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Research Article

Screening of salt tolerant endophytic bacteria with plant growth promoting characters isolated from *Acanthus ilicifolius* L., a species of mangrove ecosystem located at Corangi wildlife sanctuary, Andhra Pradesh

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Abstract
Mangroves harbour many beneficial microorganisms in their rhizosphere, phyllosphere and endophytically, which forms an ideal ecological habitation for isolating halotolerant endophytic bacteria with unique characteristics. Endophytes can produce numerous bioactive secondary metabolites and phytohormones, which may be directly or in some way beneficial to the host plant. The present study aimed to identify novel endophytes capable of producing plant growth-promoting substances. The mangrove plants *Acanthus ilicifolius* L. at Corangi Wildlife Sanctuary were selected, and their leaves and roots were collected for endophyte isolation. Eight isolates from the leaves and roots were collected, purified and preserved. All these isolates were subjected to morphological, phenotypical and biochemical studies. Isolates were grown best at 3% NaCl nutrient agar and could tolerate salinity upto 8%NaCl. Most of them could grow upto 42°C. The majority were gram's positive, motile, aerobic, rod-shaped and some were gram's negative, rod-shaped organisms. Many of the endophytic organisms had the ability to synthesize Indole-3-acetic acid(IAA) varied from 0.7 µg/ml (AIL1) to 51.0 µg/ml (AIL2) and the highest phosphate solubilizing ability was recorded with AIR3 (3.71 ppm) followed by AIR4 (3.00 ppm) and lowest was recorded by AIL4 (1.80 ppm). Among total isolates, AIL2 (51µg/ml) showed promising potential in producing IAA and had phosphate solubilization ability. Based on 16S ribosomal RNA molecular method the isolate AIL2 was identified as *Bacillus altitudinis*. This is the first to report that *B.altitudinis* strain AIL2 isolated from *A.ilicifolius* L. could produce IAA, which can be used as a bioinoculant in agriculture and allied sector.

Keywords: *Bacillus altitudinis*, Endophytes, Indole 3-acetic acid, Mangroves, Plant growth promoting hormones

INTRODUCTION

Corangi Wildlife Sanctuary near Kakinada, Andhra Pradesh, India, is an estuary with the third largest extent of mangrove forest in India. Twenty-four mangrove tree species are found in these ecosystems. *Acanthus ilicifolius* L. is a plant species found in Corangi mangrove forest and has been extensively used by local communities as a traditional medicinal plant. Extracts from flowers, fruits, bark and leaves are used for the preparation of traditional medicines by these local communities (Forest Department, Government of Andhra Pradesh).

Endophytes colonize intercellular and extracellular regions of the tissues and able to enhance the growth of the host plant. The exact methodology employed by endophytes is not well understood. However, they boost plant growth, which represents an essential step in establishing endophytic bacterial applications (Hardoim *et al*., 2008). The positive roles include the bacterial characters to improve the nutrients acquirement, acquisition of iron, nitrogen fixation, and phytohormone production (Zhang *et al*., 2009).The production of phytohormones during colonization with their host plant improves plant biomass and nutrient absorption (Shi *et al*., 2014).

Treatments of plant growth-promoting bacteria (PGPB) promotes plant growth in biotic and abiotic stress condition by fixing nutritional and hormonal balance, by solubilizing nutrients like potassium, phosphorous and

zinc, by the production of phytohormones like 1-aminocyclopropane-1-carboxylate deaminase (ACCD), Indole-3-acetic acid and in addition to protect the plant from pathogens by synthesizing hydrogen cyanide and siderophore (Jamali *et al.,* 2020). Beneficial application of PGPB includes nutrients uptake, hydration, enhancing root and shoot growth, increasing chlorophyll substance and strengthening tolerance against various diseases (Kasim *et al.*, 2016).In the quest to detect plant growth-promoting endophytic bacteria, the present study was undertaken. *A. ilicifolius* L., a mangrove plant, was selected for its ability to harbour diverse microflora having the potential to synthesize various bioactive secondary metabolites.

MATERIALS AND METHODS

Study area

This study was conducted from 2020-22 at Botany Department, Andhra University, Visakhapatnam, Andhra Pradesh.

Sample collection

Plant tissue samples were collected from leaves and roots of the mangrove plant *A. ilicifolius* located at the mangrove zone of Corangi Wildlife Sanctuary, East Godavari district, within latitude of 16°49'53.0"N and longitude of 82°20'12.0"E(16.831389, 82.336667) (Fig. 1). The collected samples were sealed in a sterile container and brought to the Laboratory.

Surface sterilization and sterility check

Samples were washed with saline water, dried and soaked in ethanol (70%) and kept for one minute. The samples were washed thrice in saline water and soaked in 1% sodium hypochlorite for about five minutes. The whole process was repeated twice. A sterility check was also carried out, as outlined by Gyaneshwar*et al*. (2001).

Segregation of endophytic organisms from *Acanthus ilicifolius* L.

After surface sterilization, 1g of each sample was grounded into the paste and diluted by serial dilution technique. 100 µl of aliquots from the highest diluted solution (4^{th} dilution) were spread on Zobell Marine Agar plates and incubated. After incubation, the individual colony of a different colony morphology were isolated and purified by the quaternary streaking method on Zobell Marine Agar plates with a code assigned. Pure cultures obtained were subjected to further studies (Sahu *et al.*, 2022).

Preservation of isolated endophytes

All the endophytic bacteria were grown on nutrient agar and kept for overnight incubation. Young cultures at the mid-log phase were taken in 20% (w/v) glycerol and stored in deep freeze (-20°C). The feasibility of the organisms was checked periodically by sub-culturing in nutrient agar every two to three months.

Phenotypic characterization of the endophytic isolates

Eight isolates were grown on nutrient agar and the cell and colony morphology were recorded as per guidelines given by Hawksworth *et al*. (1983). Pure cultures at the logarithm growth stage were microscopically observed as per the method outlined in Aneja (2006). Gram staining was done as per modified Grams staining protocols (2005).

Effect of salt concentration on growth

All the isolated cultures were grown on nutrient agar with varied salt concentrations i.e. 3%, 6%, 8% and 10% Sodium chloride. Observations on growth were recorded every 24 and 48 hours of incubation.

Biochemical characterization of endophytic bacterial isolates

Twelve hours old cultures were subjected to the catalase test, oxidase and KOH string test as per the methods outlined by Aneja (2006). IMViC test was tested as per the method summarized by Seeley Jr. (1962).

Catalase activity

Catalase test was conducted according to Aneja (2006). Twenty-four hours old culture of test isolates maintained on nutrient agar plates were transferred to clean glass test tubes containing 0.5 ml of sterile distilled water and thoroughly mixed with 0.5 of 3% of Hydrogen Peroxide solution and any effervescence produced was recorded.

Oxidase test

The oxidase test was carried out as Cappuccino and Sherman (1996) outlined. Trypticase soy agar medium was used for this test. This test was used to identify if the organism possesses the cytochrome oxidase enzyme. All the endophytic test isolates were streaked on the medium and incubated for 48 hours at 32°C in an inverted position in an incubator. 0.3ml of N', N', N', N' – Tetra methyl p-phenylenediamine dihydrochloride (TMPD) solution were added to the streaked areas after the incubation period. The plates were observed for any colour change from pink to maroon and finally to purple within 15-30 seconds, indicating a positive test for oxidase.

KOH string test

Like the Gram staining procedure, the potassium hydroxide test was based on the differential resistance to 3% KOH between grams positive and grams negative

organisms. A small amount of inoculum from the colony is mixed with a small quantity of KOH. If the cells of the isolated get lyse, then the nucleic acid content was released and made the sample viscous or stringy in appearance. Hence, a positive KOH test meant grams of negative cells and a negative KOH test meant gram-positive cells.

Indole production

Glucose tryptone broth was used to conduct the Indole Production test (Seeley and Vandemark, 1981). This test was performed to know the ability of the bacteria to decompose the amino acid tryptophane with the release of Indole in the medium. The test isolates were inoculated in sterilized glucose tryptone broth and incubated for 48 hours at 32°C. After incubation, 0.3 ml of Kovac's reagent was added to the broth and shaken well. The presence of a red alcoholic layer at the top of the tube indicates the production of Indole.

Methyl red and Voges Proskauer test

For Methyl Red and Voges Proskauer test, Glucose phosphate broth was used. The test isolated was inoculated in two sets of sterilized broth and kept for 48 hours at 32°C. After the incubation period in one set of test tubes, methyl red indicator was added, and observe for any colour change, the appearance of red colour indicates a positive test for methyl red. To another set of tubes, six drops of 5% α-naphthol and 2 drops of 40% of KOH solution were added and mixed well. The development of a red colour ring at the surface of the test tubes within a few minutes indicated a positive reaction for Voges Proskauer test (Seeley and Vandemark, 1981).

Citrate utilization test

Simmon's citrate agar was used to know the ability of the organisms to utilize citrate as the sole carbon and inorganic ammonium salt as the sole nitrogen source. All the test isolates were inoculated in sterile slants containing the Simmon citrate agar and incubated at 32°C for 48 hours. Change from green to blue due to the change in the pH of the medium indicated a positive test for citrate utilization (Seeley and Vandemark, 1981).

Property studies of isolated endophytic organisms

The isolated endophytic organisms were tested for their different properties, like production enzymes like protease and amylase; and plant growth-promoting characteristics like phosphate solubilizing ability and phytohormone Indole 3 acetic acid production.

Protease and amylase production

Protease and amylase enzyme production was screened by Gelatin and Starch hydrolysis. The organisms were grown 1% Starch and 1% Gelatin Nutrient Agar plates for amylase and protease enzyme production, respectively. After incubation, for 48 hours, the starch plates were flooded with diluted Iodine solution,

Fig. 1. *Map showing Coringa wildlife sanctuary, Kakinada, Andhra Pradesh, the selected area under study*

and gelatin plates were flooded with saturated Ammonium sulphate solution (approximately 5g per 10ml). Observation on the halo zone formed was recorded.

Production of plant growth-promoting characters
Indole 3-Acetic acid (IAA) production:
All the organisms were screened for IAA production by Colorimetric assay using Salkowski reagent (1ml of 0.5M Ferric chloride in 4.9 ml of 35% perchloric acid). Young cultures were inoculated in nutrient broth for 48 hours of incubation. After incubation, the cultures were centrifuged at 2,000 rpm for 20 min. After centrifugation, 1.5 ml of clear solution was added with 1.5 ml of Salkowski's reagent. Cultures positive for IAA production showed a different range of colour, from orange to dark purple. The colour development was calculated using Spectrophotometer at 536 nm wavelength.

Phosphate solubilizing ability
All the organisms were screened for phosphate solubilization ability by Colorimetric assay using Pikovskaya broth. All the cultures were grown in Pikovskaya broth for 48 hours, and the cultures were then treated to centrifugation at 2,000 rpm for 20 min. After centrifugation 50µl of clear solution was mixed with 3.95 ml of distilled water to make upto 4 ml. To 4 ml of sample 750 µl of phosphate reagent was added and incubated for 5 min. Development of blue colour with different intensities were measured using Spectrophotometer at 680 nm.

Molecular identification using 16S ribosomal RNA gene method
Nucleic acid was obtained from a bacterial sample and its purity was checked by using 1.0% agarose gel electrophoresis. The 16S ribosomal RNA gene fragments were amplified using 16SrRNA-Forward and 16SrRNA-Reverse primers. The PCR template of 1500 bp was purified. Using the Aligner Software tool, a consensus genome sequence of 16S ribosomal RNA gene was created from forward and reversed data sequences. Gene sequencing of 16S rRNA was carried out by BLAST with 'nr' database of the National Center for Biotechnology Information (NCBI), GenBank. On the basis of the highest identity score top, ten gene sequences were elected and lined up by the Multiple Alignment software tool Clustal W. Phylogenetic tree and Distance Matrix were constructed by MEGA10.

RESULTS AND DISCUSSION

Based on research findings, there are limited studies done on endophytic bacteria isolated from *A. ilicifolius* L. However, many studies have been carried out on endophytic *Bacillus altitudinis,* which are proven promising in producing antibiotics, plant growth-promoting hormones and many bioactive metabolites (Zhang *et al.,* 2021; Asmani *et al.,* 2020; Goswami and Deka, 2019; Sunar *et al.,* 2015) since its first discovery by Shivaji *et al.*(2006).

Endophytic bacterial cultures from *Acanthus ilicifolius* L.
Roots and leaves samples from *A. ilicifolius* L. were surface sterilized, cut into pieces and serially diluted. From the highest dilutions, isolated endophytes were grown on Nutrient Agar with 3% NaCl plates. To obtain pure cultures, a single colony was isolated by the quaternary streaking method on nutrient agar plates. Colonies with distinct morphological characters were selected for further studies. Overall eight isolates were obtained from both leaves and root samples. These isolates were subjected to phenotypic, biochemical and molecular characterization. All isolates were named in concurrence to their host plant, followed by plant parts (leaves and roots) and finally by serial number, as shown in Table1.

General morphology
All tested isolates were Gram positive except AIR-3, which was Grams negative. Cell morphology varied from strain to strain. The majority of the tested isolates were rod-shaped, appeared in diplobacilli and were chain formers. The length of the cells varied from small rods to medium and long rods. All the tested isolates showed motility and grew upto 42°C temperature.

Growth at various NaCl concentrations
Test isolates showed similar patterns of growth at different concentrations of NaCl, as shown in Table-3. All tested isolates tolerate salinity upto 8% NaCl concentration. No growth was observed in 0% and 10% NaCl. Similar findings were recorded by Vo Thi Ngoc Ha (2021) in their study revealed that 54 isolates from rhizosphere and endophytic bacteria were able to grow in the presence of up to 3M NaCl concentration isolated from soil and root samples of five native halophytes of *Rhizophora apiculate, Avicennia officinalis, Thespesia populnea, A. ilicifolius* and *Trichophorum cespitosum.*

Biochemical properties of isolated endophytes
All the isolates were subjected to biochemical studies. The results revealed that all the tested isolates were catalase positive when 3% H_2O_2 was added. KOH test showed negative without stringy appearance except for AIR3 isolates which showed positive reaction with viscous appearance. AIR3 isolate showed oxidase positive with purple colour appearance when N', N', N', N' – Tetra methyl p-phenylenediamine dihydrochloride (TMPD) solution was added, remaining isolates showed oxidase negative. Eight isolates recorded negative for Indole test except AIL-2. All the tested isolates showed negative reactions with Voges proskauer and Citrate test.

Table 1. Coding of endophytic bacterial isolates

Plant	Plant part	Isolates*
Acanthus ilicifolius L.	Root (04 isolates)	AIR1, AIR2, AIR3 & AIR4
	Leaves (04 isolates)	AIL1, AIL2, AIL3 & AIL4

*A-*Acanthus* I-*ilicifolius* R-roots, L-leaves

Table 2. Enzymatic properties of tested isolates from *Acanthus ilicifolius* L.

			Plant Growth Hormone	Enzyme production		
Plant	**Part**	**Code**	**IAA (µg/ml)**	**PSB (ppm)**	**Starch (cm)**	**Gelatin (cm)**
***Acanthus ilicifolis* L.**	Root	AIR-1	0.8	2.69	1.5	1.9
		AIR-2	-	1.00	0.8	0.9
		AIR-3	-	3.71	1.4	1.5
		AIR-4	-	3.00	1.4	2.0
	Leaf	AIL-1	0.7	2.30	2.6	2.5
		AIL-2	51.0	2.86	0.6	2.8
		AIL-3	-	-	0.5	0.8
		AIL-4	16.0	1.80	1.4	1.6

Methyl red test was positive with AIR-3& AIL-4 strains, remaining isolates showed negative reaction withthe methyl red indicator. The recorded observation is detailed in Table-3.

Identifying protease production by Gelatin hydrolysis test

All isolates tested for gelatinase production showed that the 48 hours of inoculated plates with tested isolates were saturated with ammonium sulphate and kept for 30 minutes. Hydrolysis of gelatin was examined by the formation of halo zone around the culture, indicating a positive test. Formation of the clear zone varied from strain to strain to range from 1.5 cm (AIR3) to2.8 cm (AIL2) in diameter (Table-2).

Identifying amylase production by Starch hydrolysis test

Two days old inoculated plates with tested organisms were subjected to starch test by flooding with diluted Iodine reagent. The starch present in the agar plates reacts with iodine solution forming blue colour in the plates. The positive test indicates formation of clear halo zone around the cultures. Starch hydrolysis by tested isolates showed varied results ranging from 0.6 cm (AIL2) to 2.6 cm (AIL1) in diameter (Table-2).

Production of Indole-3-acetic acid (IAA):

All the test isolates showed IAA synthesis except AIR3 and AIR4. IAA production was least in AIL1 (0.7 µg/ml) and highest in AIL2 (51.0 µg/ml) (Table-2).

Similar studies were carriedout by Shah *et al.* (2021) who reported that 5 different endophytic organisms collected from three diverse wheat varieties showed diverse levels of catalase acidity, phosphate solubilization, nitrogen fixation, ability to produce IAA and siderophores, antimicrobial activity against plant pathogens. Zhang *etal*., (2021) investigated that *B. altitudinis* strain collected from *Glyceria chinensis* uses different novelty molecular pathways and induces transcriptional changes in the host plant that enhance plant growth. However, the present finding is the first to investigate whether *B. altitudinis* strain AIL2 can synthesize IAA isolated from mangrove *A. ilicifolius* L.

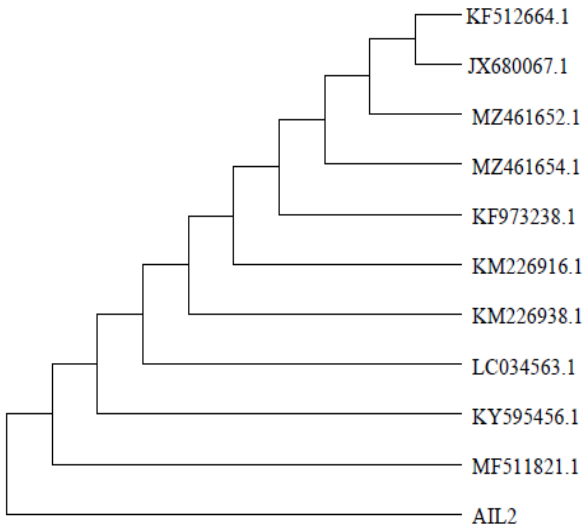

Fig. 2. *Phylogenetic tree of 16S ribosomal RNA sequence of AIL2 strain compared with those Maximum similar entries of type strains from NCBI nucleotide database*

Table 3. Cultural and Biochemical characters of tested isolates from *Acanthus ilicifolius* L.

	AIR-1	**AIR-2**	**AIR-3**	**AIR-4**	**AIL-1**	**AIL-2**	**AIL-3**	**AIL-4**
Pigmentation	NIL	NIL	NIL	NIL	NIL	**Orange**	NIL	NIL
Microscopic observation	Small rods	Medium rods exists in two	Very small rods	Long rods exist in two	Short rods	**Medium rods exits in two**	Short rods	Long rods exists in chains
Gram's staining	+ve	+ve	-ve	+ve	+ve	**+ve**	+ve	+ve
Endospore	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**
Shape and position	Oval /centre of the cell	Oval /centre of the cell	-	Oval / centre of the cell	Oval /centre of the cell	Oval /centre of the cell	Oval /centre of the cell	Oval /centre of the cell
Motility	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
KOH	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**-**
Catalase	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Oxidase	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**-**
Growth at 42°C	**++**	**+**	**+**	**+**	**++**	**++**	**++**	**++**
Growth on 0% NaCl	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Growth on 3% NaCl	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**
Growth on 6% NaCl	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**
Growth on 8% NaCl	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Growth on 10% NaCl	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Starch hydrolysis	**++**	**+**	**++**	**++**	**+++**	**+**	**+**	**++**
Geletin hydrolysis	**++**	**+**	**++**	**+++**	**+++**	**+++**	**+**	**++**
Hemolysis	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**
Indole	**-**	**-**	**-**	**-**	**-**	**++**	**-**	**-**
Methyl Red	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**+**
Voges Proskauer	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Citrate	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Bacillus selective media	No colour	Green	No colour	Green	Green	**Green**	No colour	Green
Preliminary identification	Bacillus sp.	Bacillus sp.	Pseudomonas sp.	Bacillus sp.	Bacillus sp.	***Bacillus altitudinis***	Bacillus sp.	Bacillus sp.

'+' indicates normal growth, '++' indicates luxurious growth and '-' indicates No growth

Phosphate solubilizing ability

All the test isolates showed phosphate solubilizing ability. Highest phosphate solubilizing ability was recorded with AIR3 (3.71 ppm) followed by AIR4 (3.00 ppm) and the lowest was recorded with AIL4 (1.80 ppm) (Table2).Kushwaha *et al.*(2021) in a study, isolated *B. altitudinis* strains (BT3 and CT8) from rhizosphere of chickpea. The isolates showed plant growth-enhancing properties like the synthesis of ammonia, Indole 3-acetic acid, siderophores and the ability to solubilize phosphate. They also showed outstanding abilities to solubilize insoluble zinc compounds.

Identification of isolates by using 16S ribosomal RNA molecular method

Because of its ability to produce a higher amount of IAA compared to other test isolates, AIL2 strain was selected for identification using 16S ribosomal RNA molecular studies.

The RNA was obtained from the cultures of AIL2 and was evaluated by one per cent agarose gel to obtain specific RNA band with high molecular weight. The 16S ribosomal RNA gene fragments were amplified by by16S rRNA-Forward and 16S ribosomal RNA Reverse primers by BDT on ABI 3730 X l Genetic Analyzer. From the BLAST analysis from the NCBI database revealed that 16S ribosomal RNA genome sequence of the AIL2 strain showed a higher relationship with *B. altitudinis*. The percentage of 16S ribosomal RNA sequence identity between strain AIL2(Accession no. MF511821.1) and *B. altitudinis* was 100%. The phylogenetic tree was constructed using the neighbour-joining algorithm software (Fig. 2) and also established that the strain AIL2 and *B. altitudinis* grouped together and represented a different group from the other tightly connected species.

Conclusion

The present research findings indicated that endophytes, especially organisms belonging to the genus *Bacillus* living in extreme conditions, can produce many phytohormones and bioactive substances that are beneficial to the host plant. Thus, endophytes isolated from the mangrove plant *A. ilicifolius* L., especially strain *B.altitudinis* (AIL2), might be used as a favourite candidate source for the growth of plants in sustainable agriculture and other allied sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics Statement

This research work does not contain any study involving or experimenting on humans and animals performed by any of the authors. All datasets generated or analyzed during this study are included in this manuscript.

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