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RESEARCH ARTICLE

Isolation, identification, characterization and *in vitro* assay of saline tolerant endophytes against groundnut root rot caused by *Rhizoctonia bataticola* (Taub.) Butler

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Abstract

Groundnut, known as *Arachis hypogaea* L., is India's significant oil seed crop. Dry root rot, caused by *Rhizoctonia bataticola*, poses a substantial challenge to cultivating groundnuts. During the roving survey, 60.50% dry root rot disease incidence was recorded in Namakkal district, Tamil Nadu. This study aims to acquire salt-tolerant endophytic bacteria residing in groundnuts with significant antagonistic activity against *R. bataticola*. A total of 27 bacterial strains were isolated from groundnuts. Among these strains, RMV 3 and RMV 2 are the most effective isolates, exhibiting 60.1% and 50% inhibition zones, respectively. The effective isolates were characterized through morphological, biochemical and phytostimulation activities and 16S rDNA sequencing. Among the isolates, RMV 3 and RMV 2 showed positive results for siderophore, indole acetic acid (IAA) and cellulase test. The strain RMV 3 was identified as *Bacillus subtilis* through 16S rDNA sequencing. GC-MS analysis identified twenty bioactive compounds produced by *B. subtilis* RMV 3, such as pyrrolo [12-a] pyrazine-14-dione hexahydro-3 (2-methylpropyl) and hexadecanoic acid methyl ester. The crude metabolite assay demonstrated a 96.6% inhibition of *R. bataticola* by RMV 3. This study demonstrated that *Bacillus subtilis* RMV 3, which exhibits a robust antagonistic effect on *R. bataticola*, can potentially be an effective biocontrol agent for groundnut dry root rot.

Keywords

groundnut; dry root rot; *Rhizoctonia bataticola*; endophytic bacteria; *Bacillus subtilis;* biological control

Introduction

Groundnut (*Arachis hypogaea* L.) is a crucial oilseed crop, extensively cultivated for its high oil and protein content. However, its productivity is threatened by diseases such as dry root rot caused by *Rhizoctonia bataticola*. *Rhizoctonia bataticola* (Taub.) Butler, a fungal pathogen, poses a significant threat to groundnut crops, especially in semi-arid regions, as it is responsible for dry root rot and yield loss. This fungus can affect groundnut plants through seeds and soil (1). Affected seedlings display bark shredding in the collar area, accompanied by tiny dark sclerotia within the shredded bark and root tissue. It can survive in soil even at temperatures as low as -18°C (2). Peanut crops are also vulnerable to salinity, significantly impacting their productivity (3). Among the prominent states in India where groundnut

is cultivated, the presence of soil salinity affects approximately 2.0 million hectares of land in saline and coastal regions, consequently impacting groundnut productivity (4).

Soil salinity limits plant growth and reduces yield (5). It is estimated that around 50% of arable land will be affected by salinity stress by 2050 (6). Soil salinity causes ion toxicity due to Na+ replacing K+ in biochemical reactions. K^+ is essential for enzyme function and high levels of tRNA binding are needed during protein synthesis. Na^+ and Cl^- cause protein conformational changes (7). In salt-sensitive crops, the negative impacts of salinity include abnormal seed germination, decreased crop vigour, and impaired vegetative and generative growth (8). Although breeding crop varieties that are tolerant to salt is possible, such plants' development is limited so far. Bacterial endophytes enhance plant stress tolerance by reducing ethylene production, a plant hormone triggered by biotic and abiotic stress factors, through the enzyme ACC deaminase, resulting in lower ethylene levels within the host plant(9). It was demonstrated that inoculation of *Rhizobium pusense* S6R2 and *Pantoea dispersa* YBB19B alleviated salt stress (10).

The objectives of the present study aimed to isolate and characterize salt-tolerant bacterial endophytes from groundnut plants and their effect on groundnut root rot caused by *R. bataticola* and evaluate the effective isolates for phytostimulation activities.

Materials and Methods

Survey and sample collection

A roving survey was conducted on dry root rot incidence with GPS tag in major groundnut-growing areas of Tamil Nadu viz., Tiruchirappalli, Perambalur, Cuddalore, Salem and Namakkal in various groundnut cultivars, namely TMV 2, TMV 7, TMVGn 13, VRI 2, K 6, CO 2, TMV 10, CO 6. The degree of dry root rot occurrence was evaluated using the percent disease incidence method (Wheeler, 1969).

Isolation of Pathogen

The pathogen was isolated from the diseased plants of groundnuts, showing dry root rot symptoms. Diseased plant portions were cut into 15-5mm pieces with a sterilized scalpel. Plant bits were subjected to surface sterilization with 0.1 percent mercuric chloride solution for about 1 minute, and pieces were washed twice in sterile distilled water to remove the residue of mercuric chloride and dried on sterile tissue paper. After drying, the samples were plated on PDA media amended with suitable antibiotics and for 3 days at incubated 28°C. A single hyphal tip of the pathogen was transferred to PDA culture slants to obtain the pure culture of *Rhizoctonia bataticola* (11).

Pathogenicity test

Pathogen isolates were cultured and multiplied in a sand maize medium. A mixture of sand and maize seeds was sterilized in an autoclave in a ratio of 19:1. The medium was inoculated with mycelial discs of *Rhizoctonia bataticola* and allowed to grow for 15 days. The resulting inoculum was used for subsequent experiments. A potting mixture was prepared using a 1:1:1 ratio of clay loam soil, sand and farmyard manure. Each *Rhizoctonia bataticola* isolate was incorporated into sterilized soil in earthen pots (12). Groundnut seeds were surface sterilized and sown in pots. The experiment had three replications using the CO 6 groundnut cultivar in a glasshouse. Root rot incidence was assessed at different time points.

Molecular Characterization of *Rhizoctonia bataticola*

Fungal genomic DNA was extracted by utilizing the CTAB method. For the amplification of DNA fragments, universal fungal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used (13). A thermocycler was used to conduct PCR with the cycling conditions; initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minutes and extension at 72°C for 60 seconds, with a final extension at 72°C for 5 minutes. The PCR products were analyzed on a 1% agarose gel in gel electrophoresis and visualized using a UV gel documentation system. The amplified DNA fragments of *Rhizoctonia bataticola* underwent Sanger sequencing. Nucleotide BLAST analysis was conducted with the consensus sequence, and the organism's identity was confirmed by examining the BLAST output. Phylogenetic trees were constructed using MEGA XI software.

Isolation of Bacterial Endophytes

To isolate endophytic bacterial strains, the roots stems, and leaves of healthy groundnut plants were collected from salinity-affected fields in groundnut growing regions of Tamil Nadu, specifically Tiruchirappalli, Cuddalore and Mayiladuthurai. The fresh samples were thoroughly washed under running tap water and surface sterilized using 1% sodium hypochlorite for 2 minutes, followed by 70% ethanol for 2 minutes and rinsed three times with sterile distilled water (14). The surface-sterilized samples were blot-dried with sterilized filter paper, and they were ground aseptically in a sterile pestle and motor using Phosphate Saline Buffer (PBS) (15).

Characterization of Endophytes for salinity tolerance

To assess the salt tolerance of endophytic bacterial isolates, 20 µl aliquots of a 24 h old test culture were inoculated into LB broth with sequential NaCl concentrations of 0%, 5%, 10% and 15%. The cultures were incubated under growth conditions and after 24-48 hours, their growth was measured by absorbance at 600 nm using a spectrophotometer (16).

Morphological and biochemical characterization

The bacterial endophytes were morphologically characterized, displaying various colony shapes, colours, margins, and textures. Gram staining was utilized to categorize effective strains as either gram-positive or gram -negative. The identification of antagonistic strains to the species level was based on biochemical tests such as amylase, catalase, indole tryptone, citrate utilization, methyl red, VP, urease and H2S production tests (17).

Molecular Characterization of bacterial endophytes

The endophytic bacterial isolates were identified through sequencing of the 16S rRNA gene using a primer 27-F (AGAGTTTGATCCTGGCTCAG);1492(GGTTACCTTGTTACGACTT) (18). The PCR was carried out under the following cycling conditions; initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1 minute, with a final extension at 72°C for 5minutes. The PCR products were sequenced and analyzed using the Basic Local Alignment Search Tool for 16S ribosomal RNA sequences stored in the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (19). The Phylogenetic relationships of the bacterial isolates with other bacteria exhibiting sequence similarity were analyzed using MEGA 11 software.

In vitro screening of bacterial isolates

Bacterial endophytes isolated from groundnuts underwent *in vitro* screening against the dry root rot pathogen *R. bataticola*. During the dual culture assay, a pathogen disc 8 mm in diameter from an active culture was positioned 1 cm away from the periphery of the Petri plate. In contrast, antagonistic bacteria from a 48-hour-old culture were streaked at a 1cm distance from the edge of the periphery on PDA media. In the case of antagonistic fungi, both the pathogen and the antagonistic organism were placed in opposite directions at the corner of the plate, 1 cm away from the edge. The plates were then incubated at 28±2°C for five days, with three replicates. Control plates containing only the pathogen *Rhizoctonia bataticola* were also set up separately. The inhibitory effect of the endophytes was assessed by observing the inhibition of fungal growth compared to the control plate (20). The Percent Inhibition (PI) relative to the control was calculated using the formula provided by Vincent (1925).

$$Percent\ Inhibition\ over\ control(I) = \frac{Rc - Rt}{Rc} \times 100$$

Where I = percent inhibition in growth of test pathogen

Rc = Radial growth of the *R. bataticola* in control

Rt = Radial growth of the *R. bataticola* in treatment with biocontrol agents

Assay on Plant growth-promoting activities:

Siderophore production

Endophytic bacterial isolates were tested for siderophore production using Chrome azurol S (CAS) agar medium. Calcium sulfate (60.5 mg) was dissolved in 50 mL of deionized water and the resulting solution was added to 10 mL of Fe (III) solution. This mixture was then gradually combined with 72.9 mg of Hexadecyltrimethylammonium Bromide (HDTMA) dissolved in 40 mL of water while stirring. The resulting dark-blue solution was autoclaved, cooled to 50-60°C and mixed with 15 g/L agar in 900 mL of sterile water. Five days after incubation at room temperature (28 ± 2°C), an orange halo appeared around the colony, indicating siderophore production (21).

IAA production

To quantify the IAA produced by promising endophytic bacteria in each isolate, a colourimetric assay was conducted using the Salkowski reagent (22). All the isolates were newly cultured in LB broth with 0.1% L-tryptophan and incubated at 28°C for four days. After the incubation period, the culture broth was centrifuged and equal volumes of the isolated supernatant and Salkowski reagent (0.5 M FeCl3 + 35% HClO4 solution) were mixed and incubated in the dark at room temperature. After 30 minutes, the reagent mixed culture supernatant changed to pink and the absorbance was measured at 530nm (23).

Cellulase production

CMC agar plates were soaked in iodine solution for five minutes and left to stand at room temperature. Several colonies displayed clear zones on the agar plates. CMC agar media containing 0.05% K2HPO4, 0.188% CMC sodium salt, 0.025% MgSO4, 0.02% Congo red, 1.5% agar, and 0.2% gelatin. The plates were then flooded with iodine and incubated at 28°C for 48 hours (24). After incubation, the CMC plates were stained with Congo red dye and examined for clear zones around the colonies.

Gas Chromatography-Mass Spectrometry Analysis

The bacterial isolates were cultured in Nutrient Broth (NB) and incubated at 28 ± 2°C. After 72 hours, the samples were centrifuged, and the supernatant was extracted with an equal volume of ethyl acetate (1:1 ratio v/v). The solvent fraction was dried using a vacuum evaporator, and the final product was dissolved in 1 µl methanol and filtered through a 0.2 µm bacteriological membrane filter. The purified crude methanolic extract was then analyzed. The secondary metabolites of *B. subtilis* RMV 3 were identified using a GC-MS Agilent (GC 8890/MS5977C/ Autosampler7693A) QIMA (Quality Inspection India Private Limited). The analysis was performed: the capillary column (DB- 5ms column length of 30 m/ 0.25 mm internal dia/0.25-micron film thickness) was used. The GC program started at 50 °C for 1 min and then 10 °C/min to 300 °C for 1 min. The identity of the secondary metabolites was determined using the National Institute of Standards and Technology NIST 20 database. The compounds present in the crude sample were identified by comparing the spectrum obtained through GC/MS (25).

Effect of secondary metabolites on the growth of *Rhizoctonia bataticola.*

The antimicrobial activity of endophytic bacterial isolates was assessed with the agar well-diffusion method modified by (26). Twenty millilitres of PDA medium were added to sterilized Petri plates and allowed to solidify. Then, a mycelial disc of the test pathogen was placed in the centre. A 7mm well was created in four corners of the agar plate at equal distances using a sterile cork borer. Metabolites were separately poured into wells at different concentrations (10 ul, 25 ul, 50 ul, 100 ul) per well and incubated for 72 h at 28±2°C (27). Inhibitory activity was measured by comparing the radial growth of *Rhizoctonia bataticola* in treated plates to control plates in which sterile water was poured into the wells after incubation.

Statistical analysis.

Data from the completely randomized design experiment on *in vitro* biocontrol activity were arc sine transformed before statistical analysis. The results were analyzed using one-way ANOVA with the AGRES software with a significance level set at 5% ($p \leq 0.05$).

Results

Survey and isolation of *Rhizoctonia bataticola*

A survey was conducted to assess the incidence of dry root rot (*Rhizoctonia bataticola*) in groundnuts in major groundnut-growing regions of Tamil Nadu. The roving survey was undertaken in Tiruchirapalli, Perambalur, Cuddalore, Namakkal and Salem (Fig. 1). The dry root rot disease incidence ranges from 7.78% to 60.50%. The highest incidence was found in Koothampoondi (60.50%), followed by Paganur (55.82%) in TMV 2 cultivars, and the lowest incidence was found in Anukur (7.78%) followed by Maniyanur (15%) in TMVGn 13 and CO 2 cultivars respectively (Table 1).

Groundnut crops grown under irrigated conditions exhibited lower incidences of dry root rot than those grown under rainfed conditions. The prevalence of dry conditions in rainfed areas likely favours the multiplication of pathogens, leading to higher root rot disease incidence. Five isolates of *R. bataticola* were obtained from the infected plant samples collected from different regions of Tiruchirapalli, Perambalur, Cuddalore, Namakkal and Salem. (Fig. 2).

Pathogenicity test

Pathogenicity tests were carried out on all the isolates of *Rhizoctonia bataticola.* The disease incidence with high virulence capacity was analyzed. The PDI varies from 30.05% to 55.75% for *Rhizoctonia bataticola* (Fig. 3). The most virulent isolate, MP 1, was used to reisolate the pathogens and their morphological characteristics were observed.

Table 1. Incidence of dry root rot in various groundnut cultivars

District	Location	Variety / Hybrid	PDI (%)
Tiruchirappalli	Paganur	TMV 2	55.82
	Mathur	TMV 7	30.50
Perambalur	Anukur	TMVGn 13	7.78
Cuddalore	Virudhachalam	VRI 2	30.00
	Kurinchipadi	K 6	38.25
Salem	Maniyanur	C0 2	15.00
Namakkal	Koothampoondi	TMV 2	60.50
	Pillanallur	CO 6	10.00

PDI- Percent Disease Incidence

Molecular characterization of *Rhizoctonia bataticola*

Molecular characterization of virulent isolates was performed by extracting DNA using the CTAB method. The genomic DNA of strains MP 1 and MP 4 were used to amplify a fragment coding for the ITS rDNA region, and the isolates were amplified at 560bp (Fig. 4). The accession numbers PP327216 and PP463546 was assigned to *R. bataticola* MP 1 and *R. bataticola* MP 4 upon submission to GenBank. A phylogenetic tree was constructed using the MEGA 11 software (Fig. 5) for ITS rDNA gene sequences for MP 1, grouping the related sequences into a singular cluster.

Fig. 1. Dry root rot affected fields (a). Cuddalore (b). Tiruchirappalli (c). Perambalur (d) Namakkal (e)

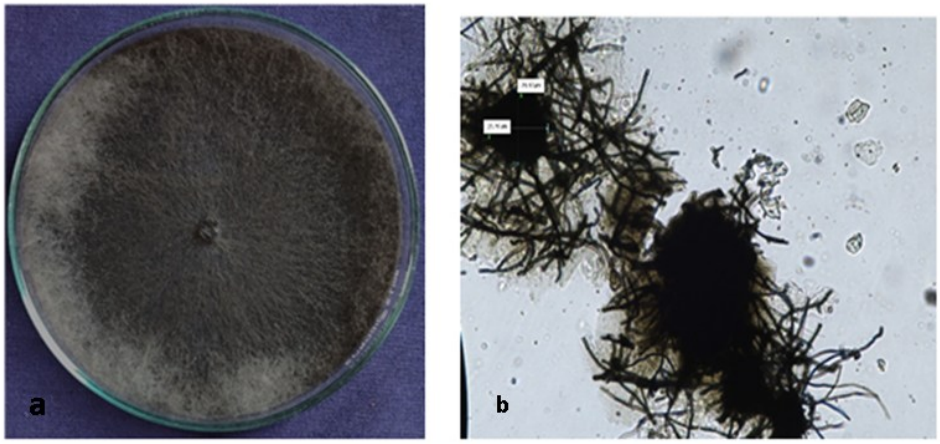

Fig. 2. (a) Cultural morphology of *R. bataticola* in PDA medium (b) Sclerotia of *R. bataticola* under phase contrast microscope at 40x magnification

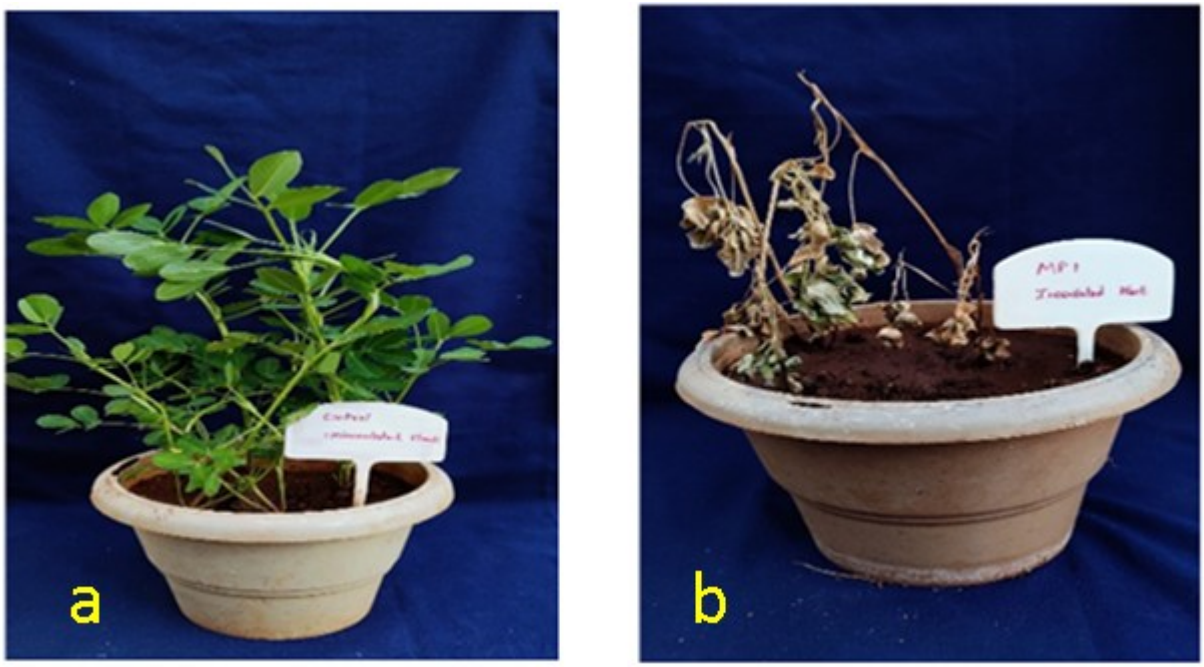

Fig. 3. Pathogenicity test **(a)** control **(b)** pathogen-infected plant

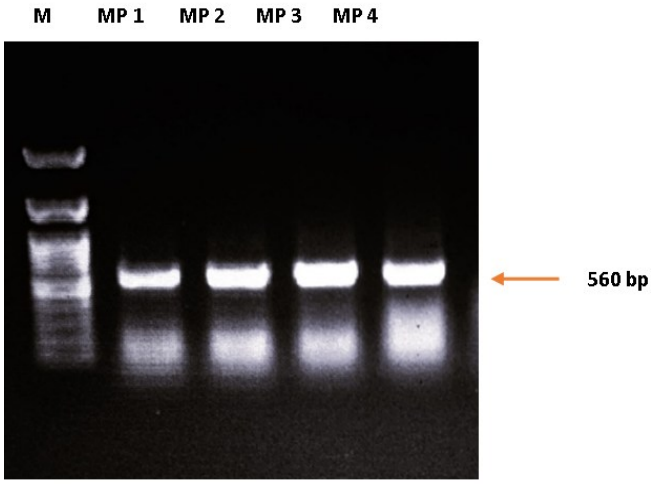

Fig. 4. PCR amplicons of *Rhizoctonia bataticola*

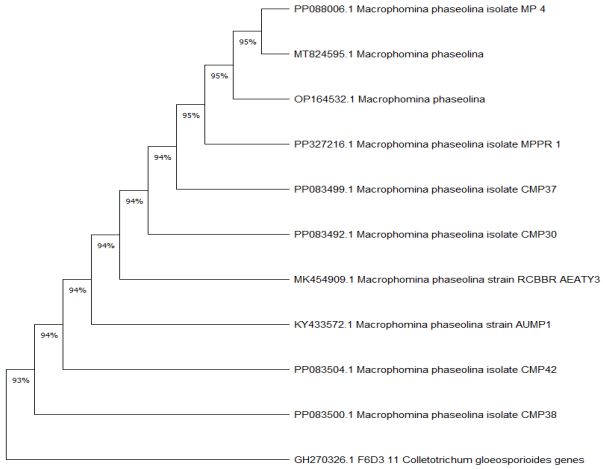

Fig. 5. Phylogenetic analysis of *Rhizoctonia bataticola*

Isolation, Morphological and Biochemical characterization of Bacterial Endophytes

Endophytic bacterial isolates were isolated from the salinity -affected fields in groundnut-growing regions of Tamil Nadu (Table 2). Isolates are obtained from healthy groundnut plants' sterilized roots, stems and leaves. A total of 27 organisms were isolated and considered as potential eco-friendly management for dry root rot disease in groundnut. These isolates were distinct in colony morphology with raised, flat, convex margins and pigmentation was obtained. Most of the colonies were observed to be whitish to dull yellow. Five bacterial endophytes are characterized biochemically, among them, the effective endophytes RMV 2 and RMV 3 showed positive results for gram staining, citrate utilization test, indole tryptone test, urease test and gelatin liquefaction (Table 3).

Characterization of Endophytes for salinity tolerance

Two effective bacterial endophytes, RMV 2 and RMV 3, were inoculated in LB broth with sequential NaCl concentrations and their growth was determined in a spectrophotometer at 600nm. The isolates could tolerate 5% salt stress, above which the growth of the bacteria started to decline. Among the two effective bacterial endophytes, maximum growth (OD600nm = 0.489) was observed in RMV 3 isolate, whereas in RMV 2 (OD600nm = 0.245). Under control conditions, RMV 2 and RMV 3 showed significant growth such as (OD_{600nm} = 0.789), (OD_{600nm} = 1.352), respectively.

Molecular Characterization of Bacterial Endophytes

Effective isolates of endophytic bacteria were molecularly characterized using the CTAB method and DNA was extracted. The genomic DNA of strains RMV 2 and RMV 3 was molecularly characterized using the bacterial universal primers 27F and 1492R. The PCR products were amplified at 1200 bp amplicon size. After amplification, the best isolate RMV 2 and RMV 3 was sequenced, and a phylogenetic tree was generated using MEGA 11 software. The results revealed that *Bacillus subtilis* RMV 2 isolate (PP835389) showed 95.23% similarity to *Bacillus subtilis* strain NBRIYE1.3 (MK168629) (Fig. 6) and the sequence results of *Bacillus subtilis* RMV 3 isolate (PP833618) showed 99.47 % similarity to *Bacillus subtilis* strain DS51(OP572156) (Fig. 7).

Table 2. Isolates of endophytic bacteria obtained from salinity-affected regions in Tamil Nadu

S. No	Isolates	Place	District	Soil PH	Geo Coordinates	
					Latitude	Longitude
1	RMV 1	Vettangudy	Mayiladuthurai	7.20	11.269684°	79.801326
2	RMV 2			7.20	11.269684°	79.801326
3	RMV 3			7.20	11.269684°	79.801326
4	RMV 4			7.20	11.269684°	79.801326
5	RMV 5			7.20	11.269684°	79.801326
6	LMV 6			7.20	11.269684°	79.801326
7	LMV 7			7.20	11.269684°	79.801326
8	LMV 8			7.20	11.269684°	79.801326
9	LMV 9			7.20	11.269684°	79.801326
10	SMV 10			7.20	11.269684°	79.801326
11	SMV 11			7.20	11.269684°	79.801326
12	SMV 12			7.20	11.269684°	79.801326
13	LMS 13	Sirkazhi		7.90	11.239088°	79.736122
14	LMS 14			7.90	11.239088°	79.736122
15	SMS 15			7.90	11.239088°	79.736122
16	STM 16	Mathur	Tiruchirappalli	8.30	10.724744°	78.594478
17	STM 17			8.30	10.724744°	78.594478
18	STM 18			8.30	10.724744°	78.594478
19	LTM 19			8.30	10.724744°	78.594478
20	RTM 20			8.30	10.724744°	78.594478
21	LTP 21	Paganur		8.11	10.725265°	78.581268
22	STP 22			8.11	10.725265°	78.581268
23	STP 23			8.11	10.725265°	78.581268
24	RCK 24	Kurinchipadi	Cuddalore	7.40	11.588293°	79.601953
25	SCK 25			7.40	11.588293°	79.601953
26	LCP 26	Periyabattu		7.90	11.589489°	79.685845
27	SCV 27	Vadalur		7.89	11.569538°	79.568658

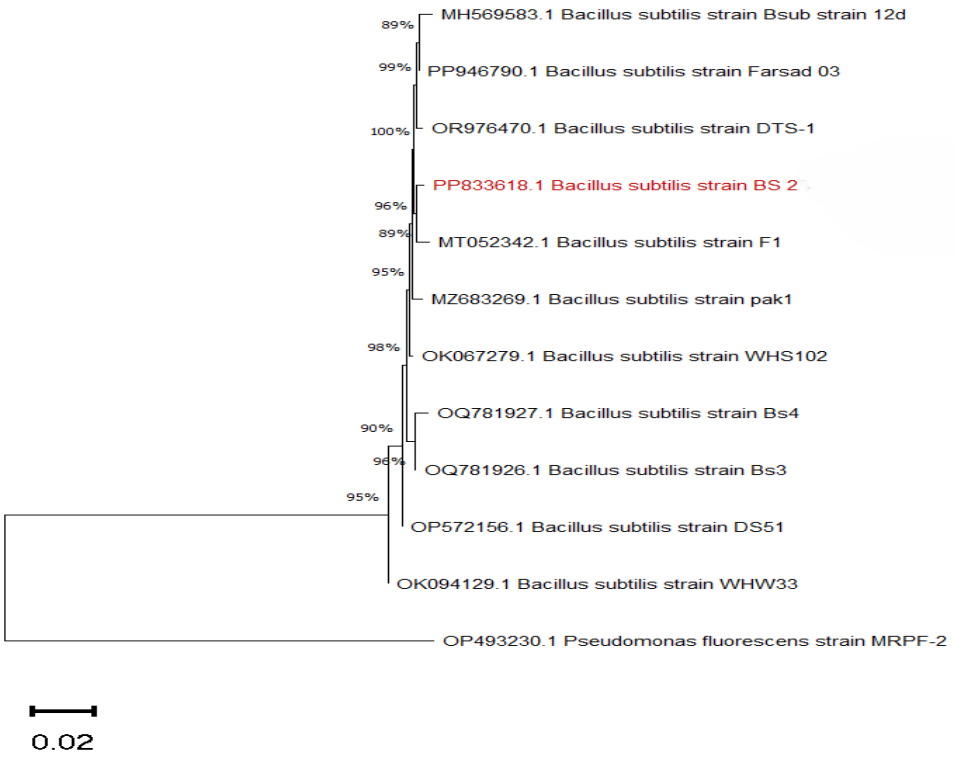

Fig. 6 Phylogenetic tree generated from 16S rRNA sequence analysis of *Bacillus subtilis* RMV 2 using MEGA 11 (version 11.0.13)

Table 3. Biochemical characterization of endophytes

S. No	Isolates	1	2	3	4	5	6	7	8
1	RMV 1	+	-	+	+	+	+	+	+
2	RMV 2	+	-	+	+	+	+	+	+
3	RMV 3	+	-	+	+	+	+	+	+
4	LTP 21	+	-	+	+	-	-	+	+
5	LMV 7	+	-	+	+	-	-	+	-

1.Gram staining, 2. KOH test, 3. indole tryptone test,4. catalase test, 5. gelatin liquefication test, 6. citrate utilization test, 8 urease test

\+ Positive reaction; - Negative reaction

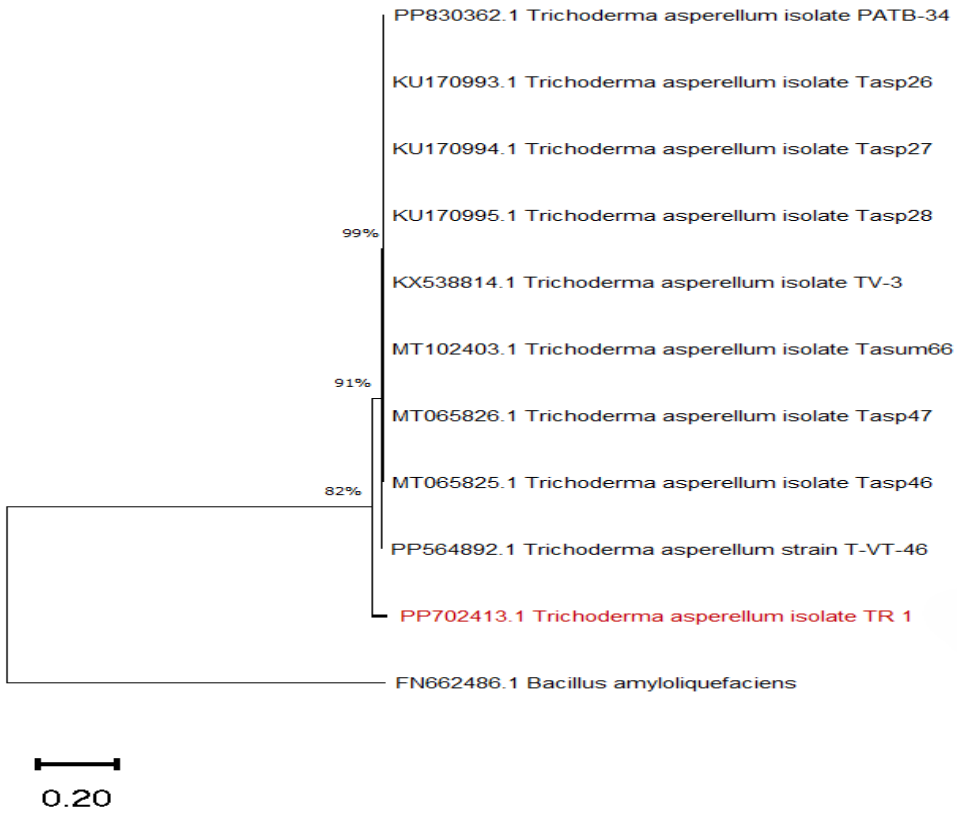

Fig. 7. Phylogenetic tree generated from 16S rRNA sequence analysis of *Bacillus subtilis* RMV 3 using MEGA 11 (version 11.0.13)

In vitro antagonistic activity of endophytic bacteria against *Rhizoctonia bataticola*

In total, 27 bacterial endophyte strains were isolated from groundnut plants, these bacterial endophytes' antagonistic effect against *R. bataticola* was assessed on dual culture technique. The results showed that the bacterial strains showed vigorous antifungal activity against *R. bataticola* with an inhibition zone of 14.81 - 61.1%. Among 27 isolates RMV 3 strain shows the highest inhibition with 61.1%, followed by RMV 2 with 50% inhibition (Table 4; Fig. 8). The triangle method of streaking was also done and the results revealed that RMV 1, RMV 2 and RMV 3 showed maximum inhibition of 100% against *R. bataticola* (Fig. 9). Although significant antagonistic activity was observed, additional studies under varied conditions and comparative analysis with established biocontrol agents are required to substantiate these findings.

Plant growth-promoting activities

The IAA and siderophore production by endophytic bacteria were estimated. The isolates RMV 1, RMV 2, RMV 3 and LTP 21 showed positive results for siderophore production. *Bacillus subtilis* RMV 3 produces a clear zone in CMC agar media, indicating cellulase activity. The production of IAA by endophytic isolate *B. subtilis* RMV 3 was confirmed by the broth's reddish-pink colour after adding the Salkowski reagent (Fig. 10).

GC-MS analysis

The GC-MS system detected twenty secondary metabolites produced by B. subtilis RMV 3. The results showed the presence of Diethyl Phthalat, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (2-methylpropyl)-, 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (phenylmethyl)-, Benzoic acid, 4-ethoxy-, ethyl ester (Table 5; Fig. 11).

Effect of secondary metabolites against *Rhizoctonia bataticola*

Radial growth of *R. bataticola* was reduced by the secondary metabolites produced by the effective isolate RMV 3, which was assessed through the agar well diffusion technique. The isolate RMV 3 showed 96.6% inhibition against *R. bataticola* at 100 µl.

Table 4. *In vitro* antagonistic activity of endophytic bacteria against *Rhizoctonia bataticola* in dual culture assay

S. No	Isolate	Mycelial growth (mm)	Inhibition (%)
1	RMV 1	44.66^{b}	50.37^{l}
2	RMV2	45^{b}	50^{l}
3	RMV 3	35^{a}	61.11^{m}
4	RMV 4	61.33^{fgh}	31.85^{fgh}
5	RMV 5	56^{def}	37.77^{hij}
6	LMV 6	54.66^{cde}	39.25^{ijk}
7	LMV 7	54.66^{cde}	39.25^{ijk}
8	LMV 8	58.66^{defg}	34.81^{ghij}
9	LMV 9	70^{ijk}	22.22^{cde}
10	SMV 10	60^{defgh}	33.33^{fghij}
11	SMV 11	60.66^{efgh}	32.59^{fghi}
12	SMV 12	61^{fgh}	32.22^{fgh}
13	LMS 13	70^{ijk}	22.22^{cde}
14	LMS 14	60^{defgh}	33.33^{fghij}
15	SMS 15	63.33^{gh}	29.62^{fg}
16	STM 16	54.33^{cd}	39.62^{jk}
17	STM 17	59^{defg}	34.44^{ghij}
18	STM 18	65.66^{hij}	27.03^{def}
19	LTM 19	61.66^{fgh}	31.48^{fgh}
20	RTM 20	73.33^{kl}	18.51^{bc}
21	LTP 21	49.33^{bc}	45.18^{kl}
22	STP 22	64^{ghi}	28.88^{efg}
23	STP 23	61.66^{fgh}	31.48^{fgh}
24	RCK 24	63^{gh}	30^{fg}
25	SCK 25	73.33^{kl}	18.51^{bc}
26	LCP 26	71.33^{jkl}	20.74^{bcd}
27	SCV 27	76.66^{l}	14.81^{b}
28	CONTROL	90^{m}	0^{a}

*Mean of three replications*Values in the parentheses are arcsine transformed values

Means in a column followed by the same superscript letters are not significantly different according to DMRT at P ≤ 0.05

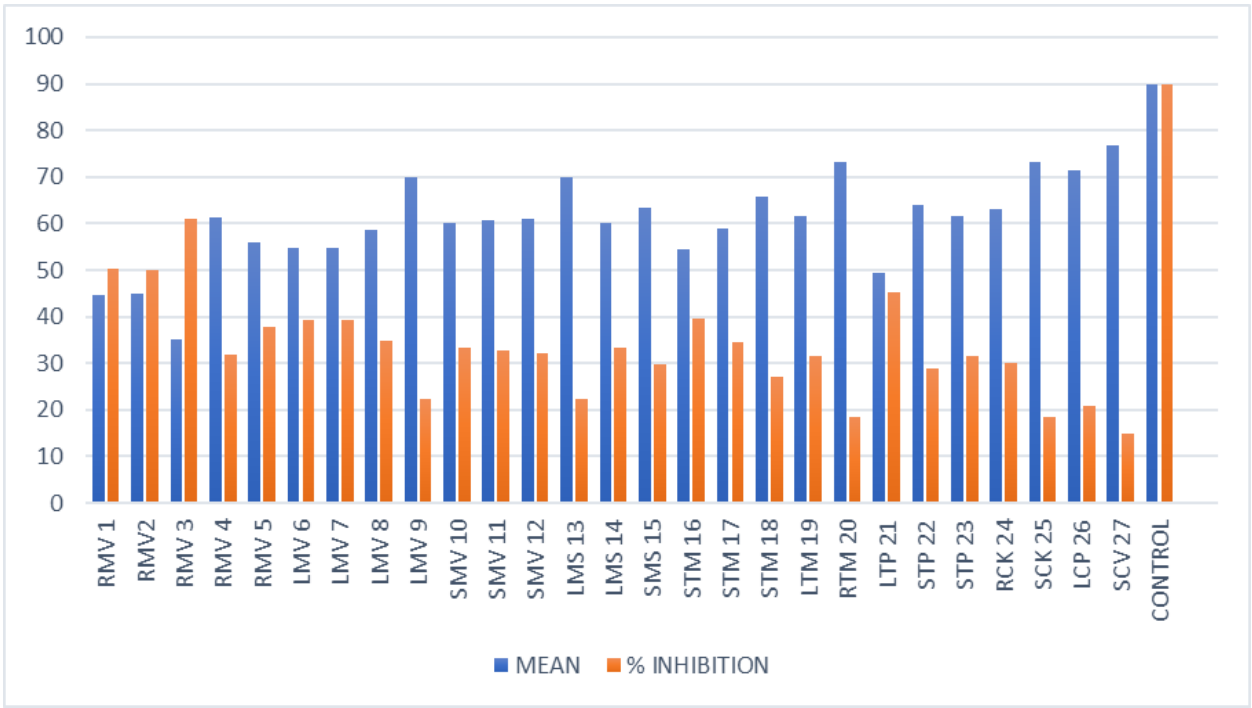

Fig. 8. Antagonistic activity of bacterial endophyte isolates against *R. bataticola in vitro* dual culture assay

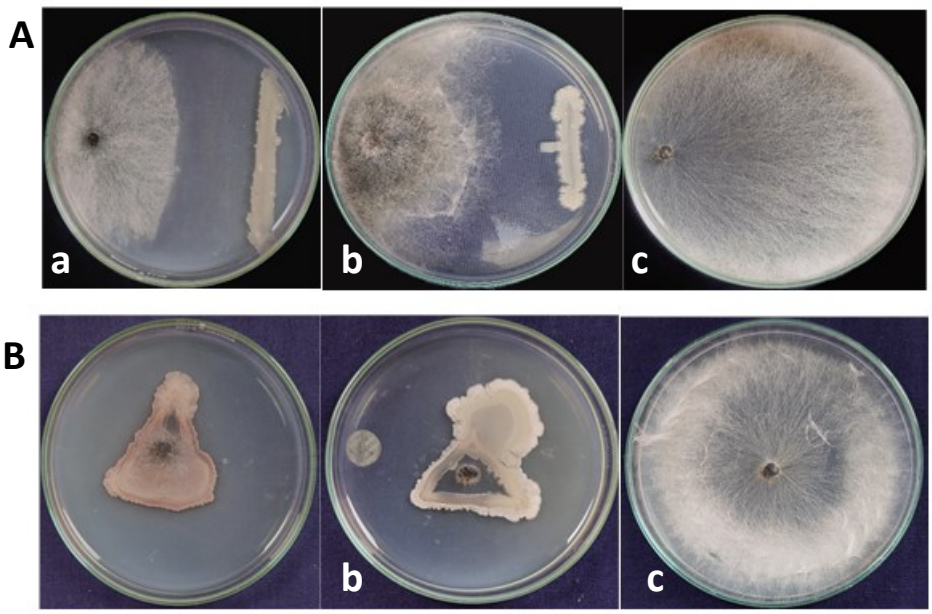

Fig. 9. *In-vitro* study of antifungal activity by antagonists bacterial endophytes against *R. bataticola* **(A.)** Dual plate assay: a- RMV 3, b-RMV 2, c- Control (B.) Triangle method of streaking: a- RMV 3, b-RMV 2, c- Control

Table 5. GC-MS profiling of secondary metabolites in *Bacillus subtilis* RMV 3

S.No	RT	Name of the Compound	Local area (Percent)	Molecular Formula	Function	Molecular weight (g/mol)
1.	6.0741	Dodecane	4.27	$C_{12}H_{26}$	Antibacterial, antifungal activity	170.33 g/mol
2.	6.8206	Octane, 5-ethyl-2-methyl-	1.50	$C_{11}H_{24}$	Antioxidant, antimicrobial activity	156.31 g/mol
3.	8.5175	1-Dodecene	0.71	$C_{12}H_{24}$	Antibacterial	168.32 g/mol
4.	9.2349	2-Bromo dodecane	0.45	$C_{12}H_{25}Br$	Antibacterial	249.23 g/mol
5.	9.2932	Pentadecane	0.53	C15H32	Antifungal	212.41 g/mol
6.	11.3615	1-Tetradecene	1.56	$C_{14}H_{28}$	Antifungal activity	196.37 g/mol
7.	25.7527	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (phenylmethyl)-	0.63	$C_{14}H_{16}N_2O_2$	Antimicrobial activity	244.29 g/mol
8.	21.0078	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (2-methyl propyl)-	1.33	$C_{10}H_{16}N_2O_2$	Antimicrobial compound	196.25 g/mol
9.	23.6370	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	1.31	$C_{12}H_{22}N_2O_2$	Antifungal	226.32 g/mol
10.	24.6930	Bis(2-ethylhexyl) phthalate	2.21	$C_{24}H_{38}O_4$	Antibacterial, Antifungal	390.6 g/mol
11.	18.2476	Eicosane	0.35	$C_{20}H_{42}$	Antifungal, Antibacterial	282 g/mol
12.	16.2375	1-Octadecene	2.66	$C_{18}H_{36}$	Antibacterial activity	252 g/mol
13.	18.3131	Hexadecanoic acid, methyl ester	1.31	$C_{17}H_{32}O_2$	Antibacterial	268.4 g/mol
14.	11.5327	2-Piperidinone	1.61	C_5H_9NO	Antimicrobial activity	99.13 g/mol
15.	13.9251	Cetene	2.66	$C_{16}H_{32}$	Catalytic activity	224.42 g/mol
16.	15.1778	Benzoic acid, 4-ethoxy-, ethyl ester	0.41	$C_{11}H_{14}O_3$	Antioxidant, antibacterial activity	194.22 g/mol
17.	15.9024	Dodecyl acrylate	0.92	$C_{15}H_{28}O_2$	Antimicrobial, Antifungal activity	240.38 g/mol
18.	16.3212	Diethyl Phthalate	0.76	$C_{12}H_{14}O_4$	Antimicrobial, Antifungal	222.4 g/mol
19.	20.1666	Docosane	0.53	$C_{22}H_{46}$	Antifungal	310.6 g/mol
20.	17.0677	Isopropyl myristate	0.93	$C_{17}H_{34}O_2$	Antimicrobial, Antifungal	270.5 g/mol
21	9.4059	Dodecane, 2,7,10-trimethyl-	0.71	C15H32	Antimicrobial activity	212.4 g/mol
22	10.6039	Naphthalene	2.75	$C_{10}H_8$	Antifungal activity	128.17 g/mol
23	11.2776	Tetradecane	1.60	$C_{14}H_{30}$	Antifungal, antibacterial	198.39 g/mol
24	26.5538	Hentriacontane	6.51	$C_{31}H_{64}$	Antifungal activity	436.8 g/mol

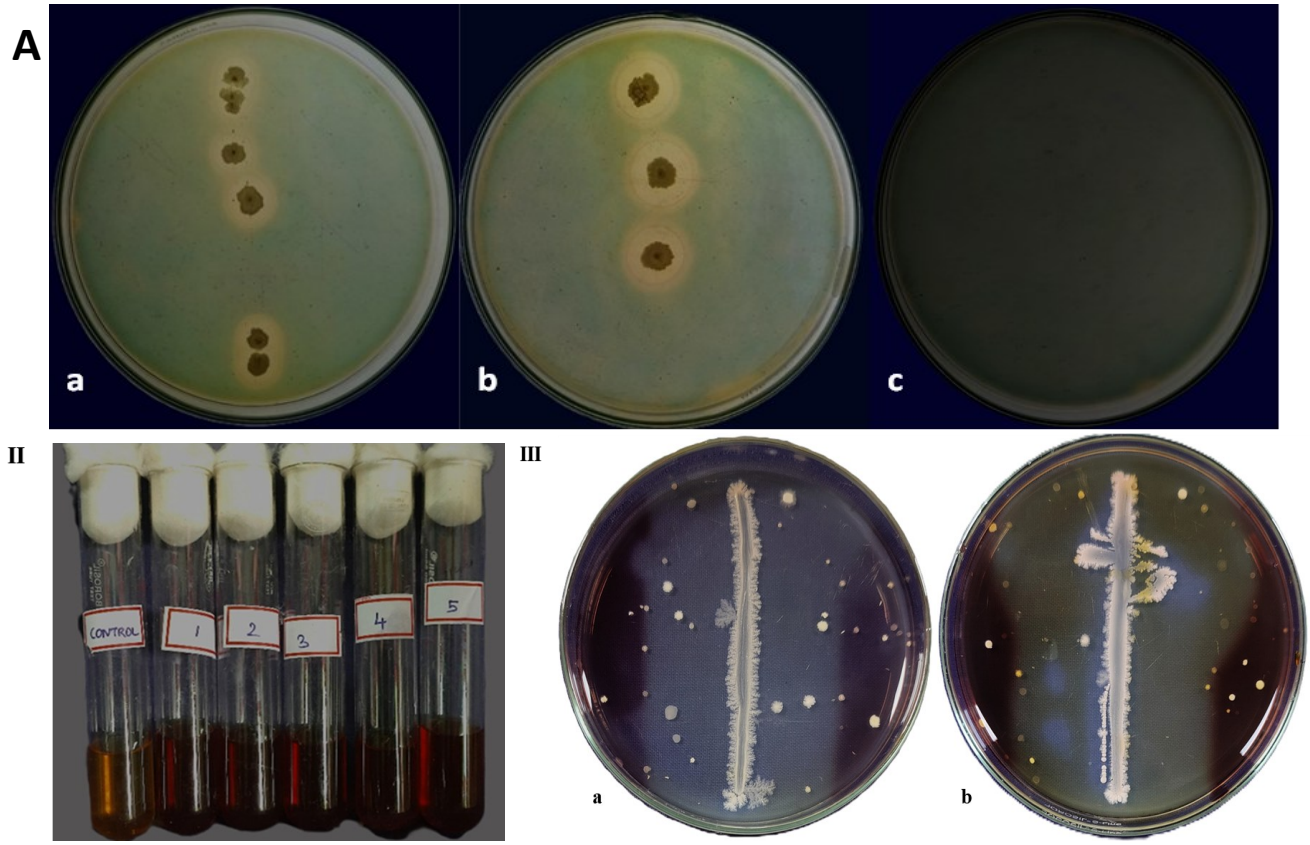

Fig. 10. Qualitative screening of (A) Siderophore production by endo phytic bacterial isolates a-RMV 3, b- RMV 2, c- Control; (B)Indole acetic acid (IAA) 1-RMV 1, 2-RMV-2, 3- RMV 3, 4- LTP- 21, 5- LMV 6 and control and (C) Cellulase production a- RMV 3, b- RMV 2

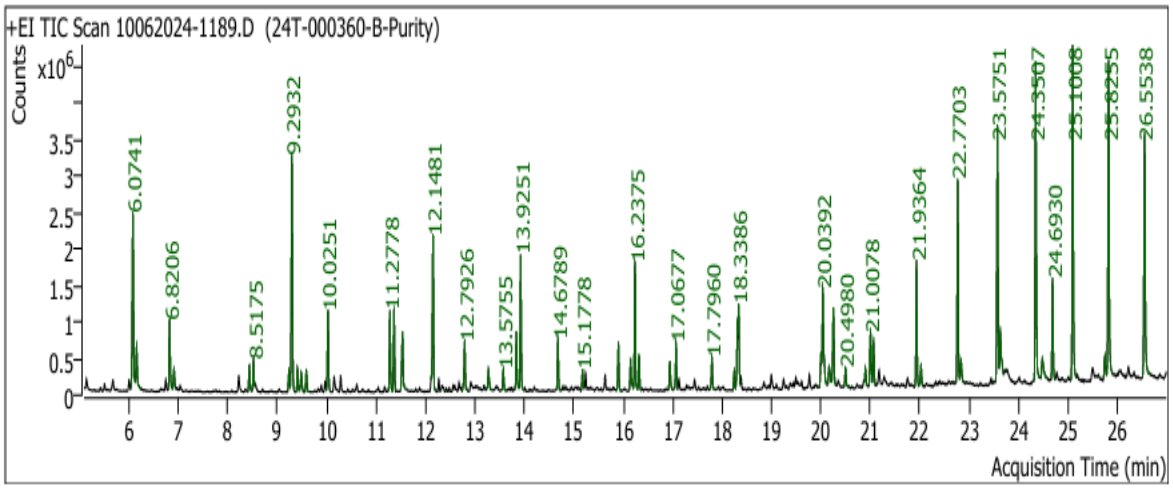

Fig. 11. Identification of secondary metabolites through GC-MS analysis

Discussion

Rhizoctonia bataticola is one of the most destructive diseases of groundnuts, causing damage to the plant and a reduction in the yield. Plant disease management using antagonistic endophytic bacteria has become significant due to their numerous advantages compared to free-living ones (28). The main objective of this study was to isolate salt -tolerant endophytic bacteria from healthy groundnut plants grown in salinity-affected regions, evaluating their antagonistic effect against the pathogen responsible for causing dry root rot disease, *R. bataticola* in groundnut. We also investigated some biochemical tests and plant growth-promoting activity of bacterial endophytes to determine the potential use as a biocontrol agent. A survey was also conducted and a 60.50% incidence of dry root rot was recorded in Namakkal District, Tamil Nadu. (29) mentioned that a similar percentage of groundnut dry root rot disease incidence was reported in the Tiruvannamalai district of Tamil Nadu. The pathogen was isolated from the infected groundnut leaves and identified as Rhizoctonia bataticola MP 1 (PP327216) and R. bataticola MP 4 (PP463546) using ITS rDNA sequence analysis. Early studies have also reported the isolation of *R. bataticola* from major groundnut -growing areas of Southern India (30). The potential endophytic bacteria were isolated by surface sterilization of plant tissues from groundnut (31-32), soybean (33), and tomato. However, (3) have isolated 56 endophytes from groundnut plants' roots, stems, and seeds grown in salinity-stressed conditions. Accordingly, in this study, 27 endophytic strains were isolated from groundnut crop's root, stem and leaf tissues grown in salinity-affected areas.

Bacterial endophytes can be effective bio-control agents against soil-borne pathogens, providing targeted protection to the host plant. These beneficial bacteria inhabit the host plant to combat various phytopathogens (34). The effective isolates RMV 3 and RMV 2 showed high inhibition rates of 61.1% and 50%, respectively, against *R. bataticola*. These isolates were identified as *Bacillus subtilis* RMV 3 (PP833618) and *Bacillus subtilis* RMV 2 isolate (PP835389). *Bacillus sp.* has demonstrated a wide range of potential in agriculture by controlling plant diseases, promoting plant growth and enhancing yield (35). However, the antagonistic activity of endophytic *Bacillus* species against *R. bataticola* has been reported in other studies, with a maximum inhibition of 79.6% observed in black gram (36), *Bacillus subtilis* ESBs 19 showed inhibition of 57.91% in cotton against *R. bataticola* (37). *Bacillus* species promote plant growth directly and indirectly by producing indole acetic acid (IAA), siderophores, antifungal metabolites and solubilizing phosphate (38).

This study demonstrated that the endophytic *Bacillus subtilis* strain RMV 3 produces several plant growth-promoting substances, including siderophores, indole-3-acetic acid (IAA) and cellulase. Indole-3-acetic acid helps provide plants resistance against both biotic and abiotic stress (39). Many bacterial endophytes can chelate iron, including *Bacillus*, *Azotobacter*, *Arthrobacter*, *Nocardia*, *Streptomyces* and *Enterobacter* (40). Furthermore, the siderophore production by *Bacillus* sp was also demonstrated by (41) reported *B. velezensis* CBRE5, *B. velezensis* CBRE5 and *B. subtilis* CFLE3 produces siderophore. We performed GC-MS analysis to identify the antifungal compounds present in *Bacillus subtilis* RMV 3, to which the antagonistic behaviour against *R. bataticola* can be attributed. The results showed the presence of Diethyl Phthalat, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (2-methylpropyl)-, 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3 (phenylmethyl)-, Benzoic acid, 4-ethoxy-, ethyl ester. These secondary metabolites contributed to the antagonistic activity of *B. subtilis* RMV 3. While bioactive compounds were detected, future studies should focus on isolating and testing these individual compounds to determine their specific contributions to the observed antagonistic activity. The *in vitro* efficacy observed with *Bacillus subtilis* RMV 3 requires validation through in vivo studies and field trials to confirm its effectiveness under varied environmental conditions and against different pathogen strains.

Conclusion

In conclusion, the present study reported effective saline-tolerant antagonistic endophytic bacteria *Bacillus subtilis* RMV 3 against *Rhizoctonia bataticola* in groundnut. Applying plant growth-promoting endophytic bacteria with biocontrol potential is a novel approach to plant health management.

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Authors' contributions

AR conducted the experiments, analyzed the data, and wrote the original manuscript. DM designed the experiment and revised the article. HS provided the resources. GKN, MV supervised the research

Compliance with ethical standards

Conflict of interest: The authors state no conflict of interest concerning this article's research, authorship and publication.

Ethical issues: None

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