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Research Article
A Study on Biocontrol and Plant Growth Promoting Efficacy of *Azadirachta indica* (Neem) Leaf Endophytic Bacteria

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Abstract

Background and Objective: Endophytic bacteria can protect host plants by producing secondary metabolites and also improve host plant growth by various direct and indirect mechanisms. This unique character makes a special role in, using endophytic bacteria in sustainable agriculture. The study aims to isolate endophytic bacteria from *Azadirachta indica* (neem) leaf and screen for their biocontrol and plant growth-promoting properties. **Materials and Methods:** Thirteen endophytic bacteria were isolated from neem leaf and it was tested for their *in vitro* antifungal activities against *Rhizoctonia solani*. A total of 3 isolates (N1B, N4B and N5B) were selected on the broad antifungal activity (50, 56 and 75% mycelial inhibition). Among different solvents used for the extraction of secondary metabolites from endophytic bacteria, only ethyl acetate extract of N5B showed maximum zone of inhibition in well diffusion method against fungal pathogen. **Results:** The GC-MS analysis of ethyl acetate extracts of N5B exhibited 14 compounds with antifungal activity, such as dimethoxyglycerol docosyl ether, pentadecanoic acid, oleic acid etc. Selected 3 endophytic isolates were identified up to molecular level using 16S rRNA sequencing and it was identified as *Bacillus haynesii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. The identified isolates were used for nursery experiments based on their strong *in vitro* antifungal and PGP activities such as P, K and Zn solubilization, N fixation, IAA, HCN and EPS production. **Conclusion:** The tested bacterial isolates significantly decreased disease severity in rice, infected with *R. solani* and increased plant biomass when compared with un-inoculated control. The findings suggested above mentioned 3 bacterial species may be promising candidates as a biocontrol agent to confer resistance to sheath blight disease of rice.

Key words: *Bacillus haynesii*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Azadirachta indica*, N fixation, endophytic bacteria

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

India is one of the world's major producers of rice (*Oryza sativa*). Rice is the most commonly consumed essential food, particularly in Asia. Its growth and yield are highly affected by different diseases[1]. More than 70 diseases were identified and it is caused by fungi, bacteria, viruses, mycoplasma or by nematodes. Among these, fungi are the major diseases causing agents in the rice field. Some economically important fungal diseases are caused by different pathogens such as *Cercospora oryzae* (Narrow brown leaf spot), *Helminthosporium oryzae* (rice brown spot), *Rhizoctonia solani* (sheath blight), *R. oryzae* (Aggregate sheath), *Sarocladium oryzae* (Sheath rot), *Fusarium* sp. (root rot), *Sclerotium oryzae* (stem rot) etc.

The sheath blight of rice is one of the economically important rice diseases. It is a soil-borne disease caused by the fungus *Rhizoctonia solani*, which belongs to the phylum Basidiomycota and family Ceratobasidiaceae)[2]. In India, the estimation of losses due to this disease has been stated up to 54.3%. They not only have a supreme role in causing dreadful epidemics in plants but also play a long-lasting role in annual yield losses which affect the economy of the country. As a way out for these problems, several methods have been engaged. The most basic technique used by farmers to control the pest and fungi is crop rotation[3]. Nevertheless, the problem is that crop rotation requires 2-3 years for dropping the fungal inoculums to accepted levels. So crop rotation alone was not adequate for fighting against the phytopathogens.

Chemical fungicides are the finest prevention against fungus and obstruct disease caused by phytopathogens but they affect soil fertility harshly[4]. The soil texture and composition gets varied on the frequent application of chemicals. Apart from that, the fungus can gain resistance against these fungicides which make the fungicide ineffective against them. Hence the application of chemical fungicides is not an eco-friendly method for getting rid of fungal pathogens and serious measure is required to verdict a new alternative technique for plant protection that is less dependent on the chemicals and is more eco-friendly.

Bio-control of sheath blight using endophytic bacteria has emerged as an effective strategy during the last 2 decades. Endophytes are obligate or facultative symbiotic microbes, mainly consisting of bacterial and fungal species. They live in healthy internal plant tissues, without causing disease or damage to the host plant[5]. They produce a wide range of compounds beneficial for plants for their growth, protection from biotic and abiotic stress. The development of biological products based on beneficial microorganisms can extend the range of choices for upholding the health and yield of crops[6]. Colonization of endophytes in the host plant has different benefits such as increased resistance to drought, flooding, pathogens as well as enhanced competitive abilities[7]. Endophytes release antibiotics or hydrolytic enzymes which prevent the colonization of microbial plant pathogens as well as prevent insects, nematodes etc from being infecting the host plant[8]. By the process of Induced Systemic Resistance (ISR), endophytic microbes secrete metabolites that trigger the host defence mechanism against different plant pathogens[9]. Numerous compounds isolated from endophytic bacteria have great application value in emerging medicine and they also act as bio-fungicides[10].

Endophytes are considered to be a promising source of novel secondary metabolites with potential for medicinal use as well as important in agriculture and industry. The anticancer compounds such as podophyllotoxin[11] and the natural insecticide such as azadirachtin[12] produced by endophytic microbes are good examples of bioactive compounds. The PGP properties of endophytes are unique and therefore it is noteworthy to study such properties from microbial populations connected with medicinally and economically important plant species. One such medicinally important native tree of India is *Azadirachta indica* (neem), it is popular for its different medicinal, insecticidal and ethnopharmacological properties. More than a hundred compounds have been isolated from different parts of the neem tree and most of the active principles like Limnoids belong to the group of tetranortriterpenoids particularly 'Azadirachtin' and its analogues[13]. The Indian peoples have already known the useful properties of neem since ancient times and only recently have other people in more developed countries appreciated the value and importance of this tree to humankind. Considering the antimicrobial and insecticidal property of neem it is pertinent to focus on the role of endophytic bacteria present in neem leaf for its bio-control activity against *R. solani*[14]. Additionally, aimed to discover the possible *in vitro* and *in vivo* mechanisms used by endophytes to control the fungal pathogen and growth improvement of rice.

MATERIALS AND METHODS

Sample collection: The study was carried out at the Department of Microbiology, Government Arts and Science College Kozhinjampara, located at Palakkad district, Kerala, South India during the year June, 2019-March, 2020. Fresh and healthy leaves of *Azadirachta indica* (neem) L. plants were collected from the college campus for endophytic bacterial isolation.

Isolation of endophytic bacteria from *Azadirachta indica* (neem) leaf sample

Surface sterilization of leaf sample: The fresh, healthy, undamaged leaves were selected and then washed under running tap water before surface sterilization. Surface sterilization was done by washing with 75% ethanol for 1 min, 3% sodium hypochlorite for 3 min followed by 75% ethanol for 30 sec and subsequently, these leaves were washed thrice in sterile distilled water and leaves were dried using sterile tissue paper and used for further work[15].

Isolation of endophytic bacteria: The surface-sterilized leaf samples were cut into small pieces and macerated in a sterile pestle and mortar. Tissue extract was then prepared for tenfold dilution in sterile distilled water. Serial dilutions were carried out and 0.1 mL of aliquots from 10^6 and 10^7 were spread onto nutrient agar medium under laminar airflow to avoid external contamination and the plates were incubated at 28°C for 24 hrs. The bacterial colonies exhibiting different colony morphology were selected, purely cultured and used for bio-control studies[16].

Fungal pathogens used for the study: The rice pathogen *Rhizoctonia solani* (Rice sheath blight) was procured from the culture repository of IFGTB, Tamil Nadu, India for the present study. Actively growing hyphae were successively transferred to the Potato Dextrose Agar (PDA) and the cultures were maintained on slants and stored at 4°C.

Screening bio-control activity of endophytic bacteria

Dual culture method: All endophytic bacterial isolates were screened for their bio-control potential by dual culture assay[17]. The fungal disc of 6 mm was inoculated in the middle of the Petri plate containing PDA medium and bacteria were streaked 3 cm away on either side of the pathogen and incubated at 28°C. The Petri plate inoculated with pathogen alone in the absence of antagonist bacteria was served as control and the experiment was done in replicate. The radial growth of fungal mycelium on each plate was measured between 2nd, 4th and 6th days and the percent inhibition of growth over control was determined using the formula[17]:

$$\text{Fungal growth inhibition (\%)} = \frac{C - T}{C} \times 100$$

where, C is the radial growth of the test fungal pathogen in the control plates (mm) and T is the radial growth of the test fungal pathogen in the test plates (mm).

Antifungal activity in liquid culture: The antifungal liquid culture method was used to test the antifungal activity in a liquid broth (PDB)[18]. Bacterial isolates that showed more than 70% antagonism in dual culture plates were selected. A hundred millilitre of PDB was sterilized in a 250 mL conical flask and inoculated with a 6 mm disc of pathogenic fungi and 1 mL of bacterial culture (OD 1.00 at 590 nm). The PD broth inoculated with fungal disc and without bacterial culture was used as a control. It was incubated at 28°C for 5 days. The dry weight of the fungal matt with bacterial strains and control (without bacterial strains) were recorded and compared.

Screening for production of volatile antifungal metabolites: The production of volatile substances by selected endophytic bacteria was determined by the method described by Battu and Reddy[19]. Selected strains and each pathogenic fungus were cultured in PDA medium in separate plates and then the plates with selected endophytes were kept over the plates with the fungus, by avoiding direct contact between the 2 cultures. Both plates were sealed face to face with parafilm to avoid the escape of volatile metabolites and incubated at 28°C for 5-6 days. The production of volatile compounds was then determined based on inhibition of the radial growth of the fungi. The percentage of fungal inhibition was compared with the control plate.

Screening for production of secondary metabolites

Preparation of inoculum and extraction of cured metabolites: As per the procedure followed by Battu and Reddy[19], the inoculum preparation and metabolite extraction from selected isolates was carried out. The selected microbial isolates were cultured in 100 mL nutrient broth and kept under shaking conditions (120 rpm) at 28°C for 4-5 days. The culture was centrifuged at 10,000 rpm for 15 min to get the cell-free filtrate. These culture filtrates were used to study the efficacy against fungal pathogens. Crude metabolites were extracted from the effective growth medium by partitioning with organic solvents viz., acetone, chloroform, ethyl acetate, hexane and petroleum ether[20].

Antifungal property of crude extract: The individual extracts were tested for antifungal activity by the well diffusion method[21]. The PDA plates were swabbed with a fungal pathogen and 100 µL extract was filled in the well, incubated for 28 for 48 hrs. The zone of inhibition was measured and the extract exhibited maximum zone of inhibition was stored for further study.

GC-MS analysis of the effective solvent extract: The most effective extract was analyzed using GC-MS to predict the presence of inhibitory compounds. It was carried out at South Indian Textile Research Association (SITRA), Coimbatore, Tamil Nadu. GC-MS equipped with a DB35-MS fused with silica capillary column and GC interfaced to an MSD with XCALIBUR software. For GC-MS detection, -70 eV ionization energy was commonly used. Helium gas was used as a carrier gas and 2 µL of the sample was injected. The injector temperature was 250°C, ion source temperature was 200°C and the oven temperature was programmed from 70-200°C at the rate of 6°C per minute, held isothermal for 1 min and finally raised to 260 at 6°C per min. The interface temperature was kept at 250°C. Total GC run time was 40.51 min. The relative percentage of the extract constituents was expressed as a percentage with peak area normalization.

Interpreting the mass spectrum of GC-MS was done using the database of the National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The unknown components mass spectrum was compared with the spectrum of the known components stored in the National Institute of Standard and Technology library. The name and molecular weight of the components present in samples were ascertained[22].

Morphological, biochemical and molecular characterization of effective isolates

Morphological and biochemical characterization: Bacteria selected for the phenotypic and genotypic identification was based on the bio-control and multifactorial PGP traits exhibited under *in vitro* conditions. Taxonomic studies of the selected bacteria were carried out according to Mendes *et al.*[23]. Identification studies such as morphological characteristics (shape and size, gram reaction, motility and spore formation), cultural characteristics were studied using nutrient agar, biochemical characteristics (Indole, Methyl red test, Voges-Proskauer test, citrate, triple sugar iron agar test, gelatin liquefaction, starch hydrolysis, catalase, urease, oxidase, nitrate reduction test, Carbohydrate fermentation test).

Molecular characterization of the effective isolates: The 16S ribosomal RNA (rRNA) analysis of effective bacteria was carried out at Yaazh Xenomic laboratory, Coimbatore, Tamil Nadu. The genomic DNA of endophytic bacterial isolates were extracted using HiPurA Bacterial genomic purification kit obtained from Hi-Media, Mumbai, India. The primers 27F (5'-CAGAGTTT GATCCTGGCT-3') and 1492R (5'AGGAGGTGATCCAGCCGCA-3') were used for amplification of the 16S rRNA gene (Sanger sequencing protocol). The amplified 16S rRNA gene was sequenced and sequences obtained were subjected to BLAST search of (National Centre for Biotechnology Information) bacterial database. Three individual bacterial sequences were submitted to GenBank and got accession numbers and its phylogenetic tree was construed using the neighbour-joining method[24].

Screening for plant growth-promoting (PGP) traits of effective endophytic bacterial isolate: Among 13 endophytic bacteria, 3 showed more than 70% inhibition against fungal pathogens. These isolates were tested for their plant growth-promoting traits.

Indole 3-acetic acid production: For the IAA production test, each isolate was inoculated to the sterile 15 mL Nutrient broth amended with L-tryptophan in test tubes and incubated at 28°C for 72 hrs in the dark[25]. Subsequently, 2 mL of this broth was centrifuged at 12,000 rpm for 10 min, followed by the addition of 4 mL of Salkowski reagent to the 1 mL of supernatant. The tubes were incubated at 28°C in the dark for 1 hr. The development of pink/red colour in the medium indicated IAA production by the organisms.

Phosphate solubilization: The phosphate solubilization ability of the isolates was detected by spotting them on the Pikovskaya agar medium containing tricalcium phosphate and incubated at 28°C for 2-3 days. The development of a clear halo zone around the strains indicated a positive result for phosphate solubilization[26].

Potassium solubilization: Potassium solubilization by endophytic isolates was studied on modified Aleksandrov agar medium plates by the spot test method[27]. Plates of modified Aleksandrov medium having mica powder (an insoluble form of potassium) were prepared. A loopful of 48 hrs old-growth of the rhizobacterial strain was spotted on the above-prepared plates. Plates were incubated at 28 for 3 days. Isolates showing halo zone of clearance was evident for K solubilization.

Zinc solubilization: The selected isolates were tested for zinc solubilization efficiency utilizing plate assay using modified Bunt and Rovira agar medium containing 0.1% of Zinc Oxide (ZnO) as the insoluble source. The plates were incubated at

28°C for 3 days. Isolates showing halo zone of clearance was evident for Zn solubilization[28].

Identification of ammonia producing isolates: Each isolate was tested for the production of ammonia in peptone water. Overnight broth cultures were inoculated in 10 mL peptone water and incubated at room temperature for 48-72 hrs. Nessler's reagent (0.5 mL) was added to each tube. The development of brown to yellow colour was noted as a positive result for ammonia production[29].

Exo-polysaccharide (EPS) production: Exo-polysaccharide production was screened out by the standard method[30]. The selected isolates were inoculated into a 10 mL basal medium and incubated at room temperature for 5-6 days. Ten millilitres of culture suspension was collected and centrifuged at 15,000 rpm for 45 min and added thrice the volume of chilled acetone to the supernatant. EPS was separated from the mixture in the form of a slimy precipitate. Precipitates were collected on a pre-dried filter paper. Allowed the precipitates to dry overnight at 50°C, reweigh the dried filter paper after overnight drying.

Hydrogen cyanide production: Production of Hydrogen Cyanide (HCN) was determined in nutrient agar supplemented with 4.4 g L^{-1} of glycine[31]. The bacterial cultures were streaked on an agar plate and Whatman No.1 filter paper strips dipped in 0.5% picric acid in 2% sodium carbonate solution were pasted on the lid of the Petri plate and sealed with parafilm, incubated at 28°C for 4 days. A change of colour to brown or reddish-brown was recorded as a positive reaction.

Evaluation of endophytic bacteria for growth promotion and disease suppression in nursery condition:

$$\text{Seed germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100$$

Treatment model:

T_1 = Control (distilled water)
T_2 = *Rhizoctonia solani*
T_3 = *Bacillus haynesii*
T_4 = *Stenotrophomonas maltophilia*
T_5 = *Pseudomonas aeruginosa*
T_6 = *Rhizoctonia solani* + *Bacillus haynesii*
T_7 = *Rhizoctonia solani* + *Stenotrophomonas maltophilia*
T_8 = *Rhizoctonia solani* + *Pseudomonas aeruginosa*

Nursery studies: Individual isolates were evaluated for the ability to stimulate the growth of rice and its biocontrol potential was also evaluated. These initial trials were done with autoclaved soil and sterilized seeds in poly bags, to remove any bacteria from the soil and on or within the seeds, to ensure that the only bacteria present would be the inoculum (except for the un-inoculated controls). Treatment T_1 got distilled water as inoculate (control). T_2, inoculated with a fungal pathogen (negative control), T_3, T_4 and T_5 are treated with antagonistic bacteria as bio-inoculant, T_6, T_7 and T_8 are treated with both antagonistic bacteria and fungi. Twenty millilitres of broth cultures were applied as bio-inoculant into respective bags as described in the treatment schedule. The seedlings were watered regularly with the controlled experimental condition[32]. After 3 weeks of growth, plant height and total weight and length of shoots and roots were measured.

RESULTS

Isolation of endophytic bacteria from *Azadirachta indica* (neem) leaf sample: This study was conducted to isolate and characterize the endophytic bacterial diversity from *A. indica* (neem) leaf tissue. Two samples were collected from the college campus (Government Arts and Science College Kozhinjampara). Sample 1 shows 17.0×10^6 and 14.0×10^7 CFU mL^{-1} of leaf sample. Sample 2 shows 25.0×10^6 and 23.0×10^7 CFU mL^{-1} of surface-sterilized leaf sample, among them, sample 2 have a maximum number of colonies (Table 1).

Totally 13 bacterial colonies showing diverse colony morphology, margin, shape and colour from samples 1 and 2 were selected, purely cultured and used for further study (Table 2). The colonies show different forms such as irregular and circular forms. The colony elevation is raised and flat. The margin was found to be lobate, entire, curled, filiform and undulate. The surface was observed as wrinkled and smooth. The opacity is translucent and opaque. Different colony colours were observed such as pale white, white, red, light pink, greenish, yellow and orange.

Screening bio-control activity of endophytic bacteria

Dual culture method: A total of 13 endophytic bacterial isolates were studied for their bio-control potential against *Rhizoctonia solani* by dual culture method. The 2nd, 4th and 6th day data of the dual culture method were recorded and tabulated in Table 3 and Fig. 1a-d. Compared with controls, radial growth of tested fungal pathogen were

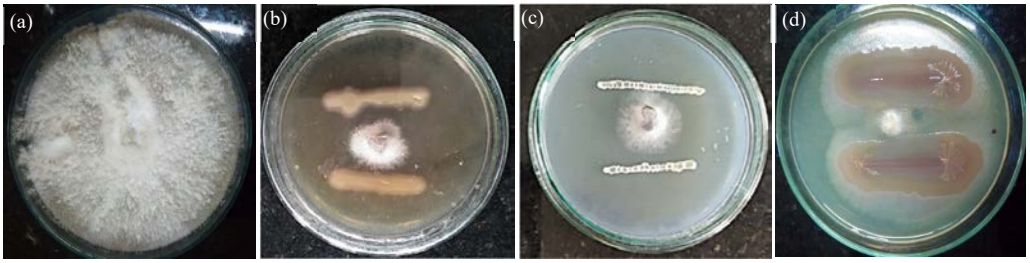

Fig. 1(a-d): Screening antagonistic activity of endophytic bacterial isolates against plant pathogen by dual culture method, (a) *Rhizoctonia solani* (control), (b) *R. solani*+N1B, (c) *R. solani*+NB4 and (d) *R. solani*+N5B

Table 1: Isolation of endophytic bacteria from *Azadirachta indica* (neem) leaf sample

	Number of cells	
Number of samples	($\times 10^6$ CFU mL^{-1})*	($\times 10^7$ CFU mL^{-1})*
1	17.0×10^6	14.0×10^7
2	25.0×10^6	23.0×10^7

*Mean of two replicate, CFU: Colony forming unit

Table 2: Different colony morphology of endophytic bacterial isolates

Isolate codes	Form	Elevation	Margin	Surface	Opacity	Colour
N1B	Irregular	Raised	Lobate	Wrinkled	Opaque	Pale white
N2B	Irregular	Raised	Undulate	Wrinkled	Opaque	White
N3B	Circular	Flat	Undulate	Smooth	Opaque	Red
N4B	Irregular	Raised	Lobate	Wrinkled	Opaque	Light pink
N5B	Circular	Flat	Entire	Smooth	Translucent	Greenish
N6B	Circular	Raised	Curled	Wrinkled	Opaque	Yellow
N7B	Circular	Raised	Entire	Smooth	Opaque	Orange
N8B	Circular	Flat	Entire	Smooth	Opaque	Yellow
N9B	Irregular	Raised	Filiform	Wrinkled	Opaque	Pale white
N10B	Irregular	Raised	Undulate	Wrinkled	Opaque	Pale white
N11B	Circular	Raised	Entire	Wrinkled	Opaque	Light orange
N12B	Circular	Raised	Entire	Smooth	Opaque	Yellow
N13B	Irregular	Flat	Entire	Smooth	Opaque	Yellow

Table 3: Testing the efficacy of endophytic bacterial isolates against *Rhizoctonia solani* using the dual culture assay

	Percentage of mycelium inhibition of *Rhizoctonia solani**								
	2nd day			4th day			6th day		
Isolate codes	Fungal control C (cm)	Test T (cm)	Inhibition (%)	Fungal control C (cm)	Test T (cm)	Inhibition (%)	Fungal control C (cm)	Test T (cm)	Inhibition (%)
N1B	5.2	3.7	28.8	6.5	4.0	38.4	8	4.0	50.0
N2B	5.2	5.0	3.8	6.5	6.5	0.0	8	8.0	0.0
N3B	5.2	4.5	13.46	6.5	5.7	12.3	8	6.5	18.75
N4B	5.2	3.0	42.3	6.5	3.2	50.7	8	3.5	56.0
N5B	5.2	2.0	61.5	6.5	2.2	66.0	8	2.0	75.0
N6B	5.2	5.2	0.0	6.5	6.5	0.0	8	7.0	12.5
N7B	5.2	4.6	11.5	6.5	6.3	3.0	8	7.0	12.5
N8B	5.2	3.3	36.5	6.5	6.5	0.0	8	8.0	0.0
N9B	5.2	4.1	21.0	6.5	5.0	23.0	8	5.3	33.75
N10B	5.2	4.6	11.5	6.5	6.5	0.0	8	7.0	12.5
N11B	5.2	3.6	30.7	6.5	3.6	44.6	8	7.0	12.5
N12B	5.2	4.4	15.0	6.5	6.2	4.6	8	7.2	10.0
N13B	5.2	2.2	57.6	6.5	2.8	56.9	8	2.5	68.75

*Mean of 2 replicates, C: Control, T: Test, Inhibition (%) = C-T/C×10

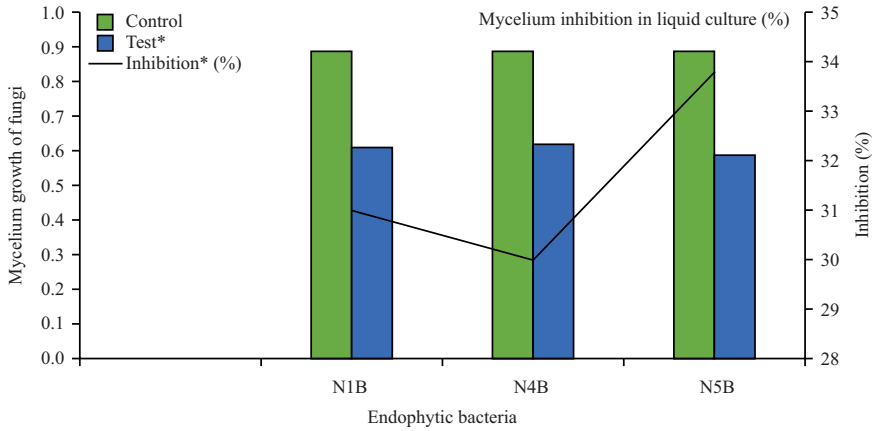

Fig. 2: Screening antifungal activity using liquid culture method

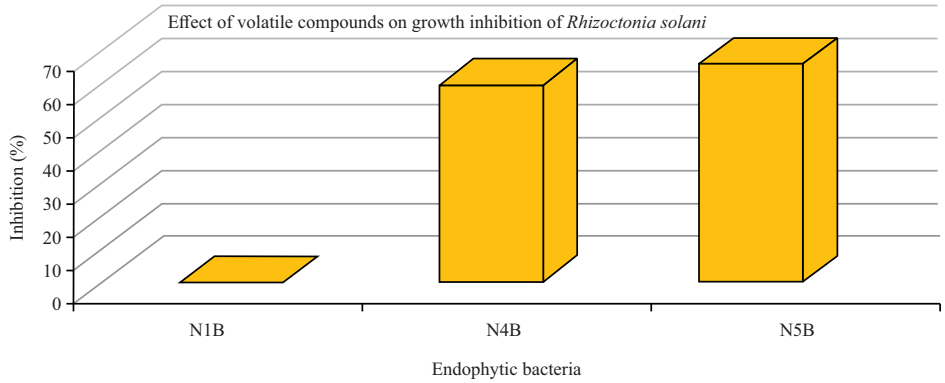

Fig. 3: Screening for production of volatile antifungal metabolites

inhibited maximum by 3 endophytic strains, among 3 isolates N5B exhibited maximum mycelial inhibition followed by N1B and N4B. N5B showed a 75% reduction in mycelium growth of *Rhizoctonia solani*.

Antifungal activity in liquid culture: Among 13 endophytic bacteria only 3 shows above 70% of fungal inhibition, it was further subjected to liquid culture assays. N5B inoculated broth showed 33.8% fungal matt reduction followed by endophytic N1B (31%) and N4B (30%) (Fig. 2).

Screening for production of volatile antifungal metabolites: Compared with controls, radial growth of *Rhizoctonia solani* was inhibited by N5B (56%) followed by N4B (40%) (Fig. 3).

Screening for production of secondary metabolites: The isolates were subjected to the production and extraction of secondary metabolites. These isolates were extracted with solvents like acetone, chloroform, ethyl acetate, hexane and petroleum ether with different polarities. After extraction, its antifungal efficacy was tested with a well diffusion method. The ethyl acetate extract of N5B showed a maximum zone of inhibition when compared with others.

Antifungal property of the crude extract and GC-MS analysis: Bacterial crude extract showed promising results by exhibiting maximum antifungal activity against fungal plant pathogen using the agar well diffusion method. Among these ethyl acetate extract showed the highest zone of inhibition and showed more antifungal activity than other solvent extracts.

The ethyl acetate extract of N5B bacteria was subjected to GC-MS analysis which showed 15 different compounds based on their Retention Time (RT). The important compounds identified having maximum probability was Dimethoxyglycerol Docosyl Ether (77.58), 13-Docosenamide, (Z)-(46.20), pentadecanoic acid (42.46) and 9, 12, 15-Octadecatrienoic acid (22.97) were represented in Table 4 and the retention time, relative abundances of different compounds mentioned above were shown in Fig. 4.

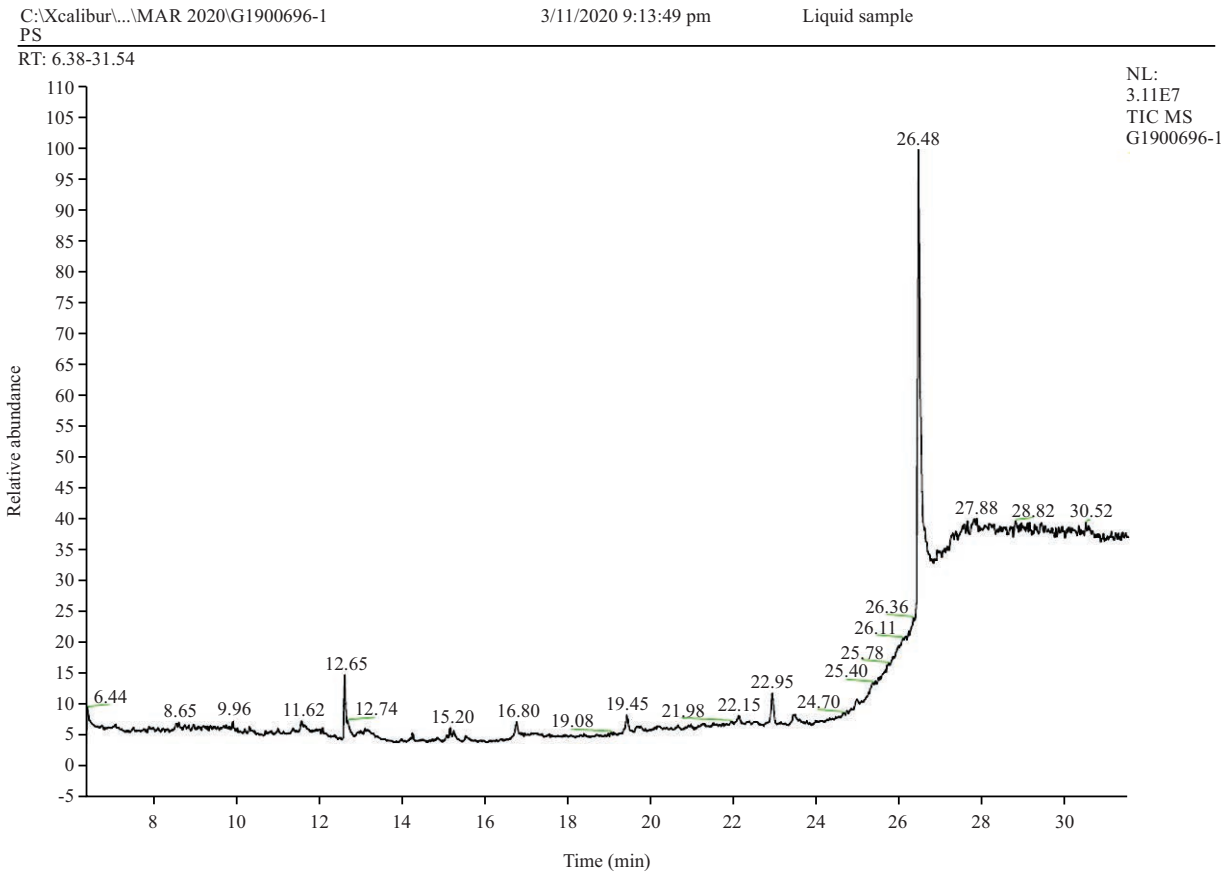

Fig. 4: GC-MS analysis of N5B ethyl acetate extract

Table 4: Bioactive compounds identified from N5B isolates using GC-MS analysis

Compound names	Molecular formula	Molecular weight (g mol^{-1})	Probability	Area (%)	Retention time
Glycocholic acid methyl ester TMS	$C_{36}H_{69}NO_6Si_3$	695	8.53	7.27	6.49
Ergosta-5,22-dien-3-ol	$C_{30}H_{48}O_2$	440	4.66	7.27	6.49
Dimethoxyglycerol Docosyl Ether	$C_{27}H_{56}O_5$	460	77.58	1.52	1.52
Hexadecanoic acid	$C_{36}H_{58}O_6$	586	1.71	1.52	1.52
Quercetin 7,3',4'-trimethoxy	$C_{18}H_{16}O_7$	344	6.21	1.40	9.96
Lucenin 2	$C_{27}H_{30}O_{16}$	610	5.56	0.77	10.38
Dotriacontane	$C_{32}H_{66}$	450	3.21	0.77	10.38
1-Eicosanol	$C_{20}H_{42}O$	298	4.12	1.42	11.64
7-Ethyl-octahydro-inden-3a-ol	$C_{10}H_{14}D_2O$	152	5.38	1.42	11.64
Pentadecanoic acid	$C_{17}H_{34}O_2$	270	42.46	5.27	12.67
9,12,15-Octadecatrienoic acid	$C_{27}H_{52}O_4Si_2$	496	22.97	0.67	14.28
Oleic acid, eicosyl ester	$C_{38}H_{74}O_2$	562	11.73	2.21	15.20
Hexa-t-butylselenatrisiletane	$C_{24}H_{54}SeSi_3$	506	5.93	3.26	22.95
3-(5-Cyano-4-methoxycarbonyl methyl-4,5-dimethyl-2-thioxo-pyrrolidin-3-yl)-propionic acid, methyl ester	$C_{14}H_{20}N_2O_4$	312	2.40	1.73	23.49
13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	337	46.20	1.23	24.1

Morphological and biochemical characterization of the Isolates: The microbial isolates revealed maximum bio-control and PGP properties were selected for identification. They were identified up to genes level, based on the result of morphological characterization, staining and biochemical reactions. The staining and biochemical properties of the isolates were summarized in Table 5. Molecular-level confirmation was done by 16S rRNA sequence analysis. A BLAST search of the sequences revealed that the isolates N1B, N4B and N5B be the closest homolog to *Bacillus haynesii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* (Table 6). The sequences of the isolates have been deposited

Table 5: Morphological and biochemical identification of endophytic bacteria

Test	N1B	N4B	N5B
Gram nature and shape	Positive rod	Negative rod	Negative rod
Spore	Present	Absent	Absent
Motility	+	+	+
Indole	-	-	-
MR	-	-	-
VP	+	-	-
Citrate	+	-	+
Catalase	+	+	+
Oxidase	+	-	+
Urease	-	-	-
Nitrate	+	-	+
Gelatin	+	+	+
Starch hydrolysis	+	-	-
Carbohydrate fermentation			
Glucose	+	+	-
Mannitol	-	-	+
Sucrose	-	+	-
Fructose	+	-	-

Table 6: Molecular characterization of the effective isolates

Isolate codes	Bacteria identified	Similarity (%)	Accession number
N1B	*Bacillus haynessi*	98	MN880435
N4B	*Stenotrophomonas maltophilia*	99	MN880434
N5B	*Pseudomonas aeruginosa*	99	MN880486

Table 7: Screening for PGP traits of endophytic bacterial isolates

	Endophytic bacterial isolates		
Sr. No	N1B	N4B	N5B
P Solubilization	-	-	-
K Solubilization	-	-	+++
Zn Solubilization	-	-	-
IAA	+	+	+++
Ammonia	+	+	+++
Exo polysaccharide (g)	0.141	0.177	0.014
HCN	-	-	+++

+: Mild production, ++: Moderate production, +++: High production, -: No production, P: Phosphate, K: Potassium, Zn: Zinc, IAA: Indole-3- acetic acid, HCN: Hydrogen cyanide production

in GenBank with accession numbers MN880435, MN880434 and MN880486, respectively. The phylogenetic tree was represented in (Fig. 5-7).

Plant growth-promoting traits of effective endophytic bacteria: The potential of endophytic bacterial isolates for PGP activities was checked. Phosphate solubilization was found to be negative in all the 3 isolates tested. Potassium solubilization was found to be positive in the N5B isolate and the remaining isolates were found to be negative, zinc solubilization was found to be negative for all 3 isolates tested. Ammonia production as observed in all the 3 isolates tested, among 3, N5B showed maximum production. HCN was found to be positive in N5B isolate only. Exo-polysaccharide production was maximum in N5B followed by N4B and N1B. The IAA production was found to be positive in all the tested isolates. These traits have been illustrated in (Table 7).

***In vitro* seed germination:** The isolates positively influence the germination of rice (Fig. 8). Following the coating of fungal infected seeds with endophytic bacteria, the percentage of germination increased, while the germination rate in the fungal infected seeds was found to be 0%. After 7 days, growth parameters of fungus+endophytic bacteria-infected seedlings were superior to those of the fungus treated seedlings. In distilled water treated seedling (T_1) the germination was 50%. In T_2 the fungus treated seed 0% germination, all seeds were infected with fungus. In the case

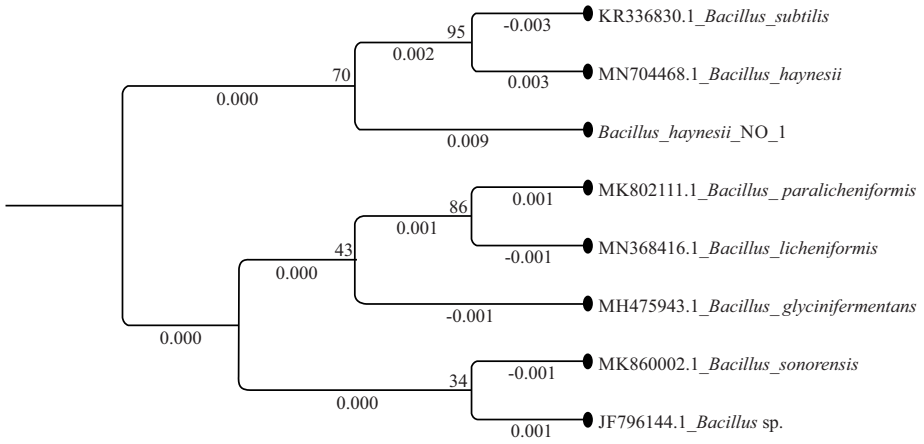

Fig. 5: Phylogenetic tree construction using neighbour-joining method of N1B isolate

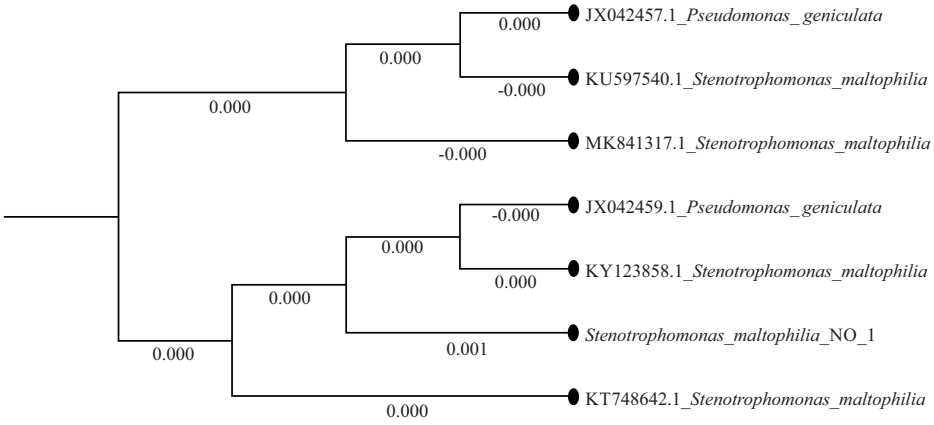

Fig. 6: Phylogenetic tree construction using neighbour-joining method of N4B isolate

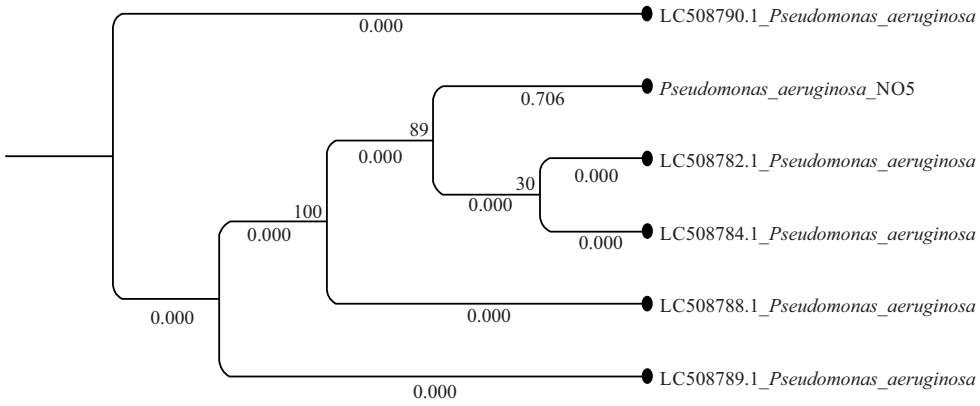

Fig. 7: Phylogenetic tree construction using neighbour-joining method of N5B isolate

of *Bacillus haynesii* (T_3), *Stenotrophomonas maltophilia* (T_4) and *Pseudomonas aeruginosa* (T_5) (endophytic bacteria treated) seeds show 54, 56 and 60% germination. The treatment T_6, T_7 and T_8 (fungus+endophytic bacteria) treated seeds exhibited maximum percentage of germination, without fungal infection 60, 61 and 65%.

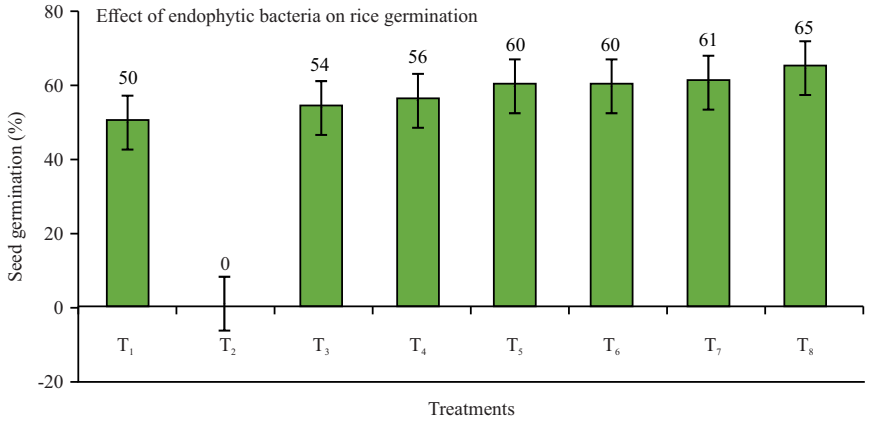

Fig. 8: Percentage of rice germination along with endophytes and *R. solani*

T_1: Control (distilled water), T_2: *Rhizoctonia solani*, T_3: *Bacillus haynesii*, T_4: *Stenotrophomonas maltophilia*, T_5: *Pseudomonas aeruginosa*, T_6: *Rhizoctonia solani*+*Bacillus haynesii*, T_7: *Rhizoctonia solani*+*Stenotrophomonas maltophilia* and T_8: *Rhizoctonia solani*+*Pseudomonas aeruginosa*

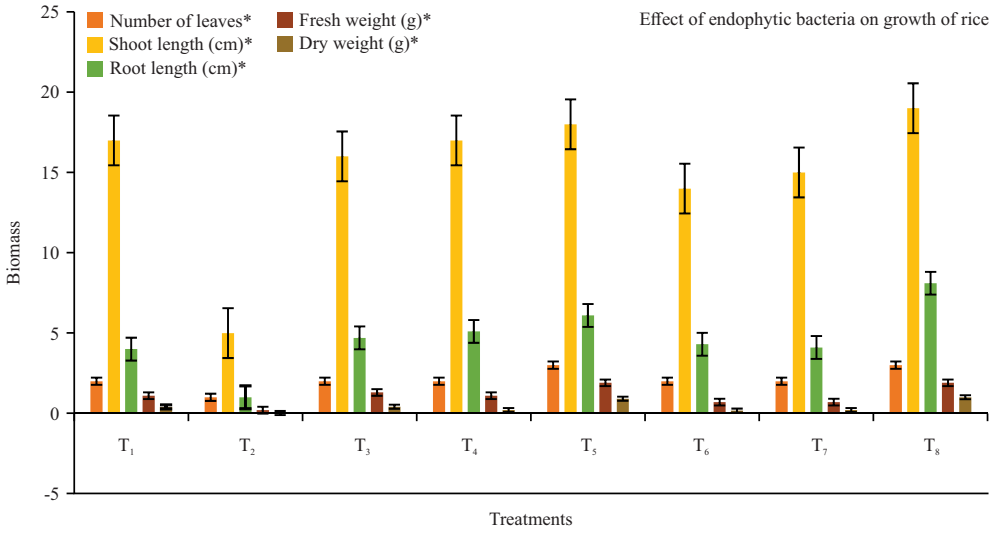

Fig. 9: Bar diagram represents the effect of endophytic bacteria on PGP activity of rice seedling

Treatment:

T_1 = Control (distilled water)
T_2 = *Rhizoctonia solani*
T_3 = *Bacillus haynesii*
T_4 = *Stenotrophomonas maltophilia*
T_5 = *Pseudomonas aeruginosa*
T_6 = *Rhizoctonia solani* + *Bacillus haynesii*
T_7 = *Rhizoctonia solani* + *Stenotrophomonas maltophilia*
T_8 = *Rhizoctonia solani* + *Pseudomonas aeruginosa*

***In vivo* plant growth promotion:** For the *in vivo* experiment, the rice plant was chosen to test the active contribution of the N1B, N4B and N5B endophytic isolates (Fig. 9). The seeds infested with fungi and endophytic bacteria possessed the significant antagonistic ability to control the disease in rice plants. The percentage increase in various growth traits over the pathogen infested plants indicated the strong disease suppression by endophytic bacteria treated seedlings. Significant effects on plant growth were observed for the plants treated with N5B isolates compared with those for the non-treated control. Root length, shoot height, fresh biomass and dry biomass increased respectively in endophytic bacteria treated seeds. In addition, the rice seed treated with this *P. aeruginosa* had more lateral roots and significant disease suppression when compared with others.

Therefore, the data obtained from *in vivo* experiments suggested that *P. aeruginosa* followed by *B. haynesii* and

S. maltophilia might be used as a Biocontrol Agent (BCA) and plant growth-promoting agent.

DISCUSSION

Endophytic bacteria exhibited a mutualistic relationship with the plants and in turn helping the plant in a different way. The present study was undertaken to isolate and identify bacterial endophytes present in leaf tissue of *A. indica* plant and studied its bio-control activity along with the identification of bioactive compounds responsible for antifungal properties.

The leaf is an ideal tissue for exploring the endophytes and their secondary metabolites. The leaf has shown the highest report on species richness and colonization of endophytes[33], which was under our study. The initial identification of the bacterial isolates was carried out based on the various morphological structure of isolated endophytic bacteria. The colony characteristics of endophytic bacteria isolated from neem are varied from one another, such as irregular and circular, flat elevation, the margin of the colonies were undulated, the surface of the growth was rough, opaque and its colour was observed as white, pink and yellowish. It was reported that the endophytic microbes were varied from plant to plant and also from different species to species. Plants under the same species may also reveal different endophytic populations in different regions. Hence the presence of endophytes was affected by different temporal and climatic changes[34]. The study revealed colonization of endophytic bacteria in the leaf tissues of neem. Similarly, several workers have also reported their occurrence in various plant species.

The pure cultured endophytic isolates were tested for antifungal activity by following the dual culture method. The bacterium, N1B, N4B and N5B exhibited significant growth reduction of *R. solani.* There were various reports on the isolation of endophytic bacteria as a Biocontrol Agent (BCA's) against different phytopathogenic fungi such as *Alternaria alternata*, *Botrytis fabae*, *B. cinerea*, *Colletotrichum gloeosporioides*, *Pythium* sp., *R. solani*, *Fusarium* sp., *Verticillium dahlia*, *Sclerotinkia sclerotiorum* and *Penicillium digitatum*[35]. Various endophytic bacteria colonize an ecological niche, which helps them to act as a suitable biocontrol agent[36]. In the *in vitro* dual culture method, the reduction of mycelial growth of sheath blight causing fungi with endophytic bacteria agreed to the statement mentioned by various authors.

The selected isolates were tested for the production of volatile compounds under *in vitro* study. As compared with antibiotics, the Volatile Compounds (VOC's) can spread over a long distance and fungistatic microenvironments develop around the antagonist communities[37]. Therefore, microbial antagonist strains capable of producing volatile compounds with potent inhibitory activity against plant pathogens are more likely to prevent pathogenic fungi, by killing spores in the soil and limiting both the production and the establishment of the disease.

Pseudomonas aeruginosa ethyl acetate extract showed maximum mycelium reduction of *R. solani* when compared with others. The important bioactive compounds identified using GC-MS analysis having maximum probability was Dimethoxyglycerol Docosyl Ether (77.58), 13-Docosenamide, (Z)-(46.20), pentadecanoic acid (42.46) and 9, 12, 15-Octadecatrienoic acid (22.97). Kanjikar and Londonkar[38], reported the presence of Dimethoxyglycerol Docosyl Ether in GC MS analysis of Petroleum ether extract of *Lepidagathis scariosa*. The occurrence of pentadecanoic acid has been reported in some species of marine macroalgae and its antimicrobial activity was proved by Agoramoorthy *et al.*[39]. The fungus *Diaporthe schini* solvent extract exhibited the presence of 13-Docosenamide with antimicrobial activity was reported by dos Reis *et al.*[40]. Psidium guajava extract analyzed by gas chromatography-mass spectrometry exhibited different compounds one of which is 9,12,15-Octadecatrienoic acid and its antimicrobial activity was well studied. These antimicrobial compounds in the endophytic bacterial extracts may be contributed to their antifungal activity in the current study.

The isolates showing maximum antagonistic activity was identified using morphological, biochemical and molecular characterization and it was identified as *Bacillus haynesii* (MN880435), *Stenotrophomonas maltophilia* (MN880434) and *Pseudomonas aeruginosa* (MN880486), respectively. The functional roles of bacterial endophytes have been well explained. They are well in increasing the host plants resistance to pathogens and promoting Biological Nitrogen Fixation (BNF)[41]. Different studies have reported that they arbitrate de novo synthesis of novel antibacterial compounds and antifungal metabolites[42]. The antifungal activity of the endophytic isolates was tested *in vitro* against sheath blight causing fungi. Bacteria of the genera *Bacillus* sp. and *Pseudomonas* sp. are dominant rhizospheric bacteria but have also been reported as endophytes in several plant species[43]. Tiwari *et al.*[44], reported the isolation of different endophytic bacterial species such as *Bacillus amyloliquefaciens*, *Burkholderia denitrificans*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Azotobacter tropicalis*,

Acetobacter xylinum and *Azospirillum lipoferum* from native neem varieties at Sanganer areas situated at Rajasthan, India. The endophytes such as *P. aeruginosa*, *P. putida* and *B. megaterium* associated with black pepper were reported as active antagonists for biocontrol of *Phytophthora* root rot which recorded over 70% disease reduction in greenhouse experiment[45]. The phenotypic and biochemical analysis of the N1B indicated that gram-positive isolate recovered from neem leaf tissue, share the characteristics of previously reported *Bacillus* sp., from the desert soil and also from Palm leaf tissue[46]. *Stenotrophomonas maltophilia* occur ubiquitously in the environment and have been isolated from a wide range of sources including water, sediment, soil, rhizosphere and plant tissues[47]. Following the current study Nagrajkumar *et al.*[48] isolated *Stenotrophomonas maltophilia* endophytic bacteria from roots and leaves of *Prosopis cineraria*. Devi *et al.*[49] isolated, *Pseudomonas aeruginosa* AL2-14B, an endophytic isolate from the aerial part of the *A. aspera* L. plant, which produced a maximum quantity of siderophore, IAA and solubilized inorganic phosphate. It improves the growth and antioxidant properties of the host plants.

The selected isolates exhibited different PGP properties under *in vitro* conditions. Production of Indole-3-Acetic Acid (IAA) by endophytic microbes is one of the important traits that enhance plant growth directly. The endophytic isolates with a significant amount of IAA production were isolated by Chandra *et al.*[50] and Ahemad and Khan[51]. This statement is following the results of the present study. It has been reported that many endophytes including *Enterobacter*, *Azotobacter*, *Serratia*, *Klebsiella* produced IAA which stimulated plant growth[52]. In plant growth-promoting analysis endophytic strains *P. aeruginosa* BacDOB-E19 and PGPR strains *B. cereus* RbacDOB-S24 produced a significant amount of IAA[50], which are the following present study.

Phosphorus is needed by plants for growth and development but its availability in agricultural soils is low[26]. Endophytes are known to improve plant growth by phosphate solubilization[37], this is in contrast to our study, 3 isolates were unable to solubilize phosphorus in a medium.

In the present study, potassium solubilization was carried out in an Aleksandrov agar culture medium. It was found that among 3 bacterial isolates, N5B isolates exhibited maximum K solubilization efficiency. Similar to the present study[27], reported that soil beneficial microorganisms such as bacteria, fungi and actinomycetes have solubilized the insoluble potassium to soluble forms of potassium by different mechanisms including acidolysis, chelation, complexolysis, exchange reactions, production of inorganic and organic acids and polysaccharides. Decomposition of organic matter in the soils leads to formic acid, citric acid and oxalic acid production. Organic acids thus produced increases the solubilization of K compounds by providing protons and calcium ion complex[53]. Zinc is a micronutrient essential for plants in very low amounts. The 3 isolates of the study were unable to solubilize Zn in an *in vitro* culture medium. Zinc solubilizing microorganisms solubilize zinc through various mechanisms. These microbes produce anions by lowering the pH and chelated zinc to solubilize[54] and different PGPR organisms involved in either of these mechanisms such as *Pseudomonas, Rhizobium* sp., *B. aryabhattai* and *Azospirillum* sp.[55].

Nitrogen-fixing bacteria can convert stable atmospheric nitrogen gas it is a biologically useful form and organisms involved in this process is known as diazotrophs. Ammonia production is another plant growth-promoting property, which has a signalling role between plant and bacterial interactions. With the help of enzyme nitrogenase, diazotrophic organisms reduce di-nitrogen to ammonia[56]. Diazotrophic bacteria can positively influence plants by improving growth and root development, which increases plant tolerance to various environmental stresses. Production of ammonia can be taken up by plants as a source of nitrogen. Here it was observed that the most prominent activity was exhibited by N5B followed by N4B which has also been reported by researchers[57].

In the case of EPS production, PGP bacteria and actinomycetes isolates exhibited significant EPS production. Exo-polysaccharide (EPS), Lipopolysaccharide (LPS), capsular polysaccharide and cyclic β-(1→2)-glucan play essential roles in the formation of the infection thread and nodule development, although the precise functions of these molecules are still being investigated[58].

Various bacterial endophytes enhance plant growth indirectly by preventing the growth and activities of plant pathogens by the production of antimicrobial substances like HCN through different mechanisms. In the current study, HCN activity was displayed by N5B isolate it is in line with the findings of other researchers[59]. Nagrajkumar *et al.*[48] reported that the production of HCN and lytic enzymes by *Pseudomonas* strains that could able to control the plant root pathogens including *F. oxysporum* and *R. solani* was following the present study.

Rice (*Oryza sativa*) is one of the staple crops for a larger part of the world's population and is produced around the globe. Rice cultivation is affected by many abiotic and biotic stresses which include fungal pathogens that attack the crop from seed to harvest and result in severe yield losses. Seed-

borne fungal pathogens drastically affect germination and yield[59]. The effects of these isolates on plant growth and soil-borne diseases of rice were evaluated in nursery experiments. We found that the isolates, not only significantly improved seed germination, seedling length and plant growth of rice but also, when used as bio-inoculant, significantly reduced disease symptoms caused by sheath blight causing fungi *Rhizoctonia solani.* Following our study, Tika *et al.*[58] reported that selected endophytic bacterial strains such as *Pseudomonas fluorescens*, *Pseudomonas tolaasii*, *Pseudomonas veronii* and *Sphingomonas trueperi* have the potential for control of seedling disease of rice and plant growth promotion. Since the first step of bacteria invasion into the plant root involves the attachment of bacteria onto epidermal cells of the root surface, where root hair zone is one of the major locations of primary colonization, mostly on the basal area of developing hairs. The IAA producing isolates can provide an increased root system and can colonize plant roots better than other isolates. As similar to the current study the presence of a significant no: Of lateral roots and the occurrence of more root hairs were observed in response to colonization by the strains when compared with non-inoculated rice plants.

Plant growth promotion and bio-control activities are the main requirements for commercial microbial agents used in sustainable agriculture. Our study indicated that strain *B. haynesii*, *S. maltophilia* and *P. aeruginosa* introduced into the rice rhizosphere might play essential roles in disease control and also be involved in plant growth. Subsequently, molecular characterization of 3 microbes is necessary for confirming their identity, so the nucleotide sequence of these isolates was analysed and the data was submitted to the repositories of NCBI. These accession numbers can be utilized by other researchers in the same field for citation purposes and sequence analysis. Also, analysis can be commenced to know about the genetic diversity of these isolates.

Hence, we suggest that these strains had the potential to be used in sustainable agriculture. Although these isolates need further testing under field conditions, we are confident that our findings can be transferred to the field, because the strains were isolated from the same environment in which they are intended to be used. To the best of our knowledge, this is the first report on screening neem endophytic bacteria viz., *B. haynesii*, *S. maltophilia* and *P. aeruginosa* as potential BCAs and it comprehensively elaborates the mechanisms of biocontrol of soil-borne diseases. Integration of multiple microbial strains as a single consortium can offer different benefits to crop plants. Further investigations about outcomes of plant-endophyte interactions in different ecological settings and under field conditions would be beneficial to utilize the full plant growth-promoting potential of endophytes.

CONCLUSION

The endophytic bacteria can improve plant tolerance to various biotic and abiotic stresses. Bacteria with multiple PGP traits could be used as a bio-control agent to improve the fertility of the soil and it is a better alternative to synthetic chemicals. Hence, it is essential to identify the potential multifaceted beneficial microbes for their bio-control properties. In this context, the present study was undertaken to explore the diverse status of different endophytic microbes in neem plants. The present study brought to light that the neem endophytic isolates such as *B. haynesii*, *S. maltophilia* and *P. aeruginosa*. Up to our knowledge, these microbes were reported for the first time in neem leaf. Different plant growth promotion activities by these PGP microbes revealed that these isolates can be mass-produced for the growth improvement of rice plantations. The present investigation is one step forward in this direction to fill up the lacuna.

SIGNIFICANCE STATEMENT

This study discovers the occurrence of endophytic bacteria, *B. haynesii*, *S. maltophilia* and *P. aeruginosa* in neem leaf. This study will help the researcher to uncover the critical areas of endophytic microbes, that many researchers were not able to explore. Thus a new theory on endophytes in host plant protection and its PGP activities may be brought to knowledge.

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