptomyces* sp.	99%	681	PQ424990
	Streptomyces sp. strain huaiR 18 (MK641414.1)	*Streptomyces* sp.	100%		
Tap10	*Streptomyces celluloflavus* strain D4-17 (KP235209.1)	*Streptomyces celluloflavus*	99.79%	1401	PQ424989
	Streptomyces sp. HNA39 (CP077652.1)	*Streptomyces* sp.	99.79%		
Met9	*Streptomyces parvus* strain LSA-14 (KJ020692.1)	*Streptomyces parvus*	99.93%	1388	PQ479225
	Streptomyces sp. SZU-1 (KJ589645.1)	*Streptomyces* sp.	99.93%		
Ha1b	*Streptomyces hydrogenans* strain Kris3 (MT588802.1)	*Streptomyces hydrogenans*	98.69%	761	PQ424985
	Streptomyces sp. strain HBUM206419 (MT540570.1)	*Streptomyces* sp.	100%		
Tap10	*Streptomyces* sp. strain AUR2 (KX427130.1)	*Streptomyces* sp.	99.85%	690	PQ424989
	Streptomyces cavourensis strain TA23 (MH327527.1)	*Streptomyces cavourensis*	100%		

Table.4 NaCl tolerance, PGP grade and molecular identification of selected actinobacterial isolates

Isolates	NaCl Tolerance		PGPR Grade	Assigned Scientific name	NCBI Accession number
	7.5%NaCl	10%NaCl			
Tap3c	+	+	**7+**	*Streptomyces mutabilis* strain Tap3c	OK284534
Tap7	+	+	6+	*Streptomyces tendae* strain Tap7	OK284535
Tap2 V100	++	-	**7+**	*Streptomyces mediolani* Tap2 V100	OK284536

Figure.2 Quantitative estimation of PGP traits of selected salt stress tolerant rice-rhizosphere actinobacterial isolates (A) IAA production in the presence of L-tryptophan (Trp^-), (B) IAA production in the absence of L-tryptophan (Trp^+), (C) ACC consumption/deaminase activity. Values are the mean ± standard error of repeated experiments with 3 replications. The bars in graph denoted by the same alphabet indicate non-significance at P≥0.05 based on Duncan's Multiple-Range Test (DMRT).



The pH of the growth media (broth) with growing actinobacterial cultures was measured after incubation, with greater pH reduction indicating higher production of organic acids, correlating with enhanced phosphate solubilization. In the present study, only one endo-rhizosphere isolate Tap6 showed P-solubilization by reducing pH of the media under 5% NaCl salt stress which is consistent with the results of previous study that halotolerant *Streptomyces* isolate CLV179 showed P-solubilization under different concentration of NaCl of 300, 500, and 700 mM (Nozari *et al.,* 2022). Many of our isolates also showed siderophore production which is an iron chelating complex that competitively inhibit pathogen growth and improve plant survival under biotic stress conditions. These findings are consistent with the report by Verma *et al.,* (2011) who highlighted the biocontrol potential of endophytic *Streptomyces* from *Azadirachta indica* A. Juss. IAA is a derivative of the

plant hormone auxin, which promotes plant growth under salt stress conditions by supporting root elongation which could help in uptake of nutrients and water under stressful environmental condition (Egamberdieva *et al.,* 2009; Djebaili *et al.,* 2021). In the present study, IAA production was noticed a significantly increase in production when L-tryptophan is added into medium with a range of 11.65±0.36 to 60.88±4.57 µg/ml µg/ml. Similar findings by Sreevidya *et al.,* (2016) and Khamna *et al.,* (2010), who showed that the IAA production levels of rhizospheric actinobacteria varied from 3.6-14.6 µg/ml and 11.03 -144 µg/ml, respectively.

HCN production is also reported to play a role in disease suppression. Some of the recent studies indicated that metabolites produced by microbes such as HCN may enhance plant establishment in rhizosphere (Bhosal and Kadam, 2015). Previous studies also reported that siderophore-producing soil *Streptomyces* spp. which could inhibit the growth of phytopathogens through competition for iron in plant rhizosphere soils (Rungin *et al.,* 2012). All the isolates tested in this study showed siderophore production and 61% of isolates showed HCN production. These results in agreement with those reported by Boubekri *et al.,* (2021) where nine actinobacteria of genus *Streptomyces* and *Nocardiopsis* able to produce IAA, siderophore, HCN, and ammonia and significantly improved the germination rate and the vigor index of wheat.

Endophytes from roots of plants growing in saline soil producing ACC deaminase could facilitate plant growth by conversion of ACC to ammonia and α-ketobutyrate, which bacteria can consume and consequently lower the ethylene level in plants. We identified certain possible endophytes from the roots of rice plants grow in different nitrogen regime had substantial plant growth promoting properties such as Nitrogen fixation, IAA production and ACC deaminase production. In our study seven isolates were showing ACC deaminase activity ranging from 2.66-2.79 mmol of ACC consumption.

However, the results for ACC deaminase activity by endo-rhizosphere actinobacteria were consistent with previous work showing that different root-endophytic strains of genus *Streptomyces* spp isolated from cactus plant exhibits ACC deaminase activity (Govindasamy *et al.,* 2008). Passari *et al.,* (2016) also reported that eighteen endophytic actinobacterial isolates recovered from *Solanum lycopersicum* showed a ACC deaminase activity. therefore, production of different PGP traits by endo-rhizosphere actinobacteria under salt stress may enhance plant establishment under salt stress (Mohamad *et al.,* 2022; Thokchom *et al.,* 2017). In recent study by Xiong *et al.,* (2019), a halotolerant plant growth-promoting actinobacterium *Glutamicibacter halophytocola* KLBMP 5180, isolated from the root of a coastal halophyte *Limonium sinense* which showed EPS production was promoted tomato plant growth under salt stress.

In the present study, among the 13 endo-rhizosphere actinobacteria, six isolates were identified based on their partial 16S rRNA gene sequencing. The results demonstrated that most of the endo-rhizosphere actinobacterial isolates belonged to genus *Streptomyces.* The predominance of genus *Streptomyces* sp. among the root associated endophytic actinobacteria found in the present study has been reported in several previous studies from various plant species, that is, rice, wheat, sugarcane, sunflower, cacti, herbaceous and medicinal plants (Mingma *et al.,* 2018; Rungin *et al.,* 2012; Tian *et al.,* 2007; Kruasuwan *et al.,* 2016; Zahra *et al.,* 2020; Zhao *et al.,* 2011; Kim *et al.,* 2012; Govindasamy *et al.,* 2023).
Based on their ability to exhibit multiple plant growth-promoting (PGP) traits and ability to tolerate high concentrations of NaCl, three actinobacteria—*Streptomyces mutabilis* strain Tap3c, *Streptomyces tendae* strain Tap7, and *Streptomyces mediolani* strain Tap2 V100—were selected for further study to enhance rice growth under salt stress. This research lays the groundwork for the characterization and selection of potential root-endophytic actinobacteria from rice plants, aiming to develop effective inoculants for promoting rice growth in saline and salt stress conditions.

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Authors' Contribution

T Kavya: Methodology, Investigation, Formal analysis, Original draft, Writing – review & editing. Venkadasamy Govindasamy: Conceptualization, Investigation, Supervision, Writing – review & editing, Project administration, Funding acquisition. Gerard Abraham: Supervision, Writing – review & editing. Archna Suman: Investigation, Supervision, Writing –

review & editing.

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Data availability

Data will be made available on request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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