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Isolation, characterization and metabolic profiling of seed endophyte *B. licheniformis* against *Sarocladium oryzae* in rice

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Abstract

Rice is a vital staple food crop widely cultivated across diverse Asian agroclimatic zones. However, in recent years, the emergence of sheath rot disease, caused by *Sarocladium oryzae*, has severely impacted rice yields, devastating approximately half of rice production. Traditional methods of controlling plant diseases often have harmful effects on the environment and have led to the development of pathogenic resistance to various agrochemicals. In contrast, endophytes have shown great promise in managing plant diseases while enhancing plant growth and yield. The seed-associated endophyte *Bacillus licheniformis* has demonstrated remarkable efficacy, exhibiting a 76.47% inhibition rate against *S. oryzae*. Beyond its antibiotic properties, this endophyte also promotes biostimulant activities, including the production of indole-3-acetic acid (IAA), siderophores, and the utilization of ammonia (NH_3). Additionally, the analysis of secondary metabolites using gas chromatography-mass spectrometry (GC-MS) revealed a diverse array of compounds, including 9,12-Octadecadienoic acid (Z, Z)-TMS derivatives, Elaidic acid-TMS, Bis(2-ethylhexyl) phthalate, Caproic acid-TMS, Diethyl phthalate, Ricinoleic acid-2TMS derivatives, Mandelic acid-2TMS, and others. These compounds exhibit significant antifungal, antiviral, larvicidal, and antibacterial activities against various plant pathogens, highlighting the potential of *B. licheniformis* as a sustainable and effective biocontrol agent in rice cultivation. This research underscores the critical role of endophytes in promoting sustainable agricultural practices, offering an environmentally friendly alternative to chemical control methods while effectively combating emerging plant diseases.

Keywords

biochemical test; endophyte; GC-MS; plant growth promotion activities

Introduction

Rice is a stable food consumed throughout Southeast Asia and used as animal food. India is the world's second-largest producer of rice, following China, contributing nearly 24% of global production (1). The populations of developing countries, particularly India and China, have been growing exponentially, while rice production and productivity have only increased arithmetically. In 1960, the Green Revolution significantly changed rice cultivation methods by introducing high-yielding semi-dwarf varieties, applying a high rate of nitrogenous fertilizer, photoperiod insensitive

cultivars, and excessive water usage. These changes happen in the rice ecosystem, and maintaining prolonged conducive micro-macro climate conditions favors the plant's susceptibility to different kinds of plant stress (2). The average production and productivity per hectare were low due to the biotic and abiotic challenges and stresses. These stresses hit to bombard high-level yield losses. Among those stresses, biotic stress is very challenging to all crop cultivation. The annual yield loss of 15% is due to biotic stresses in global rice production (3). Once confined to limited regions, sheath rot has emerged as a major disease in rice ecosystems.

Sheath rot is caused by *S. oryzae.* In 1922, in Taiwan, sheath rot was first reported as *Acrocylindrium oryzae* by Ditjen Pangan in 1987 (4). In India, this disease was first recorded by Agnihothrudu in 1973 and Amin in 1974 (5). The entire flag leaf was impacted, resulting in partially filled grains, discolored grains, and sterile seeds. It produces helvolic acid, cerulenin, and SO-toxin, which is important for the aggressiveness of the pathogen (6). Globally, the disease can cause yield losses of 20 to 85% (7). In Indonesia, losses as high as 85% have been recorded (8), while in West Java, 10-15% yield losses have been monitored (9). Southeast Asian countries have reported yield losses of up to 80% (10).

To overcome these challenges, farmers are advised to implement several key management practices, *viz*., growing resistant varieties, crop rotation, nutrient management practices, and chemical management to control plant disease and improve crop productivity (11). However, challenges remain in the practical application of these methods, including pathogen variability, the durability of resistance mechanisms, the resistance of pathogens to chemical fungicides, and global climate change, all contributing to severe disease outbreaks at regular intervals (2). Using beneficial biocontrol microorganisms (BBM) to enhance plant activities is an alternative approach to adapt to the current sustainable developments in the rice system. Endophytic microorganisms interact either mutualistic or parasitically with the host. The beneficial endophytes live in plants without abnormal changes to the host plants. The seed conserves holobiont microbes. As a reproductive organ, seeds provide a niche exclusive to a particular microbiota, which possesses a variety of adaptations necessary for successful colonization. The environment within the seed may change throughout germination, affecting the microbial population. The Endophytes have developed unique traits to overcome these environmental changes, traits which are rarely found in other plant endophytes (12). Seed core microbiota helps in nutrient solubilization, phytohormone production, and synthesis of antimicrobial compounds, as well as help maintain homeostasis and reduce plant stresses (13). These endophytes act as a protective layer to seeds throughout the life cycle and help to reduce plant stress. The secondary metabolites actively reduce the colonization of plant pathogens. Their secondary metabolites actively reduce pathogen colonization and inhibit the growth of plant pathogens, often leading to lysis or shrinkage of mycelium (14).

This research underscores the critical role of endophytes in promoting sustainable agricultural practices, offering an environmentally friendly alternative to chemical control methods while effectively combating emerging plant diseases.

Materials and Methods

Isolation of sheath rot

The disease-infected leaves are cut into 0.5 to 1 cm in size. These samples were surface sterilized with 70% ethanol, followed by washing sterile water three times and dried in a sterile filter paper, then transferred with the help of forceps placed in a potato dextrose agar media plate. After 2-5 days, the hyphal tip of the pathogen was transferred onto a new plate to obtain pure cultures for sheath blight, brown spot, sheath rot and blast (15).

Pathogenicity test for sheath rot

The ADT 57 seed material was collected from TRRI in Aduthurai. Seeds were sowed in pot, and plants were grown until the flowering stage. Chaffy grains of CO 51 were collected from Tamil Nadu Agricultural University (TNAU), Coimbatore. These grains were sterilized at 121^0C for 15 psi. Then the freshly grown culture was inoculated in the sterilized chaffy grains under controlled conditions and incubated at ambient temperature for 15 days to complete sporulation. Individual mycelial coiled grains were inoculated into the boot enclosed by flowering without damaging host plants. Symptoms appeared after 5 to 7 days (15).

PCR amplification and agarose gel electrophoresis

The molecular conformation of the fungal pathogen by using ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'TCCTCCGCTTA TTGATATGC-3') with following condition initial denaturation 95^0C for 2 min, followed by 35 cycles of denaturation 94^0C for 30 sec, annealing 56^0C for 1 min, extension 72^0C for 2 min and final extension 72^0C for 8 min was conducted in Bio radar PCR with 35 cycles. The PCR reaction contains 5 µL master mix, 1 µL each forward and reverse primer, 1 µL DNA and 2 µL water, for a total volume of 10 µL.

Agarose gel electrophoresis was performed using the standardized protocol to detect the qualitative DNA after the completion of the polymerase chain reaction (16). A 1.2% (w/v) agarose gel was prepared in 1X TAE buffer and cooled before adding 5 µL of ethidium bromide. Then the amplified PCR product was resolved into Agarose gel electrophoresis with 1.2% (w/v) agarose gel. In the first lane, load 5 µL of 100 base pair ladders followed by series lane with 5 µl genomic DNA. Then run the gel electrophoresis unit for 45 min, 75 volts. After the completion gel run was followed by a visualized UV detector and documented. The pathogens were amplified at 560 bs purified, and sent for sequencing in Biokart, Bangalore. Then the gene sequences were compared with the National Centre for Biotechnology Information (NCBI) database sequences.

Seed sample collection and isolation of bacterial endophytes

Traditional rice seeds were collected from various rice-growing areas of Tamil Nadu, India. In the lab, 15 to 25 seeds were selected for endophyte isolation. The glassware, phosphate buffer, and Tryptic soya agar were sterilized by autoclaving at 121^{0}C, 15 psi. The seed samples were surface sterilized with 2% sodium hypochlorite for 3 to 4 min and rinsed with 70% ethanol for 2 min. They were then washed three times in double sterile autoclaved water and dried in sterilized filter paper. The dried seeds were crushed in a sterile phosphate buffer solution using a pestle and mortar. The resulting suspension was serially diluted, and 1 mL of the diluted solution was plated onto TSA in triplicate. A control plate with water was maintained. Plates were incubated at 28 ± 2^{0}C for 2-3 days. After 2 to 3 days, the individual morphologically distinct colonies were purified and finally stored in glycerol stock at -20^{0}C (17).

Dual culture plate for sheath rot

A Dual culture plate method was employed to determine the biocontrol efficacy of isolated endophytic bacteria against pathogens. The isolated endophytic bacterial culture was streaked 1 cm from the edge of the PDA plate, and a 5 mm disc of the pathogen was placed perpendicular on the opposite side. The plates were incubated at 28±2^{0}C until the control plate was completely grown. Each treatment was replicated thrice, and the percent inhibition zone was calculated based on the formula (18):

$$\text{Percent inhibition Zone (PIZ)} = \frac{X - Y}{X} \times 100 \qquad \text{Eqn.1}$$

where, X is the growth of the test pathogen (mm); Y is the growth of the test pathogen (mm) in the presence of the endophytic strains

PCR amplification and agarose gel electrophoresis

Molecular characterization of bacterial isolates will be done by extraction of DNA and characterization was performed by 16 rDNA regions using universal primers 27F (5-GAGTTTGATCCTGGCTCA-3) and 1492 (R-TACGGYTACCTTGTTACGACTT) following condition initial denaturation 94^{0}C for 4 min, followed by 35 cycles of denaturation 94^{0}C for 1 min, annealing 55^{0}C for 1 min, extension 72^{0}C for 2 min and final extension 72^{0}C for 10 min in a Bio Rader thermocycler with 35 cycles.

The amplified PCR product was resolved in 1.2% (w/v) agarose gel. In the first lane 5 µL of 1kb ladder was loaded, followed by a series lane with 5 µL amplified PCR products, and the gel unit was run for 45 min at 75 volts. After completing the gel run, the amplified PCR product was visualized and documented under a UV detector. The amplicon size greater than 1500 bp was purified and sequenced (Biokart, Bangalore). The gene sequences were blasted in the National Centre for Biotechnology Information (NCBI) database and compared in the online NCBI database.

Morphological and biochemical characterization of endophytes

Gram staining was performed to understand the morphological character of the bacterial endophytes. Various biochemical tests were also assessed, such as urease, methyl red, Voges Proskauer, citrate, gelatinase, indole and glucose utilization tests (19).

Urease test: The 24 hrs old bacterial colony was inoculated in urea broth (urea 20 g, sodium chloride 5 g, monopotassium phosphate 2 g, peptone 1 g, dextrose 1 g, phenol red 0.01 g in 1 liter distilled water followed by adjusted with p^H 6.7). A bright pink color formed after 3 to 4 days of incubation indicated a positive reaction for the urease test.

Methyl red test: The 24-hour-old bacterial colony was inoculated in Buffered peptone broth (peptone 7 g, glucose 5 g in 1 liter distilled water followed by adjusted with pH 6.9). This test is used to determine acid production during anaerobic fermentation. The inoculated test tube was incubated at room temperature for 24 hrs After incubation, 2-3 drops of 0.02% methyl red indicator were added and kept for 2 to 3 min to produce red, which indicated a positive result, and yellow color is a negative result.

Voges Proskauer (VP) test: The same broth of methyl red was used for the VP test. After inoculating the 24-hour-old culture in broth, it was incubated at room temperature for 2-3 days. Following incubation, 2 to 3 drops of Baritts reagent (α-Naphthol 5%) was added. A positive result was indicated by forming a red lipid layer, while the yellow lipid layer indicated a negative result.

Citrate utilization test: The 24 hrs old bacterial colony was inoculated in citrate broth (ammonium dihydrogen phosphate 1 g, dipotassium phosphate 1 g, sodium chloride 5 g, sodium citrate 2 g, magnesium sulfate 0.02 g and bromothymol blue 0.08 g in 1 liter distilled water). The inoculated test tube was incubated at room temperature for 3 to 4 days. The color change from green to blue indicated a positive result.

Gelatinase test: A loop of colonies were inoculated in a gelatinase broth containing (peptone 5 g, beef extract 3 g, and gelatin 120 g in 1 liter distilled water). Then the test tube was incubated at room temperature for 2 to 3 days. After the tubes were inserted in an ice box containing ice crystals, the liquefication tube had results, and the solidified tube showed negative results.

Glucose utilization test: A loop full of colony inoculated in a glucose broth containing (peptone 2 g, sodium chloride 5 g, dipotassium hydrogen phosphate 0.3 g, bromothymol blue solution 15 mL with 1 percent glucose in 1 liter of distilled water). The production of yellow acids indicated positive glucose utilization.

Indole test: The 24 hrs old bacterial colony was inoculated in SIM broth containing (peptone 30 g, beef extract 3 g, ferrous ammonium sulfate test 0.2 g and sodium thiosulphate 0.025 g in 1 liter distilled water). The presence of a black color indicated a positive result.

In vitro evaluation of plant growth-promoting traits of effective Bacillus subtilis

The effective endophytes were further studied for direct and indirect plant growth-promoting traits.

Protease production: The activity was performed by spot inoculating isolated bacteria on 1% skimmed milk agar medium plates and incubating at 30°C for 5-7 days. The appearance of a clear halo zone around the culture spot will be treated as positive for protease production (20).

Lipase activity: The 24 hrs bacteria were streaked in a tween 20 agar plate media containing 10 g of peptone, 0.1 g of $CaCl_2.2H_20$, 5 g of NaCl, agar 20 g, 10 mL of tween 20 in 1 liter distilled water and incubated for 5 days. Milky precipitation around the bacterial colony indicated positive results (21).

Plant growth promoting activity effective endophytes

Ammonia utilization: The ability of bacteria to utilize NH_3 is performed (22). Spot inoculation 24 hrs old effective endophytes in a test tube containing 10 mL of peptone broth. Then, it was incubated in a mechanical rotary shaker at 120 rpm for 96 hrs at 30^0C and maintained the control as without inoculation. Then 2 to 3 drops of Nessler's reagent (potassium iodide 7 g, mercuric chloride 2 g, sodium hydroxide 4 g and distilled water 100 mL) was added and allowed for 5 min. The color change from yellow to brown color indicated a positive result.

IAA production test: The IAA was examined qualitatively by using Salowski's reagent (a mixture of 0.5 M ferric chloride ($FeCl_3$) and 35% of perchloric acid ($HClO_4$)). The bacteria were spot inoculated in NA amended with L- Tryptophan (1 mg/1 mL medium) and then incubated in a mechanical shaker for 3 days at 37^0C. After incubation, the broth was centrifuged at 10000 rpm for 20 min and the pellet was discarded. Finally, 2 mL of Salowski's reagent was added to 1 mL of broth, and the development of a dark pink color indicated a positive result (11).

Siderophore production activity: The effective endophytes are screened qualitatively in a chromo azurol S (CAS) agar medium. Streak 24 hrs old potential endophytes in CAS medium and incubate at room temperature for 2 to 4 days for chelating iron. The observation of orange to yellow indicates that the bacteria can chelate the iron, which is a positive result (23).

Assessment of growth promoting activities of endophytes in rice seedling by paper towel method

The paper towel method was used to evaluate the growth promotion properties of bacteria (ISTA, 1999). The potential endophytes were inoculated in a Nutrient agar broth and kept in a shaker for 24 hrs at 27°C. Then the broth was centrifuged at 10000 rpm for 20 min. The cells were suspended in phosphate solubilizing buffer, and 0.5% of carboxymethyl cellulose was added. The seeds are disinfected in 5% sodium hypochlorite solution and rinsed with sterile water thoroughly. The disinfected seeds are soaked with endophyte culture for 2 hrs and kept in a shaker, and the seeds are soaked in water to serve as a control. Endophytes treated and control seeds were arranged in a germination paper at the rate of 25 seeds per sheet. On the seventh day, the seedlings' germination percentage, root length, and shoot length were recorded in each treatment and the seedling vigor index was calculated (24):

$$\text{Vigour Index (VI)} = X \times Y \qquad \text{(Eqn.2)}$$

Where, VI= Vigour index, X= Mean root length+ mean shoot length, Y= Germination percentage %.

Detection of secondary soluble volatile compounds through gas chromatography-mass spectrometry

The secondary crude antibiotic metabolites were extracted from the potential endophytes. The bacteria were inoculated in LB broth and incubated at 28^0C±2 for 78 hrs with continuous shaking (25). After incubation, an equal volume of HPLC-grade ethyl acetate was added, and the mixture was shaken continuously overnight. The solvent was dehydrated using a vacuum flask evaporator, and the dehydrated solvent was gathered by adding 1 mL of methanol. Followed by the derivatization process is processed. Then, samples were completely dried in an evaporator concentrator at 45 for 3 hrs. After the sample was derivatized by mixing with 50 µL methoxamine hydrochloride (20 mg/1 mL of pyridine), then incubated in a shaking water bath for 2.30 hrs at 37^0C. Then add 80 µL N -Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) incubated water bath at 37^0C for 30 mins. Finally, the samples were centrifuged at 10000 rpm for 3 min, and the supernatant was transferred into GCMS vials. (Department of Plant Biotechnology, TNAU Coimbatore).

Statistical Analysis

All the experiments were analyzed independently. The treatment means were compared using Duncan's Multiple Range-Test (DMRT), and the data were analyzed using SPSS software.

Result

Isolation of sheath rot

The microscopic observation showed cottony growth with white-colored septate mycelium. The *Sarocladium* spore is oval-shaped and colorless in nature.

Pathogenicity sheath rot

After 5 to 6 days of pathogen inoculation, a brown-colored oval-shaped spot was produced on the leaf sheath. The spot enlargement caused complete browning of the leaf, leading to a lack of chlorophyll and the abortion of seeds or discolored seeds due to toxin production. Later, the brown-colored spot spread throughout the entire plant, causing brown-colored discoloration.

Molecular characterization of plant pathogen

The pathogen was molecularly confirmed by using ITS1 and ITS4 primers for fungi. The amplified products of more than 560 bp were sequenced and blasted in the NCBI database, compared the sequence, and submitted to

the National Centre for Biotechnology Information (NCBI) Accession number *S. oryzae* (PP709063).

Isolation of endophytes

The seed samples of four traditional rice varieties were collected from different rice-growing areas *viz.,* Sivan Samba, and Thuya Malli from Erode district, Kotchi Samba, and Arupathankurai were collected from Kanyakumari district. Eighteen endophytes were isolated from those seed samples, from RE- 1 to RE- 18.

Screening of endophytes against plant disease in rice under in vitro condition

Antagonistic study of rice sheath rot disease: The seed endophytes were screened against *Sarocladium oryzae* under in vitro conditions. From that, 18 seed-borne endophytes were potentially screened against *S. oryzae.* In those 14 endophytes RE- 18 shows the highest percent inhibition against *S. oryzae* at 76.47% with radial mycelial growth of 20 mm, followed by the second highest inhibition against *S. oryzae* is RE-15 which shows 64.31373 with 30 mm of mycelial growth. The least inhibition out of those 8 endophytes is RE-40, which shows 35.68627% of mycelial reduction and 54 mm of mycelial growth. The control treatment shows a cent percent mycelial growth. The highest inhibition of RE-18 was chosen as an effective endophyte for further study (Table 1 & Fig. 1).

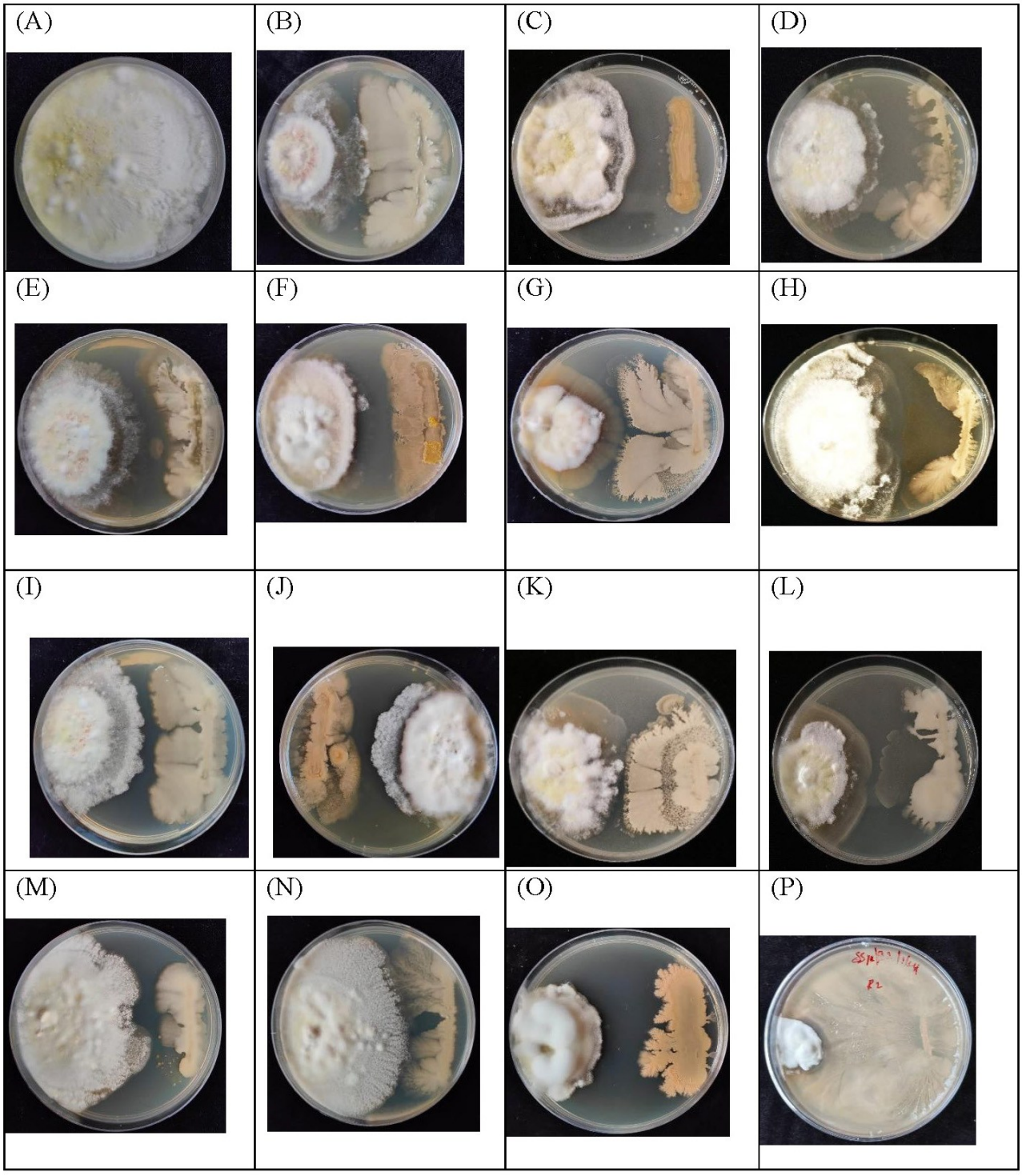


Fig. 1. Antagonistic activities against sheath rot, A) Control, B), RE-1, C) RE-2, D) RE-4, E) RE-5, F) RE-6, G) RE-16, H) RE-7, I) RE-8, J) RE-9, K) RE-10, L) RE-13, M) RE-15, N) RE-16, O) RE-17, P) RE-18.

Table 1. *In vitro* screening of antagonistic against sheath rot

Treatment	Mycelial growth (mm)	Inhibition Zone (%)
RE1	52.67^{h}	38.039^{h}
RE2	52.00^{h}	38.823^{h}
RE4	44.67^{ef}	47.451^{ef}
RE5	47.33^{g}	44.314^{g}
RE6	45.00^{ef}	47.059^{ef}
RE7	51.67^{h}	39.216^{h}
RE8	47.33^{g}	44.314^{g}
RE9	39.67^{cd}	53.333^{cd}
RE10	38.33^{c}	54.902^{c}
RE13	42.67^{de}	49.804^{de}
RE14	38.33^{c}	54.902^{c}
RE15	54.67^{h}	35.686^{h}
RE16	40.33^{cd}	52.549^{cd}
RE17	30.33^{b}	64.314^{b}
RE18	20.00^{a}	76.471^{a}
Control	85.00^{i}	0.00^{i}
CD	1.573	1.851
SED	0.898	1.056
SD error	0.275	0.323
CV	4.173	4.835

Biochemical test for effective endophyte RE-18: RE- 18 shows a series of red with dull creamy brown color colony growth, and gram staining showed gram-positive reaction. The biochemical test showed a positive result for citrate utilization, urease test, and glucose utilization test followed by negative results on the indole test, VP test, methyl red test, gelatinase test, and hydrogen sulfide production test.

Qualitative enzyme activity and *in vitro* efficacy of RE-18 isolate: The RE- 18 isolate showed a positive reaction for IAA production, siderophore production, and NH_3 utilization test. The bacteria produced a dark pink color after adding the Salowski's reagent. Effective endophytes in the CAS agar plate produce a yellow hallow zone around the colony, indicating a positive result. In the 72-hour-old bacterial tryptone broth, adding Nessler's reagent changed from yellow to brown, which indicated a positive result. Also, RE-18 showed positive protease activity results and significantly produced a clear hallow zone around the bacterial colony (Fig. 2).

Molecular characterization of endophytes: The isolate further goes for R.E- 18 for molecular characterization. These isolates were molecularly confirmed using primer 16S rRNA using universal primer 27F and 1492R primers. The amplicon size of the PCR product was more than 3000 bp, the sequenced nucleotides were compared in the online National Centre for Biotechnology Information (NCBI) database, and sequences were submitted in the gene bank with Accession number (PQ056583). RE-18 isolate matched with *B. licheniformis*.

Growth promoting activity of RE-18 isolate using roll towel paper method: The plant growth-promoting activity of the RE-18 isolate was evaluated using the roll towel paper method. Seed bacterization with RE-18 isolate, out of 25 seeds, 24 seeds were germinated, which shows a 96% germination rate with significant increases in root growth of 11.525 cm, shoot growth of 4.612 cm, and seedling vigor 1601.28. In control, 23 seeds were germinated and exhibited a 92% germination rate with seedling vigor of 1549.2. Compared to the control, the RE-18 treated seeds showed significant increases in the secondary root growth.

Detection of secondary metabolites through gas chromatography-mass spectrometry

The endophytes RE-18 produced a total of 79 compounds, out of which 48.10% were unique compounds found in the bacteria compared to the control. The remaining 51.89% of the compounds were similar to those in the control. Of that unique compound, 39.47% showed antifungal, antibacterial and antimicrobial activities. The remaining 60.52% reports no antimicrobial activity and is considered a new compound produced by bacteria RE- 18 isolates. The activities of these unique compounds are listed in Table. 2. From Table 2, 12-Octadecadienoic acid (Z, Z)-,

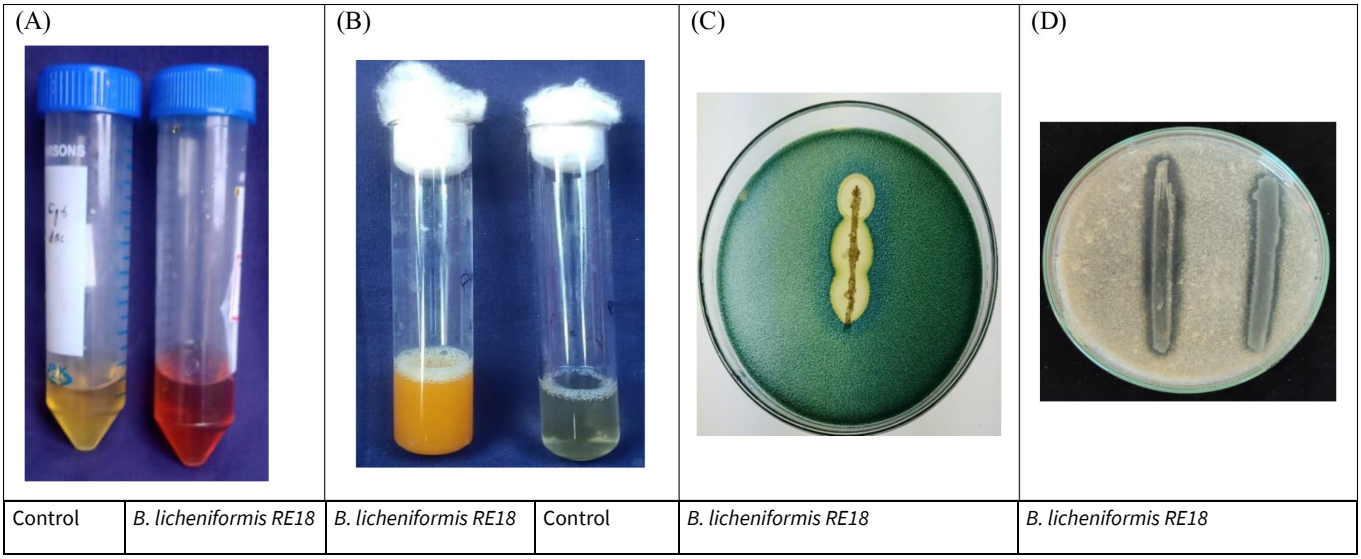
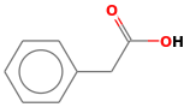
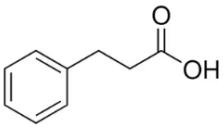
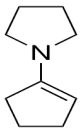
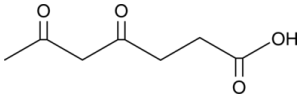
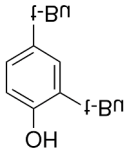
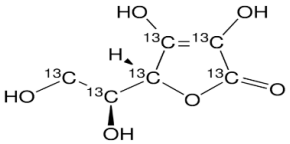
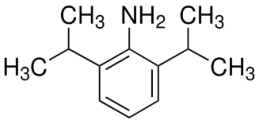
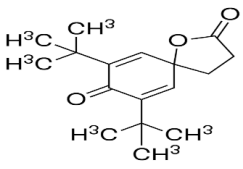
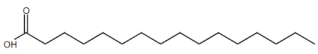
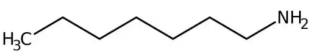
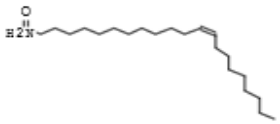
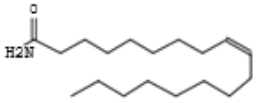
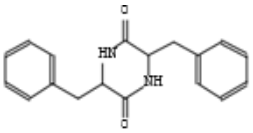
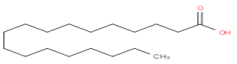
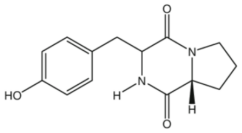
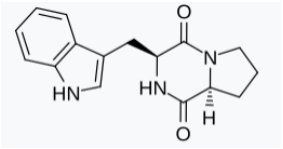


Fig. 2. Plant growth promoting activities A) IAA, B) Ammonia utilization test, C) Siderophore, D) Protease activity.

Table 2. GC-MS result of B. *licheniformis* RE18 and its activity

Compound	R/t	Area %	Type of compound	Activity	Structure
Benzene acetic acid	12.908	1.03	Organic compound	Antifungal and antibacterial activity	
Hydro cinnamic acid	14.373	0.21	Phenolic compound	Antioxidant and antibacterial activity	
Pyrrolidine, 1-(cyanoacetyl)-	16.573	0.14	Organic compound	Antibacterial activity	
1-Dodecanol	19.71	0.7	Fatty alcohol, organic acids	Antibacterial activity	
2,4-Di-tert-butylphenol	18.355	0.3	Phenolic compound	Antifungal, antioxidant, antibacterial and antiviral activity	
Succinylacetone-meto-TMS(3)	18.611	0.24	Organic compound	Antibacterial activity	
1-Heptadecene	20.298	0.17	Lipid	Antibacterial and antifungal activity	
Cyclo(L-prolyl-L-valine)	25.338	1.95	Amino acids	Antifungal and antibacterial activity	
1-(+)-Ascorbic acid 2,6-dihexadecanoate	25.792	2.87	Polyphenolic compounds	Antimicrobial activities	
3,6-Diisopropylpiperazin-2,5-dione	26.145	0.58	Diketopiperazine c	Antifungal activity	
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien	26.996	0.71	Diketone derivate	Antioxidant and antifungal activity	
n-Hexadecanoic acid	28.293	1.44	Fatty acids	Antibacterial and nematicidal activity	
Eicosane	28.857	0.26	Alkane organic compound	Antifungal activity	
n-Heptylamine, N-acetyl-1-cyano-	29.032	0.07	Alkylamine	Antifungal activity	

13-Docosenamide, (Z)-	29.438	1.01	Fatty acids	Antimicrobial activity	
9-Octadecenamide, (Z)-	30.583	0.91	Fatty acids	Antibacterial and antifungal activity	
2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	32.422	12.57	Peptide	Antifungal activity	
Octadecanoic acid	35.663	0.32	Fatty acids	Antimicrobial activity	
Cyclo(prolyltyrosyl)	43.419	2.08	Amino acids	Antibacterial activity	
Brevianamide F	49.126	0.12	Peptide compound	Antifungal activity	

TMS derivatives showed the highest area percentage of 0.89 and retention time of 32.681, followed by Elaidic acid-TMS shows 0.84 area percentage and 32.803 retention time. The lowest area percentage of 0.21 is 5-Hydroxy-2-(hydroxymethyl) pyridine, 2TMS, and 18.927 retention time. The secondary metabolites are listed in Table. 2, and the corresponding gas chromatogram is shown in Fig. 3.

Discussion

Rice is an important cereal food crop around the world. It has been devastated by various kinds of pests and diseases worldwide. In the present scenario, sheath rot (*Sarocladium oryzae*) is one of the emerging threats to crop cultivation. Previously, fungicides were usually used to control plant diseases. Nowadays, in the biological era, especially with host-associated endophytes, which significantly improve plant health and help for sustainable cultivation of crops. Seeds are the selective vehicle for many biocontrol agents (26). The seed can profit from the seed-associated microorganism and favor the seed for preservation and germination. Though the endophytic microbes are sustained in the internal environment for the specific niche, they build the dominant community during maturation from seed to seedling (26).

Eighteen culturable endophytes from four traditional endophytes viz., Sivan Samba and Thuya Malli from Erode district, Kotchi Samba, and Arupathankurai were collected from Kanyakumari district, were selected for isolation, and their crucial role in improving plant growth promoting activities (24). They use endophytes to protect economically important plants from harmful microbes and consistent performance with symbionts (27).

The bacterial community, especially *Bacillus sp* is the dominant genera in endophytes and rhizosphere (28). the endophyte *B. licheniformis* RE-18 shows a maximum of 76.47% inhibition activity against *S. oryzae. Bacillus sp* has a crucial role in antibiosis activity to reduce the plant's pathogen activity of *R. solani, M. grease, Pythium myriotylum, Gauemannomyces graminis,* and *Helerobasidium annosum* in rice (13, 29) and *Fusarium moniliformae* in maize (27). Similar reports *Bacillus sp* and *Pseudomonas* show high in inhibition activities against *Sarocladium oryzae. Bacillus subtilis, P. fluorescens* and *T. viride* all demonstrated inhibitory effects on *S. oryzae* that ranged from 10.90 to 82.18 percent and 76.25% inhibition, *T. harzianum* was shown to be the most detrimental to *S. oryzae* growth (30-32).

The endophytic bacteria must be distinguished based on different biochemical and plant growth-promoting activities *viz*., glucose utilization test, urease test, citrate utilization test, etc (33, 34). Similarly, the isolate RE-18 shows positive results. Urease hydrolyses the C-N bonds in the linear amide. When urea is hydrolyzed, these endophytic bacteria produce urease enzymes to convert urea into NH_3 and carbon dioxide. The medium becomes alkaline due to the creation of NH_3, and the color shift of phenol red from light orange indicates the pH change. The NH_3 form of conversion is the uptake from plants (18). The protease enzymes help break down

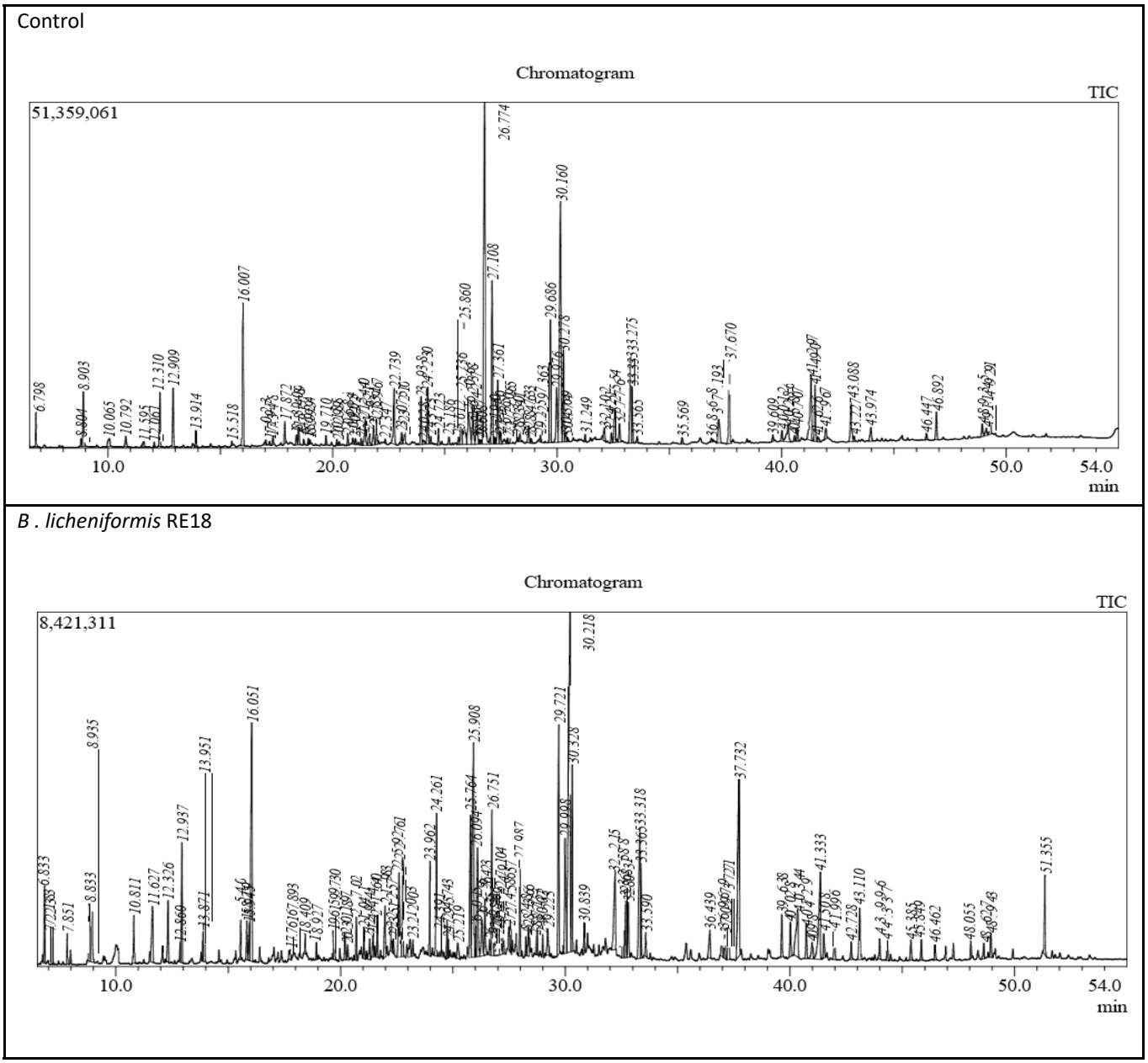


Fig. 3. GC-MS chromatogram of *B. licheniformis* RE18.

proteins into smaller peptides, subsequently converted to amino acids. Protease enzymes are a class of industrial hydrolytic enzymes used in biotechnological research, medicine, leather, textile, and agri-food industries because of their simple and inexpensive synthesis (35, 36).

In addition, endophytes potentially increase growth -promoting activity in different crops like tea (33) and rice (13). Biostimulant activity, such as IAA, NH_3 utilization activity, and siderophore production, can improve nutrient uptake and enhance colonization in plant tissue (22, 31, 33, 37). Indole acetic acid is a natural compound synthesized by many bacteria that helps in apical shoots and root growth. Indole-3-acetic acid produced by effective endophytes significantly increases secondary root in the roll towel method, in this mediated pathway also helps to sustain the plant in abiotic conditions. Ammonia utilization helps increase the availability of the uptake form of nitrogen. In the soil colloids, nutrients of the positively charged ions adhere to negatively charged particles and make them unavailable to plants (31, 38). The iron-chelating bacteria help to secrete low-molecular weight ferric iron compounds and make them available in limiting conditions. The siderophores play a dual role in the biocontrol mechanism and plant growth-promoting activities. *Pseudomonas aeruginosa* produces siderophore as a biocontrol against chili fruit rot and root rot. *Bacillus sp* potentially reduces the *Rhizoctonia bataticola, Macrophomina phaseolina* and *Fusarium udam* in soybean crop, *Pseudomonas sp* against sheath rot (30, 39). Like the *B. licheniformis*, the RE-18 isolate shows strong siderophore activity against the sheath rot to compete with the iron compounds in the surrounding area. These plant growth hormones are thought to have deleterious effects against plant pathogenic microflora (40).

Recent studies reported that the secondary metabolite of *Bacillus sp*. has high antimicrobial activity against plant pathogens *viz., R. solani* (13), *Fusarium graminearum* (41), *Fusarium solani* (42, 43) and *Botrytis cinerea* (44). These secondary metabolic compounds are highly toxic, leading to the lysis of the fungal cell wall. The secondary metabolites of *B.*

licheniformis were analyzed using gas chromatography-mass spectrometry, and the identified bioactive compounds include fatty acid, organic compound, benzoic acid, carboxylic acid, phenolic compound, cyclohexane, ketone, alkene, long chain fatty acids and koljic acid.

Among these, several compounds exhibit significant secondary metabolic activity, including 9,12-Octadecadienoic acid (Z, Z)-, TMS derivatives, Elaidic acid-TMS, Bis(2-ethylhexyl) phthalate, Caproic acid-TMS, Diethyl Phthalate, Caproic acid-TMS, Ricinoleic acid, 2TMS derivative, Mandelic acid-2TMS, Tetrapentacontane, 1,2-Propanediol, 3,3-di-1H-indol-3-yl-, 4TMS, 4-Hydroxyphenylacetic acid-2TMS, 2,5-Piperazinedione, 3-methyl-6-(1-methyl pro, Dotriacontane, 7,9-Di-tert-butyl-1-oxaspiro(4,5)Deca-6,9-dien, n-Pentadecanol, 5-Hydroxy-2-(hydroxymethyl) pyridine-2TMS. This compound shows high antifungal and antimicrobial activities against pathogenic fungi. Additionally, Bis(2-ethylhexyl) phthalate has been reported to possess antimicrobial, antifungal, and larvicidal activities (45). The seed core endophyte has multiple antibiosis, plant growth promoting activities, and nutrient solubilization activities to help consistently improve the wealth of farming societies.

Conclusion

In summary, exploring rice endophytes, particularly *B. licheniformis* RE-18, highlights their significant role in combating sheath rot caused by *Sarocladium oryzae*. This research demonstrates that these endophytes exhibit strong antifungal activity and enhance plant growth through various mechanisms, including nutrient uptake, production of growth-promoting hormones like IAA , and secretion of siderophores. The diverse biochemical activities of these microorganisms underscore their potential as sustainable biocontrol agents in rice cultivation. As the agricultural landscape shifts towards more eco-friendly practices, harnessing the capabilities of endophytic bacteria may provide an effective strategy for managing plant diseases and promoting healthy crop growth. Further studies are needed to optimize their application and fully understand their interactions within the plant microbiome.

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Authors' Contributions

KS carried out the isolation, characterization and metabolic profiling studies and drafted the manuscript. KK involved in designing the study, methodology and editing. IN participated in the sequence alignment. MP carried out the gas chromatography-mass spectrometry studies. TK performed the statistical analysis. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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