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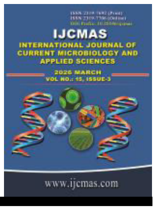

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Identification and Characterization of *Actinobacteria* from Rice-Rhizosphere for Salt Stress Tolerance and Plant Growth Promotion

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ABSTRACT

In this present study, 27 rice-rhizosphere actinobacterial isolates were screened for salt (NaCl) stress tolerance and plant growth-promoting (PGP) traits. Significant growth was observed in 24, 12, and three isolates at 7.5%, 10%, and 12.5% NaCl, respectively. Quantitative screening revealed nitrogen fixation in 13 isolates (0.65±0.04 to 28.68±0.58 µmoles ethylene/hr), phosphorus solubilization in six isolates, and siderophore, HCN, and exopolysaccharide production in 20, 25, and 16 isolates, respectively. The phytohormone, auxins (IAA) production was varied from 96.05±0.03 to 4.72±0.05 µg/ml and 90.58±0.02 to 7.52±0.01 µg/ml in the presence (19 isolates) and absence (20 isolates) of precursor L-tryptophan, respectively. Among the 18 actinobacterial isolates identified, ACC deaminase enzyme activity was reported in terms of ACC consumption in the range of 2.41±1.56 to 3.07±0.97 mmol. Among the 27-actinobactreial isolates, molecular identification of 8 isolates revealed them they are belonging to different species of actinobacterial genus *Streptomyces*. Based on the results of NaCl tolerance and PGP traits screening, three rice-rhizosphere actinobacterial isolates were selected as promising salt stress tolerant and possessing multiple-PGP traits, which were phylogenetically identified as *Kitasatospora* sp. strain Met24, *Streptomyces tritolerans* strain V04a and *Streptomyces* sp. strain Humic 11a. The halotolerant rice-rhizosphere actinobacteria identified and characterized in this study could be a promising candidate for utilization as growth stimulating bioinoculants subject to their further evaluation/investigations in the rice crop under salt stress conditions.

Keywords

Actinobacteria; *Streptomyces* spp.; PGPRs; Rice (*Oryza sativa* L.); Salt stress tolerance

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Introduction

The rhizosphere soil is hub for many of the plant growth promoting rhizobacteria as it provides a distinct ecosystem than normal soils due to root exudation which helps to proliferate higher microbial growth around the rhizosphere soils (Grayston *et al.,* 1996). In recent years, rhizosphere microorganisms, particularly the Phylum *Actinobacteria* which belongs to Gram-positive bacteria with a high G+C content in their DNA is considered as one of the key plant growth promoting rhizobacterial group. Among the actinobacterial genera *Streptomyces* spp. are most commonly found in the rhizosphere of many plants (Franco-Correa *et al.,* 2010). Other actinobacterial genera species detected in the rhizosphere of plant include *Microbispora, Nocardia*, *Kitasatospora*, *Thermobifida*, *Micromonospora, Saccharomonospora*, *Saccharothrix, Promicromonospora,* and *Streptosporangium* (Ting *et al.,* 2009; Malisorn *et al.,* 2020). Actinobacteria are also known to exhibit many PGP traits like N_2 fixation (Valdes *et al.,* 2005), siderophore production (Wang *et al.,* 2014), HCN production, phosphorus solubilization (El-Tarabily *et al.,* 2009), Phyto hormone production like indole acetic acid (IAA), cytokinins, and gibberellins (Vijayabharathi *et al.,* 2016) and also have capacity to exhibit antagonistic activity by producing antimicrobial agents and hydrolytic or detoxifying enzymes (Hu *et al.,* 2021). They have also emerged as key players in enhancing plant tolerance to various environmental stresses, including salinity, drought, high temperature and heavy metal stress (Kavya *et al.,* 2024). Actinobacteria also reported to exhibits ACC deaminase activity which helps plants to survive under environmental stress full condition (Afridi *et al.,* 2019).

Climate change-related challenges, including drought caused by water scarcity, soil salinity, salt induced edaphic stresses, and high temperature are the major abiotic stresses that will significantly reduce crop production by severely impacting the growth and yield of various crops, such as maize, rice, and sugarcane. Among cereals, tolerance to salt toxicity varies, with barley (*Hordeum vulgare* L.) being the most tolerant, followed by bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* ssp. durum), while rice (*Oryza sativa* L.) is the most sensitive to the salt induced stresses (Flowers and Yeo, 1981; Munns and Tester, 2008). Rice's high sensitivity to soil salinity and salt-induced stresses has been extensively shown to negatively impact seedling growth, seedling establishment, and key grain yield components, including spikelet and tiller numbers. These adverse effects culminate in a substantial decline in overall grain yield.

Various strategies are employed to mitigate the adverse effects of soil salinity and salt induced edaphic stresses, including the development of salt-tolerant crop varieties through classical plant breeding as well as plant genetic engineering or genome editing. These approaches aim to enhance plant resilience and improve crop productivity under abiotic stress conditions. However, a major drawback of some of these strategies is that they are time-consuming and may not provide cost-effective solutions. The use of plant growth promoting rhizosphere microorganisms as biofertilizers or their metabolites for plant growth promotion as biostimulants is considered an environmentally friendly, cost-effective, and sustainable solution for improving crop production. Owing to their plant growth-promoting characteristics, rhizosphere actinobacteria hold great potential in enhancing crop resilience under salt stress conditions. Therefore, this study focuses on the identification and characterization of actinobacterial isolates from the rice rhizosphere for their salt stress tolerance and various plant growth-promoting attributes.

Materials and Methods

Source of actinobacterial isolates

Actinobacterial isolates in the laboratory collection, which were previously isolated from rhizosphere soils of two rice varieties, BPT-5204 and Varadhan (Table S1). Both of these varieties were grown in field experimental 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.

The growth media used for the isolation of actinobacteria were, Humic acid Vitamin Agar (HVA), Tap water Yeast Extract Agar (TWYA), Tryptone Soy Agar (TSA) and Nitrate Mineral Salts Agar (NMSA) (HiMedia). These isolates were selected and used in this study by picking isolated pure colonies from the isolation media and streaking in to purification medium, Half Potato Dextrose Agar (HPDA) and further catalogued based on their morphological and cultural characteristics (Adam *et al.,* 2018).

Morphological and cultural characterization of actinobacterial isolates

The Morphological and cultural characters of all actinobacterial isolates were studied by growing 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). The morpho-cultural characterization was recorded on the Petri plates streaked with individual actinobacterial isolates and incubated for 7 days at 28+2° C in the BOD incubator. The growth of actinobacterial colonies was observed for their colony morphology and cultural characters such as colony shape, the surface texture of colonies, the colour of aerial and substrate mycelium, presence or absence of spores and its colour and soluble pigments production on the respective growth media (Coombs and Franco, 2023).

Screening of actinobacterial isolates for salt stress tolerance

The ability of all the purified actinobacterial isolates to grow at different concentrations of sodium chloride (5%, 7.5%, 10%, 12.5% and 15% NaCl w/v) was screened on Petri plates containing ISP-2 medium supplemented with respective concentration of NaCl. The osmotic potential of ISP-2 medium containing different concentration of NaCl was measured using psychrometer (PSψPRO, WESCOR INC). The actinobacterial inoculum was taken from freshly grown Petri plates and streaked on the Petri plates and incubated at 28+2° C for 7 days in the BOD incubator. The salt stress tolerance ability of actinobacterial isolates were recorded based on their growth in ISP-2 media with different concentration of NaCl.

Qualitative and quantitative screening of plant growth promoting properties/traits of actinobacterial isolates

Ammonia production

Screening of actinobacterial isolates for ammonia production was carried out by using the method followed by Islam *et al.,* (2009). All the actinobacterial isolates were tested for the production of ammonia by growing them individually in the peptone water medium. Cultures were inoculated in 5 ml peptone water and incubated at 28+2 °C for 7 days. After the incubation period was over, Nessler's reagent of 0.5 ml was added to each test tube, mixed well and kept at room temperature for 30 min. The development of yellow to orange or deep pinkish red colour was considered as a positive for ammonia production.

Actinobacterial growth on N-free media and Nitrogen fixation

The qualitative screening of all actinobacterial isolates for nitrogen fixing ability was carried out by growing them on Nitrogen free Jenson medium. The Petri plates containing this medium were streaked with freshly grown actinobacterial isolates and incubated at 28+2° C for 7 days. The actinobacterial colonies which were able to grow in the form of mucoid and slimy growth on this N-free medium were considered as putative nitrogen fixers. These positive cultures were selected and later subjected to quantitative assay for nitrogen fixation through acetylene reduction activity using Gas Chromatography (Hardy *et al.,* 1968; Dahal *et al.,* 2016). Samples for ARA were prepared in slants containing N-free Jensen medium on which the actinobacterial isolates were streaked and immediately covered with cotton plugs and incubated at 28+2°C for 5-7 days.

Then, 10% air from the tubes was replaced by acetylene through a syringe, and incubated for 24 h, 1 ml of gas was withdrawn from the tube using the syringe, and ethylene formation was analysed through a gas chromatograph (GC-2014, SHIMADZU) equipped with a flame ionization detector and a capillary column. Non-streaked slants injected with acetylene were used as a negative control.

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

Phosphate solubilization

Screening of actinobacterial isolates for mineral phosphate solubilization activity was carried out by using the method followed by Sylvester- Bradley *et al.,* (1982) as given in Beneduzi *et al.,* (2008). Plates containing GY (glucose yeast) medium were streak inoculated with actinobacterial isolates and incubated at 28+2° C for 7 days. The formation of clear halo zones on

GY medium around the actinobacterial colonies was considered as positive for mineral phosphate solubilization. Further positive cultures are evaluated for quantitative P-solubilization. Selected actinobacterial isolates are inoculated in 5ml of GY broth which was adjusted to pH 7.2 ± 0.2 and incubated in the shaker at 28+2ºC for 7 days. Then, the pH of the broth was measured. More the reduction in the pH by isolates indicates more production of organic acids which relates indirectly to high phosphate solubilization ability.

Siderophore production

Siderophore production by actinobacterial isolates was detected by the universal method using blue (CAS) agar plates containing the dye chrome azurol S (CAS). The molten medium was poured into sterilized Petri plates and allowed to solidify. After 24hrs the actinobacterial isolates was spotted (streaked) on the CAS agar containing grid plates and incubated at 28+2º C for 7 days. The presence of iron chelator (siderophore production) was indicated by decolourization of the blue-coloured ferric dye complex, resulting in to yellowish orange halo zones around the colonies by the actinobacteria positive for siderophore production (Schwyn and Neilands, 1987).

Hydrogen Cyanide (HCN) production

Hydrogen cyanide producing activity was determined according to the protocol of Baker and Schippers, (1987). Actinobacterial isolates were tested for their ability to produce hydrogen Cyanide production using king's B medium, Petri plates containing king's B medium supplemented with 4.4 g of glycine per litre were streaked with individual actinobacterial isolates and closed with a lid containing filter paper soaked in picric acid solution (0.5% picric acid prepared in 2% sodium carbonate).

The parafilm sealed plates were incubated at 28+2º C for 5 to 7 days. A change in the colour of filter paper from yellow to orange-brown indicated as positive for hydrogen cyanide production by the actinobacterial isolates. Then, filter paper was cut into small pieces and dipped in a 5 ml of autoclaved distilled water and kept in the shaker at 28+2º C for 3-5 days. After the incubation period was over, spectrophotometric absorbance (HALO DB-20S, Dynamica) for the supernatant of filter paper immersed tubes was measured at 625 nm.

Auxins or Indole 3-acetic acid (IAA) production

Actinobacterial isolates for their ability to produce auxins or indole acetic acid (IAA) were quantified by inoculating the cultures in two set of tubes, one set containing 5ml of ISP-2 broth supplemented with 10 mg ml^{-1} L-tryptophan and another set without L-tryptophan. The inoculated tubes were incubated at 28+2º C for 7 days under shaking conditions. After incubation, bacterial cultures were transferred into sterile Eppendorf tubes and centrifuged at 5000 g (3K30, Sigma) for 15 min. Subsequently, 2ml of the supernatant was collected and transferred to 15ml test tubes to this 2-3 drops orthophosphoric acid, and 4ml of Salkowsky reagent was added. The contents in the tubes were incubated at room temperature under dark conditions for 20-30 min. After the incubation time was over, spectrophotometric absorbance (HALO DB-20S, Dynamica) for the reaction mixture was measured at 530 nm (Ndeddy Aka and Babalola, 2016).

ACC deaminase activity by ACC-Ninhydrin assay

ACC deaminase activity of actinobacteria was indirectly estimated by measuring the consumption of ACC-provided as a sole N-source in the medium. Actinobacterial isolates were inoculated in ISP-2 broth and incubated in incubator shaker (180 rpm) at 28+2º C for 5 days. The fully grown cultures were centrifuged at 8000 rpm (3K30, Sigma) at room temperature for 10 min and actinobacterial cell pellets were washed thrice with sterile DF medium. Washed cell pellets were resuspended in DF medium supplemented with ACC solution (3 mmol L^{-1}) and incubated at 30º C in an incubator shaker at 200 rpm for 48h. From each of these cultures, 1mL of culture fluid was centrifuged at 8000 rpm at room temperature (25 ºC) for 10 min and 100µL of supernatant was diluted to 1 ml with DF medium. To this, 2ml of ninhydrin reagent was mixed in the test tubes and kept in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min, and spectrophotometric absorbance (HALO DB-20S, Dynamica) was measured at 570 nm. Leftover ACC in the bacterial grown DF liquid medium was quantitatively estimated by developing a standard curve for ACC. The amount of ACC consumption by the individual actinobacterial isolates was calculated from the initial ACC concentration (3.0 mmol L^{-1}) of the DF medium (Li *et al.,* 2011).

Biofilm production

The actinobacteria grown in ISP-2 broth was pelleted by centrifugation at 8000 rev.min^{-1} for 2 min and washed with sterile distilled water. Next these cells were resuspended in the same medium and maintained at OD600 = 0.2. An aliquot (150 µL) of bacterial cell suspension was added to individual wells in a 96-well polyvinyl chloride (PVC) plate, this was incubated at 28°C for 48 h. After the incubation the medium was removed and the wells were washed with sterile distilled water and treated with 150µL of 0.001% crystal violet for 15 min. The excess of dye was removed and the wells were again washed with sterile distilled water. The retained stain was solubilised with 150µL of 95% ethanol and amount of dye was quantified by plate reader at absorbance of 570 nm (Verhoef *et al.,* 2003).

Molecular identification and phylogenetic analysis of the selected actinobacterial isolates

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

The selected actinobacterial isolates were grown in ISP-2 medium over laid with cellophane in Petri dishes for 7 days and then 2-3 loop full of actinobacterial mycelium was transferred and resuspended in a 1.5 ml Eppendorf tube containing 500 µl of Tris-EDTA (TE) and total DNA was extracted following the method by Coombs and Franco (2003). Genomic DNA extracted from all the selected actinobacterial was confirmed by agarose gel electrophoresis (0.8%) and observed under UV transilluminator. Further the purity and yield (ng/µl) were determined using a NanoDrop Spectrophotometer.

Actinobacterial identity was confirmed by amplification of the 16S rRNA gene using the universal 16S rRNA primers for bacteria 27F (5' AGAGTTTGATC MTGGCTCAG 3') and 1492R (5' TACGGYTACCTTG TTACGACTT 3') (Lane, 1991). Amplifications were performed in a final volume of 25 µl consisting of 1µl DNA template, 5µl of 5X Taq buffer, 2.5 µl of 1mM dNTPs mixture, 0.5 µl of each primer, 2.5 µl of 25mM $MgCl_2$, 0.2 µl of 2 U Taq DNA polymerase and 12.2 µl of nuclease-free water. The PCR mixture was subjected to 35 cycles in a thermal cycler (HiMedia) with an initial denaturation temperature at 95°C for 5 min; denaturation at 94°C for 1 min; annealing temperature at 57°C for 1 min; extension at 72°C for 1.30 min and a final extension at 72°C for 7 min.

16S rRNA gene sequencing and phylogenetic analysis

Purification and sequencing of PCR products were performed by Barcode Biosciences company. 16S rRNA genes sequence of selected actinobacterial isolates homology analysis was carried out using the Basic Alignment Search Tool (BLASTn) search obtained in the NCBI database (www.ncbi.nlm.nih.gov) for the identification of actinobacteria. Sequences obtained were deposited in GenBank. For the phylogenetic analysis, 16S rRNA gene sequences of relevant and previously described actinobacterial strains (type species or type strains) from the GenBank-NCBI were selected for comparison. All the 16S rRNA gene sequences were aligned by ClustalW and sequence errors were checked manually. Phylogenetic analyses were conducted using MEGA 11 software (Tamura *et al.,* 2007) and a Maximum-Likelihood (ML) phylogenetic tree with a bootstrap value 1000 replicates was generated.

Data analysis

All quantitative data obtained from this study were analysed by one-way analysis of variance (ANOVA) using the software WASP version 2.0. The significant mean differences among the treatments of different experiments in this study were compared by performing New Duncan's Multiple Range Test using the software WASP version 2.0.

Results and Discussion

Morphological and cultural characterization of actinobacterial isolates

Twenty-seven actinobacterial isolates purified from rice rhizosphere soils (Table S1) were characterized based on their different morphological and cultural characters using six- different media such as Half Potato Dextrose Agar (HPDA), Yeast Malt Agar (ISP2), Oatmeal Agar (ISP3), Inorganic Salt Starch Agar (ISP4), Glycerol Asparagine Agar (ISP5), and Tyrosine Agar (ISP7) and the results are given in the Table S2. Most of the rhizosphere actinobacterial shown slow growth and are rough colony in nature. The colony shapes were varied viz., round elevated, raised irregular, minute sunken, irregularly raised and so on. In parallel, the surface texture of the colonies also varied from rough to smooth, powdery, and slimy. The colour of substrate mycelium observed were pink, white, green, yellow to light orange. Likely, the colour of aerial mycelium was like pinkish with white spore, pure white, grey spores and yellow to light orange. On HPDA medium, three actinobacterial isolates Met11a, Met26 and Burk2 produced soluble pigments of Pink, Yellow and Greenish in colours, respectively. On ISP-2 medium, three isolates TSA-1, TSA-2 and Burk 2 produced soluble pigment of beige, light brown and green colour, respectively. Only one isolate Met11a was shown pinkish soluble pigments production in the ISP-3 medium and none of the actinobacterial shown any visible soluble pigments production in the ISP-4 medium. While in the ISP-5 medium, the two isolates (Vo4a, Vo4b) were shown to produce soluble pigments of the colour brownish and dull yellow, respectively. In the ISP-7 medium, soluble pigments of Pink, Yellow and Brownish in colours was produced by Met11a, Met11b and Met26, respectively (Table S2).

Screening of rice-rhizosphere actinobacterial isolates for salt stress tolerance

All the 27 rhizosphere actinobacterial isolates were screened for their salt tolerance based on their growth in ISP-2 medium supplemented with 5%, 7.2%, 10%, 12.5% and 15% of NaCl concentrations (Fig. 1). All the 27 rhizosphere actinobacterial isolates were able to grow at 5% NaCl in the ISP-2 supplemented growth medium. Among the 27 actinobacterial isolates 24 isolates were able to show growth under 7.5% NaCl concentration. Only 12 and 3 actinobacterial isolates were shown a significant growth at 10% NaCl and at 12.5% NaCl concentrations, respectively. The three actinobacterial isolates which shown a growth at high NaCl concentrations of 12.5% were Tap14, Met26 and Ha10. None of the actinobacterial isolates were shown growth at 15% NaCl concentration (Table 1).

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

Ammonia Production

In the present study, 26 rhizosphere actinobacterial isolates were able to show ammonia production under salt stress of 5% NaCl (Fig. 2). Among them, 11 isolates were observed as highest ammonia producers as they changed peptone water colour from yellow to deep pinkish or red and 15 isolates were lowest to medium producers of ammonia as they shown the colour change from yellow to orange or pink/purple. The ammonia production was negative for actinobacterial isolate, Ha2 as there was no detectable colour change observed in the inoculated peptone water as it remains yellow even after 7 days of incubation (Table 2).

Actinobacterial growth on N-free media and N-fixation by Acetylene reduction assay

In this study, initially all the 27 rhizosphere actinobacterial isolates were screened for their ability to fix nitrogen by using N-free Jensen medium in presence of 5% NaCl salt stress (Fig. S2). Among the 27 isolates, 13 isolates were showing considerable growth on N-free Jensen medium on 7th day of incubation (Table 2). All these 13 isolates putatively positive for N-fixation trait were further confirmed and quantified for nitrogen fixing ability through acetylene reduction assay using gas chromatography. The significantly highest ($p<0.05$) nitrogenase activity was recorded in the actinobacterial isolate Burk2 (28.68±0.58 µmoles of ethylene/hr) followed by isolate Met5 (27.43±0.72µmoles of ethylene/hr), Met24 (11.07±0.77µmoles of ethylene/hr) and Ha11 (10.35±1.34µmoles of ethylene/hr). While isolates Humic11b (0.52±0.03µmoles of ethylene/hr), Humic11a (0.58±0.03µmoles of ethylene/hr) and Met22a (0.65±0.04µmoles of ethylene/hr) were showed a significantly lower ARA activity (Fig. 3A).

P-solubilization

All the actinobacterial isolates were screened for their mineral phosphate-solubilization potential using GY medium (Fig. S2). Among the 27 rhizosphere actinobacterial isolates, six isolates namely Tap6, V07, Met11, Burk1, Burk2, and Humic11a were able to solubilized the phosphorous by forming a clear zone around the colonies on GY media plates (Table 2).

Among these six-positive P-solubilizing actinobacterial isolates, the isolate Burk2 showed highest reduction of growth medium pH from 6.89±0.51 to 5.22 ±0.07 followed by Humic11a, Met11 and Tab 6 with the reduction in growth medium pH of 5.04±0.19, 4.97±0.17 and 4.95±0.37, respectively. The least reduction in growth medium pH was observed with the isolate Burk1 and the result was shown in Fig. 3B.

Siderophore and HCN production

Siderophore producing ability of all the actinobacterial isolates was ascertained in solid CAS agar medium under *in vitro* conditions. Out of 27 rhizosphere actinobacterial isolates, 20 isolates were able to produce siderophores as shown by formation of yellow to orange colour hallow around the colony as shown in the Fig. 2 & Table 2. Among the 27 rhizosphere actinobacterial isolates, 25 isolates were shown positive for HCN production in the presence of 5% NaCl salt stress (Fig. 2 & Table 3).

All the isolates showed the significant variation in supernatant colour orange-brown-deep brown by their ability to produce HCN as depicted by their OD value at @625nm**.** The significantly highest HCN production was observed by the isolate Met22b (1.06±2.31) followed by Met11b and Ha2b (0.41±1.69, 0.42±0.06). The 5 isolates Met11a (0.13±1.20), Tap9 (0.14±0.02), Tap14 (0.14±0.03), Tap6 (0.14±0.30) and V07 (0.12±0.79) were similar in their ability of HCN production. The significantly lowest HCN production was observed by the two rhizosphere actinobacterial isolates TSA-1 (0.10±0.03).and Tap25 (0.10±0.06) (Fig. 3C).

Auxins (IAA) Production

All the rice-rhizosphere actinobacterial isolates are quantitatively analysed for auxins (IAA) production both in the presence and absence of precursor metabolite, L-Tryptophan under 5% NaCl salt stress condition (Fig. 2). IAA production by the tested actinobacterial isolates was varied from 96.05±0.03 to 4.72±0.05 µg/ml and 90.58±0.02 to 7.52±0.01 µg/ml in the presence and absence of precursor L-tryptophan, respectively. Two of the isolates Tap 6 and TSA-1 showed a maximum indole-3-acetic acid production of 96.56± 0.23 µg/ml and 96.05± 1.20 µg/ml in the presence of L-tryptophan, respectively (Fig. 4A). Whereas in the absence of L-tryptophan, the isolate Tap6 showed maximum indole-3-acetic acid production (90.58±0.41 µg/ml) followed by the isolates Ha10 (79.00± 1.84 µg/ml) and TSA-1 (74.61±1.45 µg/ml) (Fig. 4B). However, significantly lowest IAA production was observed for the isolates, Met11a (4.72±0.05 µg/ml) and Tap17 (7.52±0.14 µg/ml) in the presence and absence of tryptophan, respectively.

ACC deaminase activity

Among the 27 rice-rhizosphere actinobacterial isolates, there were 18 isolates found positive for ACC deaminase activity (Table. 3). In the quantification based on ninhydrin assay for the ACC deaminase activity, there was no significant variation in the utilization of ACC as a sole nitrogen source was recorded in all these actinobacterial isolates (Fig. 2). The amount of ACC consumed by these isolates was ranged from 2.41±1.56 mmol to 3.07±0.97 mmol of ACC. Highest or full concentrations of ACC (3 mmol) consumption was observed for the actinobacterial isolates Met11b (3.00±0.29 mmol ACC), Met11 (3.00±0.23 mmol ACC) and Met 5 (3.00±0.24 mmol ACC) followed by the isolates Ha2b, Humic11b, Tap17, Ha10 and V04a with the consumption of 2.99±0.35, 2.91±0.21, 2.89±0.01, 2.87±0.18 and 2.54±0.34 mmol of ACC, respectively. The actinobacterial isolate V07 was consumed lowest ACC concentration of about 2.41 mmol (Fig. 4C).

Biofilm production

Among the 27 actinobacterial rhizosphere isolates, 16 were able to produce biofilm (Fig. 2 & Table. 2). Among them, two of the isolates Tap9 and Met24 were shown highest biofilm production production with the OD value of 0.22±0.008 and 0.20±0.01, respectively, and the remaining 14 isolates were shown low to medium amount of biofilm production with the OD value ranging from 0.17±0.008-0.10±0.007. The least amount of biofilm production was observed in the two isolates, Met11a and Ha2b (Fig. 4D).

Taxonomic identification and Phylogenetic analysis of actinobacterial isolates by 16s rRNA Gene Sequencing

Among the 27 rhizosphere isolates, eight rice-rhizosphere actinobacterial isolates *viz*., Met24, Vo4a, Humic11a, Burk2, Tap9, Ha11, V07, and Humic11b were selected based on their high salt stress tolerance along with possessing multiple PGP traits for taxonomically identification. From these isolates the total genomic DNA were extracted and the 16S rRNA gene (~1500bp length) were amplified using PCR. Further, molecular identification for these isolates were carried out through 16S rRNA gene sequencing and BLASTn analysis (Table 3). BLASTn homology results using NCBI-GenBank database revealed that the 16S rRNA gene sequences of these isolates identity to their closest phylogenetic type strains of actinobacterial genera such as *Kitasatospora* sp and *Streptomyces* spp.

with high sequence similarity. The isolate Met24 showed a highest similarity to the closest phylogenetic type strain of *Kitasatospora* sp. strain SKW16 (99.79% sequence similarity). Whereas, the isolate Vo4a showed a highest similarity to the closest phylogenetic type strain of *Streptomyces tritolerans* strain YF11 (99. 69% sequence similarity). Another, isolate Humic 11a showed highest similarity to the closest phylogenetic type strain of *Streptomyces* sp. strain with 99.86% sequence similarity. The isolate Burk2 showed highest similarity to its phylogenetic type strain *Streptomyces nigra* strain 452 with 99.93% sequence similarity. Isolate Tap9 was showed a highest sequence similarity to *Streptomyces fimicarius strain* IHB B 12014 (97.54%). Both partial and near complete 16S rRNA gene sequences of all these eight actinobacteria isolates were submitted to GenBank database of NCBI under the following accession numbers: OK284531 (Met 24), OK284532 (Vo4a) and OK284533 (Humic 11a) PQ479223 (Burk2), PQ496492 (Tap9) (Table.5).

The near complete 16S rRNA gene sequences of the five actinobacterial isolates selected in this study along with the already known isolates and type strains were used to construct the molecular phylogeny. The phylogenetic tree of the rice-rhizosphere actinobacterial isolates was rooted to *Bacillus subtilis* strain AHV-KH18 and *Nocardia* sp. strain YC222 as an out-group taxon and evolutionary history was inferred based on the Maximum-Likelihood (ML) method (Fig. 5). The molecular phylogeny revealed that all the five isolates selected in this study formed clades distinct from the other actinobacterial isolates and type strains of *Streptomyces* spp. Molecular evolutionary lineage analysis discloses that *Streptomyces* sp. strain Humic 11a and *S. fimicarius* strain Tap9 identified in this study exhibited closest phylogenetic relationship with *Streptomyces* sp. strain STR2 and *S. fimicarius* strain IHBB12014, respectively and grouped in an distinct clade (clade-II) suggesting their monophyletic origin. The three rhizosphere actinobacterial isolates of *Kitasatospora* sp. strain Met24, *Streptomyces nigra* strain Burk2 and *Streptomyces tritolerans* strain V04 formed distinctly a separate clade (Clade-III) (Fig. 5). Based on the results of NaCl tolerance and PGP traits screening, there were three rice-rhizosphere actinobacterial isolates selected as promising salt stress tolerant and possessing multiple-PGP traits. These three selected rice-rhizosphere actinobacterial isolates, Met24, Vo4a and Humic11a were phylogenetically identified as *Kitasatospora* sp. strain Met24, *Streptomyces tritolerans* strain V04a and *Streptomyces* sp. strain Humic 11a, respectively (Table 4).

Among the plant niches, rhizosphere in particular is known as a diversity hotspot with the range of beneficial microbial interactions happening over there. Thus, helps in maintaining soil health, promoting plant growth and fitness under the stressed environments such as drought, salinity. Rice is one of the most important cereal crops and extensively distributed plant species. Next after to drought, the edaphic stresses including soil salinity and salt induced stresses are major limiting factor for rice production both under upland and low land system (Kumawat *et al.,* 2022). Rice plant-rhizosphere also harbours many actinobacteria which are of significance growth promotion in rice plants and are of great importance for ecological reconstruction of rice-soil affected with salinity and salt induced stresses. However, studies on rice-actinobacterial interactions especially salt-stress tolerance coupled with plant growth promotion by the actinobacteria associated from rice-rhizosphere are still scarce.

In this present study, we used 27 rice-rhizosphere actinobacterial isolates of our laboratory collections, which were previously isolated and purified from two rice-genotypes *viz*., BPT-5204 and Varadhan. Initially, these isolates were characterized for their morphological and cultural differences with respect to colony growth and shape, colour of the substrate and aerial mycelium, spore colour and production of soluble pigments by growing them on 6 different actinobacterial growth media HPDA and ISP-2 to ISP-7 (Mohamed *et al.,* 2017; Adam *et al.,* 2018). Chukwuneme *et al.,* (2020) studied with seven actinobacteria, which were isolated from maize rhizosphere soils and characterized based on the morphology of the colonies, their ability to form aerial hyphae, and substrate mycelium as well as their biochemical properties on ISP-3 medium. The result of the study of 9 actinobacteria isolated from soil of Ujung Pancu area, Aceh Besar conducted by Fitri *et al.,* (2021) showed that actinobacteria grown on ISP-2 medium had brown, red, white, yellow, and tawny colour substrate and aerial mycelium, isolates grown on ISP-3 medium had grey, cream, pink, white brownish white, blackish green and black colour.

Table.1 Screening of rice-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)
Met11a	+	+	+	-	-	-
Met11b	+	+	+	-	-	-
Ha4	+	+	-	-	-	-
Tap9	+	+	+	+	-	-
Tap14	+	+	+	+	++	-
Met26	+	+	+	+	+	-
Tap6	+	+	+	+	-	-
Ha2	+	+	++	-	-	-
TSA-1	+	+	+	+	-	-
Vo 7	+	+	++	-	-	-
Met11	+	+	+	+	-	-
Vo4a	+	+	+	+	-	-
Vo4b	+	+	+	+	-	-
Ha2b	+	+	-	-	-	-
Met5	+	+	+	-	-	-
Tap25	+	+	-	-	-	-
TSA-2	+	+	++	-	-	-
Burk1	+	+	++	-	-	-
Burk2	+	+	+	+	-	-
Tap17	+	+	+	-	-	-
Met24	+	+	+	+	-	-
Ha11	+	+	++	-	-	-
Ha10	+	+	+	+	+	-
Humic11a	+	+	+	+	-	-
Humic11b	+	+	+	-	-	-
Met22a	+	+	++	-	-	-
Met22b	+	+	+	-	-	-

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

Table.2 Qualitative screening of rice-rhizosphere actinobacterial isolates for different PGP traits

Isolate	Ammonia production	Growth on N- free media	P-solubilization	Siderophore production	HCN production	IAA production		ACC deaminase activity	Exopolysaccharide production
						Trp +	Trp -		
Met11a	+	-	-	-	+	-	+	-	+
Met11b	+	-	-	-	+	+	+	+	-
Ha4	++	-	-	-	-	+	+	-	+
Tap9	++	-	-	-	+	+	-	-	++
Tap14	++	-	-	+	+	++	+	-	-
Met 26	++	-	-	-	-	++	++	-	-
Tap6	+	-	+	+	+	+++	+++	-	+
Ha2	-	-	-	+	+	+	-	-	-
TSA-1	+	++	-	+	++	+++	+++	-	+
V07	++	-	+	+	++	+	+	+	-
Met11	++	++	+	+	+++	+++	++	+	-
Vo4a	++	+	-	+	+	++	+	++	-
Vo4b	+	-	-	-	+	++	-	+++	-
Ha2b	++	-	-	-	+	+	-	+++	+
Met5	+	+	-	+	+	+	+	++	+
Tap25	+	-	-	+	+	+	+	++	-
TSA-2	++	++	-	+	+	++	++	++	+
Burk1	+	++	+	+	+	+	-	+++	+
Burk2	+	++	+	+	+	-	-	-	+
Tap17	+	++	-	+	+	+	-	+++	-
Met24	++	+	-	+	+	+	+	++	++
Ha11	+	++	-	+	+	+	+	+++	+
Ha10	+	-	-	+	+	++	+++	+++	+
Humic11a	+	+	+	+	++	+	++	+++	-
Humic11b	++	+	-	+	++	-	+	+++	+
Met22a	+	+	-	+	++	-	-	+++	+
Met22b	+	-	-	+	+	-	-	-	+

Note: +Less efficient, ++ efficient, +++More efficient, - No activity

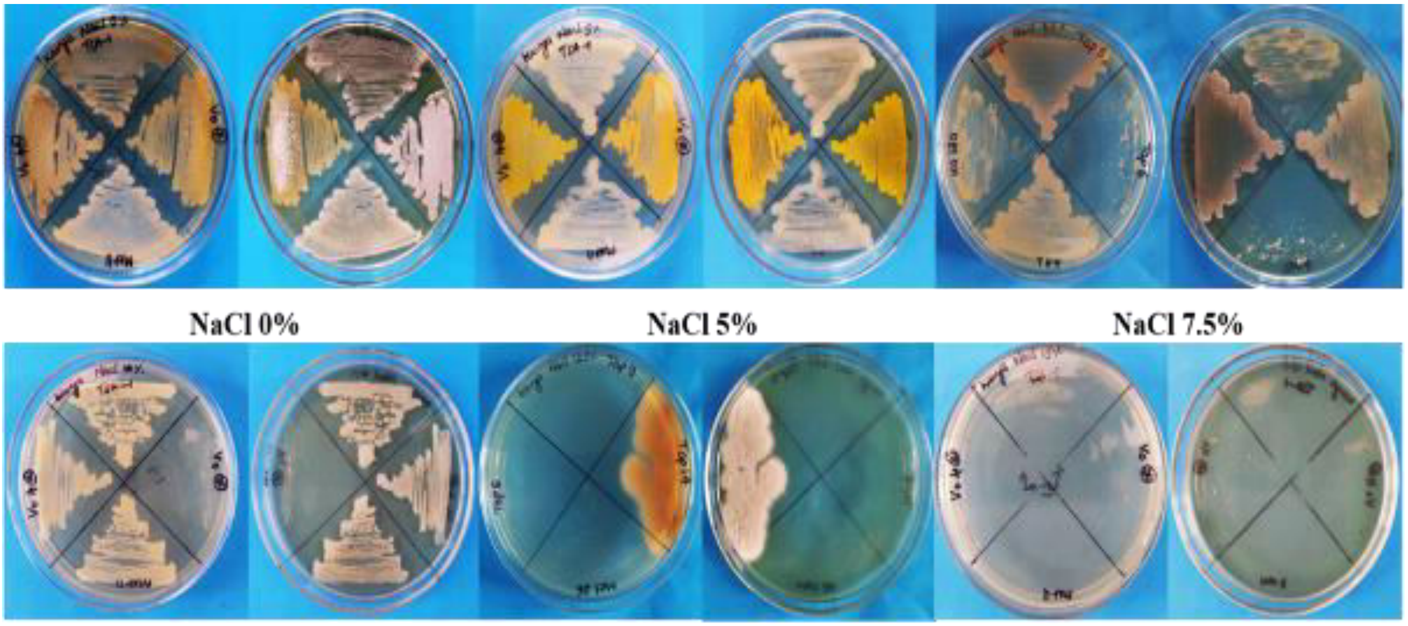
Table.3 BLAST analysis of 16S rRNA gene and taxonomic identification of the selected salt stress tolerant rice-rhizosphere actinobacterial isolates

Isolates	Closest Phylogenetic type strain (NCBI-GenBank accession)	Scientific Name	Sequence similarity (%)	Sequence length (bp)	Accession number
Met 24	*Kitasatospora* sp. strain SKW16 (MN116554.1)	*Kitasatospora* sp.	99.79	1415	OK284531
	Kitasatospora albolonga strain NBRC 13465 (NR_041144.1)	*Kitasatospora albolonga*	99.72		
V04a	*Streptomyces tritolerans* strain YF11 (MG334128.1)	*Streptomyces tritolerans*	99.69	1291	OK284532
	Streptomyces sp. UKMCC_PT6 (JF950275.1)	*Streptomyces* sp.	99.61		
Humic 11a	*Streptomyces* sp. strain STR2 (MT568583.1)	*Streptomyces* sp.	99.86	1428	OK284533
	Streptomyces sp. strain AUR2 (KX427130.1)	*Streptomyces* sp.	99.86		
Burk2	*Streptomyces nigra* strain 452 (MG572975.1)	*Streptomyces nigra*	99.93	1400	PQ479223
	Streptomyces sp. strain HNS4 (MF397911.1)	*Streptomyces* sp.	99.93		
Tap9	*Streptomyces fimicarius* strain IHB B 12014 (KF475818.1)	*Streptomyces fimicarius*	97.54	1398	PQ496492
	Streptomyces sp. IP51-3 (EU181253.1)	*Streptomyces* sp.	97.34		

Table.4 NaCl tolerance, PGP traits grade and molecular identification of three selected salt stress tolerant rice-rhizosphere actinobacterial isolates

Isolates	NaCl Tolerance		PGP traits Grade	Assigned Scientific name	NCBI Accession number
	7.5%NaCl	10%NaCl			
Met24	+	+	6+	*Kitasatospora* sp. strain Met24	OK284531
Vo4a	+	+	7+	*Streptomyces tritolerans* strain Vo4a	OK284532
Humic 11a	+	+	7+	*Streptomyces* sp. strain Humic11a	OK284533

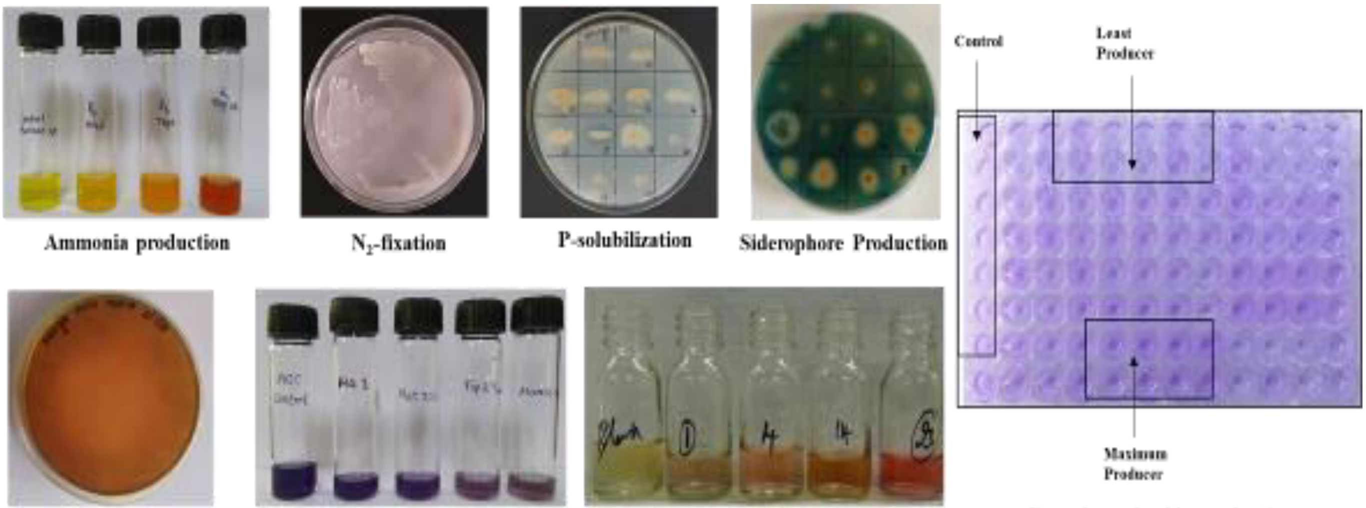
Fig.1 Representative images of screening rice-actinobacterial isolates for salt stress tolerance on ISP-2 medium supplemented with different concentrations of NaCl



NaCl 0% **NaCl 5%** **NaCl 7.5%**

NaCl 10% **NaCl 12.5%** **NaCl 15%**

Fig.2 Representative images showing qualitative screening of rice-actinobacterial isolates for different PGP traits.



Ammonia production **N_2-fixation** **P-solubilization** **Siderophore Production**

HCN Production **ACC deaminase activity** **IAA Production** **Exopolysaccharide production**

Fig.3 Quantitative estimation of PGP traits of selected salt stress tolerant rice-rhizosphere actinobacterial isolates (A) N-fixation by ARA, (B) P-solubilization 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).

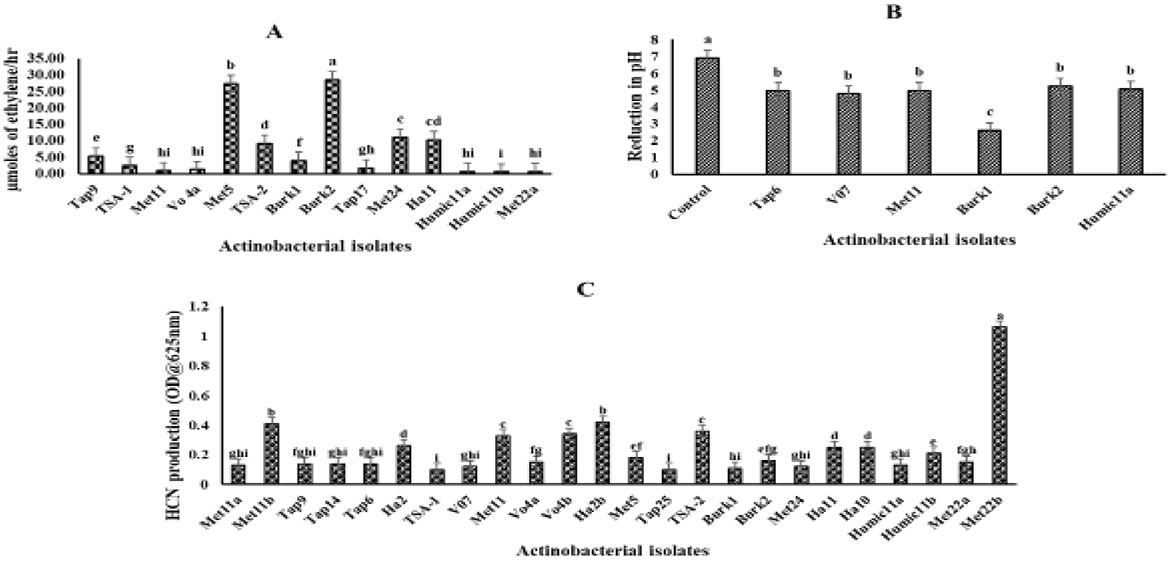


Fig.4 Quantitative estimation of PGP traits of selected salt stress tolerant rice-rhizosphere actinobacterial isolates (A) IAA production in the presence of L-tryptophan (Trp$^+$), (B) IAA production in the absence of L-tryptophan (Trp$^-$), (C) ACC consumption/deaminase activity and (D) Exopolysaccharide 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).

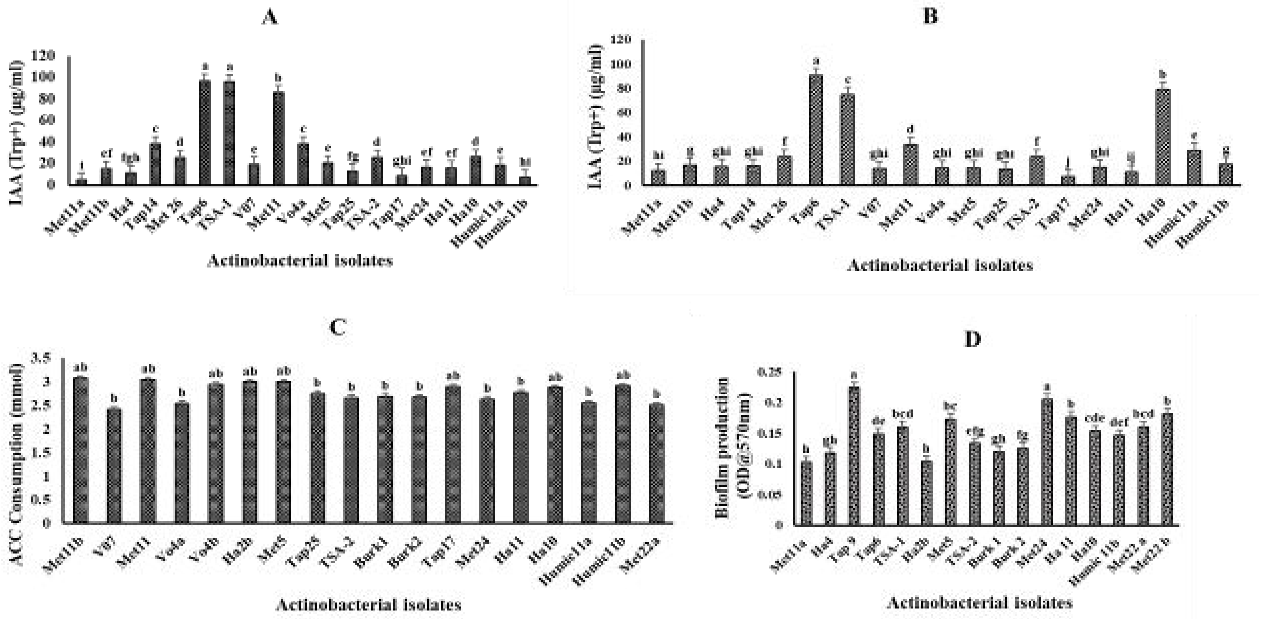
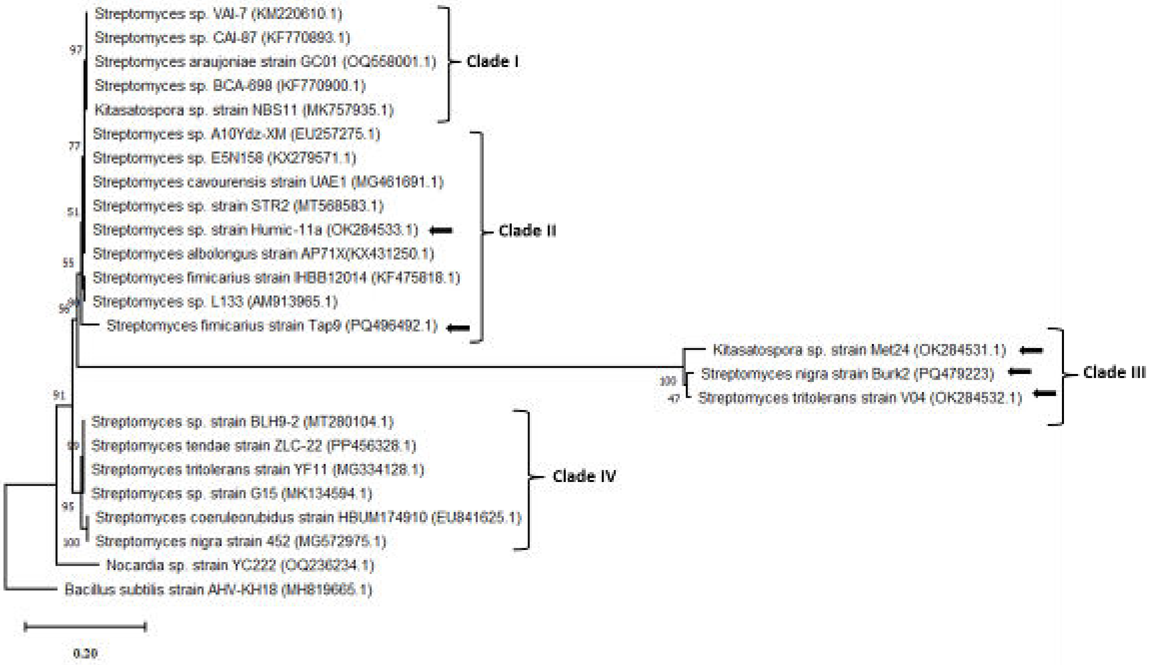


Fig.5 Maximum-Likelihood (ML) tree drawn based on 16S rRNA gene sequences showing the phylogenetic relationship among the selected salt stress tolerant rice-rhizosphere actinobacterial isolates along with their closest type strains and other actinobacterial genera from NCBI-GenBank database.



Similar results on morphological character were also observed by Priya *et al.,* (2020) while studying actinobacterial culture *Streptomyces matensis* (JJCTSPA1) and *Streptomyces chilikensis* (JJCTSPA2) on different ISP medium from ISP1-ISP7.

Further in the screening for salt (NaCl) stress tolerance, these 27 rice-rhizosphere actinobacterial isolates exhibited their growth differences in ISP2 medium supplemented with different NaCl concentrations from 5% (Ψ_S = -2.667) to 15% (Ψ_S = -4.568) and results highlighted the variation in salt stress tolerance ability among the rice-rhizosphere actinobacteria. For example, in this study 89% and 44% actinobacterial isolates shown salt stress tolerance ability by their growth at 7.5% and 10% of sodium chloride concentration, respectively. Interestingly, only three actinobacterial isolates shown salt stress tolerance ability at high NaCl concentrations of 12.5% and none of the actinobacterial isolates were shown growth at 15% NaCl concentration. Many previous studies by different researchers have reported salt tolerance of actinobacteria from 0-15% NaCl (Jiang *et al.,* 2011; Thumar and Singh, 2009). Some *Streptomyces* strains isolated from coastal rhizosphere *Streptomyces* sp. KLBMP S0051 was tolerated up to 5%NaCl (Gao *et al.,* 2022). *Streptomyces* strain D2-8 isolated from the rhizosphere of the halophyte *Phragmite australis* was able to show growth in the 10% NaCl and 120 mM soda saline and alkaline solution (Gong *et al.,* 2018). Considering our rice rhizosphere actinobacteria 11.1% of the isolates were grew profusely at different high NaCl concentrations (up to 12.5%). Therefore, these isolates may possess genes that give them the ability to synthesize compatible solutes or any other osmolytes to balance the osmotic potential of their cells under highly salt stress conditions (Empadinhas and Costa, 2008; Romano-Armada *et al.,* 2020).

In the present investigation, PGPR traits-characterization of all the 27 rice-rhizosphere actinobacterial isolates exhibited multiple plant beneficial activities with varying potential, such as the ammonia production (96.29%), growth on N-free media (48.1%), phosphate-solubilization activity (22.2%), siderophore production (74.04%), HCN production (92.6%), IAA production (88.8%), ACC deaminase activity (62.96%), exopolysaccharide production (59.25%). The present findings are in consistent with prior researcher Djebaili *et al.,* (2021), who investigated 14 strain of the genus *Streptomyces* and *Nocardiopsis* for different PGP traits as mentioned above under different concentration of NaCl. Ammonia production and atmospheric N-fixation is one of the most common plant beneficial mechanisms shown by PGP rhizobacteria (Govindasamy *et al.,* 2017). Furthermore, it is also reported that PGP rhizobacteria able to produce ammonia as secondary metabolite, also playing a major role in plant growth promotion and antagonistic effects (Elshahat *et al.,* 2016). In this present study, majority of the rice-rhizosphere actinobacterial isolates shown to possess ammonia production and nitrogen fixation activities. Zang *et al.,* (2023) reported that 6 *Streptomyces* species (*S. galilaeus, S. avidinii, S. albogriseolus, S. albidofavus, S. spororaveus*, and *S. cellulosae*) isolated from rhizosphere of different plant like rice, reed and canna showed strong nitrogen fixing ability.

Soil salinity or salt induced stresses reduces the already low availability of phosphorus in soil and further hinders its uptake by plant roots. Phosphate-solubilizing rhizobacteria as well as rhizosphere fungal colonizers can transform insoluble phosphorus ($PO4^{3-}$) into soluble forms and bioavailable forms (e.g., $HPO4^{2-}$, $H_4PO_4^{-}$) for the easy uptake of plant roots. In our study, there were six rice-rhizosphere actinobacterial isolates exhibited the P-solubilization activity and shown significance reduction in the growth media pH through organic acids production. This result is in consistent with Chouyia *et al.,* (2020) who reported a strong P-solubilization ability by the two strains *S. roseocinereus* MS1B15 and *S. natalensis* MS1B13 isolated from Moroccan oat rhizosphere.

The strain *S. roseocinereus* MS1B15 also exhibited other PGP traits like siderophore production and IAA as well as ACC deaminase activities. Siderophore compounds and HCN are potential plant growth promoters and disease suppressors. As siderophore can chelate Fe^{3+} to form a soluble iron–siderophore complex and competitively inhibit pathogen by limiting iron in the rhizosphere and HCN also inhibit the phytopathogen by affecting the electron transport chain, causing cell death (Mohamed and Gomaa, 2012; Ahmed and Holmström, 2014). Results obtained in the present study concurred with the rhizosphere actinobacteria isolated from the hyper-arid Atacama Desert, *S. niveoruber* ATMLC132021 and *S. lienomycini* ATMLC122021 showed a PGP trait, siderophore production (Gonzalez *et al.,* 2023). Many rhizosphere colonizing bacteria were reported to produce phytohormones, which promote cell proliferation in the root system and increase the root-surface area for water and nutrient uptake through the overproduction of root hairs (Bhise *et al.,* 2019). In this study, Nineteen of 27 halotolerant rice rhizosphere actinobacterial isolates were positive for IAA production in the range of 9.11-96.56 µg/ml in the presence of L-tryptophan. Additionally, some of these actinobacterial isolates showed auxins (IAA) production in the absence of L-tryptophan indicating operation of tryptophan-independent pathways of auxins production in them. This was observed in agreement with the results of former studies reported by Zang *et al.,* (2023). However, these results are as higher as compared to IAA produced by *Streptomyces* isolate C (4.7 µg/ml) under 300 mM NaCl condition (Sadeghi *et al.,* 2012) and 29.1 µg/ml by *S. paradoxus* D2-8 (Gao *et al.,* 2022).

Apart from the phytohormone productions, most of the rhizosphere colonizing bacterial members are also prosses the enzyme ACC deaminase activity. The enzyme ACC deaminase is most important for protecting plant under stressful environment as this enzyme has the ability of sequestering and cleaving plant-produced ACC into α-ketobutyric acid and ammonia. Due to this activity, the rhizobacteria down grade or modulate the stress hormone ethylene production, which in turn reduces the damage to plants caused by the ethylene biosynthesis under abiotic stress conditions (del Carmen Orozco-Mosqueda *et al.,* 2020). In our study some of the actinobacterial isolates also showed the phosphate solubilization, a key PGP trait, plays a crucial role in enhancing plant tolerance and improving crop growth and yield under abiotic stresses. The utilization of ACC by actinobacteria has also been supported in other studies illustrating enhancement of productivity of chickpea, Gray mangrove (El-Tarabily *et al.,* 2021) and also found to enhance salt tolerance of rice (Yoolong *et al.,* 2019); tomato and soyabean tolerance under Soda Saline-Alkali Stress (Gao *et al.,* 2022). Nozari *et al.,* (2021) reported the six of the 10 *Streptomyces* isolates which are able to grow up to 300 mM NaCl concentration and exhibited ACC deaminase activity. Further, the production of biofilm is an important character of many PGPR to survive under salinity stress in rhizosphere region of plants as it forms rhizosheath around the plant's roots, preventing the entry of Na^+ into plants, thereby reducing the negative effect of salt stress on plants. In this study, sixteen actinobacteria showed biofilm production under salt stress. These results confirm the findings of Yasmeen *et al.,* (2020), who observed halotolerance biofilm forming rhizosphere bacteria can enhance plant growth under the salt stress. Similar result was reported by Chen *et al.,* (2023) on EPS producing actinobacteria, *Glutamicibacter halophytocola* KLBMP 5180 could improve seeds germination of tomato under salt stress.

In the present study based on high NaCl salt stress tolerance and having multiple plant growth promoting traits, eight rice-rhizosphere actinobacterial isolates were selectively identified with more than 99 % similarity with *Streptomyces* spp. through PCR-amplified 16S rRNA gene sequencing and sequence analysis. The phylogenetic identity of these rice-rhizosphere actinobacterial isolates were reported as follows; Met24 as *Kitasatospora* sp., V04a as *Streptomyces tritolerans*, Humic 11a as *Streptomyces* sp., Burk2 as *Streptomyces nigra*, Tap9 as *Streptomyces fimicarius*, Ha11 as *Streptomyces cinerochromogenes*, V07 as *Streptomyces violascens* and Humic 11b as *Streptomyces coelicolor*. Akond *et al.,* (2016), isolated actinobacterial strains belong to *Streptomyces* spp. and *Nocardia* spp. from different straw, compost and plant samples. Sreevidya *et al.,* (2016) also isolated actinobacterial isolates from vemicompost and chickpea rhizospheric soils and reported with the dominance of *Streptomyces* spp.

Out the eight rice-rhizosphere actinobacterial isolates phylogenetically identified, three were selected *Kitasatospora* sp. strain Met24, *Streptomyces tritolerans* strain V04a and *Streptomyces* sp. strain Humic 11a as promising salt stress tolerant and multiple-PGP traits containing isolates.

Further, these promising actinobacterial isolates will be investigated for their salt stress tolerance imparting ability along with potential plant growth promotion in rice seedlings under appropriate salt stress conditions. Limited number of studies have already been conducted on PGP rhizosphere actinobacteria and their effect on plant growth in few crops under the condition of salt stress. Some of the rhizosphere colonizing actinobacteria, especially the genus *Streptomyces* and *Nocardiodes* were known to show positive effect on plant growth and health under saline condition reported in recent studies (Meena *et al.,* 2020; Liu *et al.,* 2022). In addition, the halotolerant actinobacteria identified and characterized from rice-rhizosphere in this study may be the subject of a future in-depth study on their potential applications as biofertilizers to improve growth and yield of rice crops in soils affected by salt stress and salinity.

Abbreviation

PGP - Plant growth promotion; ACC - 1-Aminocyclopropane-1-carboxylate;

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Author Contributions

T Kavya: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. Venkadasamy Govindasamy: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. Gerard Abraham: Writing – review & editing, Supervision. Vinutha T: Writing – review & editing, Supervision. Aundy Kumar: Writing – review & editing, Supervision. Archna Suman: Writing – review & editing, Methodology, Investigation, Conceptualization.

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Data availability

Data will be made available on request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

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