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Culture-dependent analysis of seed bacterial endophyte, *Pseudomonas* spp. EGN 1 against the stem rot disease (*Sclerotium rolfsii* Sacc.) in groundnut

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Abstract

Background: Groundnut, *Arachis hypogaea* L., crop is an important oil seed crop in India and is prone to attack by numerous fungal and viral diseases, among the soil-borne diseases, stem rot caused by *Sclerotium rolfsii* Sacc. is economically important.

Main body of abstract: Incidence (86.6%) of the stem rot disease was recorded in Coimbatore district, Tamil Nadu, India, during the roving survey. For its management, an attempt was made to isolate bacterial endophyte from peanut seeds of different groundnut cultivars by culture-dependent analysis. Totally, 16 bacterial endophytes (endophytes groundnut (EGN) 1 to EGN 16 along with standards TNAU-Pf1 and EPC 5) were obtained and characterized through morphological, biochemical and molecular studies and also phytostimulation activities were performed. Among the isolates, EGN 1 and EGN 4 showed positive results for indole acetic acid (IAA), siderophore, phosphate solubilization and protease tests in vitro. The dual culture analysis showed inhibition rates of 60.1% (dual plate assay), 68.23% (filter paper disc assay) and 100% (triangle method of streaking and culture filtrate assay) for EGN 1 against *S. rolfsii*. Further, the crude metabolite assay showed 97.7% inhibition in EGN 1, followed by 87.7% in EGN 4. The roll towel study showed a high vigour index of 4286.7 in EGN 1; hence, this isolate was chosen. Further, thin-layer chromatography (TLC) analysis showed various bands at 0.72 R_f , whereas GC-MS analysis indicated the prominent peaks of hexadecanoic acid and cis-vaccenic acid that may responsible for antifungal activity. In a molecular approach, the genomic DNA of EGN 1 strain was used to amplify a 1200 bp PCR-fragment and sequenced.

Short conclusion: The overall outcome of this study showed that *Pseudomonas* spp. EGN 1 had a great potential as a bio-stimulant and biocontrol agent to manage effectively the stem rot in peanut.

Keywords: Groundnut, Stem rot, *Sclerotium rolfsii*, Seed bacterial endophytes, *Pseudomonas* spp., Antagonist, Phytostimulation activity

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Background

Groundnut crop known as “King of oilseeds” is an important oil seed crop in India and the fourth most important edible oil source in the world. It originated in South America and contains 48–50% oil and 26–28% protein (Janila et al., 2013). In India, Gujarat is the largest producer contributing 25% of the total production, followed by Tamil Nadu (22.48%). It is valued as fourth most important source of edible oil and third most important source of vegetable protein. It is also an important agricultural export commodity from India. Several factors are responsible for lower productivity among which leaf spot, collar rot, stem rot and bud necrosis, etc. are very important. Out of them, stem rot caused by *Sclerotium rolfsii* Sacc. is a major problem and is an important soil-borne pathogen. Characteristic symptoms include production of brown-coloured mustard-sized sclerotia, resting structure (0.1 to 3.0 mm) and causes pod yield losses up to 80% under favourable conditions (Deepthi, 2014). As an alternative to judicious use of fungicides, which is creating adverse effect on environment, eco-friendly disease management strategies have to be evolved, especially using endophytes from seeds. Seed endophytes resulting in colonization of embryo and endosperm are gaining importance. Some of these bacteria can benefit plant growth and defence against abiotic and biotic stress, as well as vertical transmission of endophytic bacteria in seeds has also been reported (Frank et al., 2017). This process of vertical transmission results in a weakening of microbial pathogenic strength in order to support plant growth and development. Truyens et al. (2015) reviewed studies on seed endophytes and reported that the bacteria found in seeds mostly belong to the genera, especially *Bacillus* and *Pseudomonas*, and also *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter*. Many seed-borne bacteria produce plant hormones such as auxins, cytokinins and gibberellins; others produce ACC deaminase. This confers plant protection by the induction of plant defence mechanisms and production of lytic enzymes and antibiotics. Moreover, alongside their vital role in plant growth and defence, these seed-borne bacterial and fungal endophytes benefit the host plants through providing their progeny with valuable endosymbionts. Plant growth promotion by endophytic bacteria-inoculated plants has been frequently reported and this has attracted research toward their use in phytoremediation or as bioinoculants in agriculture.

The study aimed to isolate and characterize endophytes that naturally colonized peanut seeds and their effect on soil-borne plant pathogen.

Main text

Materials and methods

Survey and stem rot pathogen

Survey was conducted in stem rot-infected fields during the growing seasons in different groundnut cultivars,

namely COG 0549, VRI 7, COG 0539, TMV 7, TMV 2, CO 6, TAG 24, CG 20, AK 303, TG 37, K6, COG 0537, Dharani, TMV 14, BSR 2, CO 7 and VRI 8. The cultivars were grouped into group 1 (resistant), group 2 (moderately resistant), group 3 (susceptible) and group 4 (highly susceptible cultivars) based on percent disease incidence (PDI) in groundnut cultivars. The pathogen namely *Sclerotium* was isolated from the infected stem regions of groundnut through tissue segmentation method (Adhilakshmi et al., 2014) from Aliyar, Coimbatore, Sivaganga and Madurai region.

Seed bacterial endophytes

Seeds were surface disinfected in 5% commercial bleach and 0.01% Tween 20 for 10 min and rinsed 10 times with sterile distilled water. The efficiency of the procedure was checked using an aliquot of the water used and in the final wash was plated on nutrient agar. Surface-sterilized seeds were split into two and placed in a nutrient agar plates at an inverted position and incubated at 28 °C in the dark for 24–48 h. The rest of the isolates were obtained by plating dilutions of seed macerates on nutrient agar (NA). Surface-sterilized seeds were triturated with a pestle and mortar in PBS (phosphate-buffered saline, pH 7.0) and 60 µl of the suspension were placed onto NA, followed by incubation for 2 days at 28 ± 2 °C. After 24 h of incubation, colonies with distinct growth features were isolated (Xu et al., 2014).

In vitro antagonistic activity

All of the endophytic bacterial isolates were evaluated against *S. rolfsii*. A full loop of endophytic bacteria isolates (48 h old) grown on NA was streaked at the distance of 1 cm from the periphery of a Petri plate. Nine millimetre mycelial discs of *S. rolfsii* were taken from the 4-day-old culture and placed at the other end of the Petri plate containing potato dextrose agar (PDA). The plates were incubated for 7 days at 28 ± 2 °C (room temperature) and the radial growth of *S. rolfsii* was observed for the development of inhibition zone and the inhibition of mycelial growth was assessed by measuring the radial growth of *S. rolfsii* in the treated plate. The mycelial disc of *S. rolfsii* was also inoculated on the plate without endophytic bacteria as a control (Bodhankar et al., 2017). Four replicate plates were measured per isolate and the experiment was repeated twice to confirm the results. The percentage inhibition radial growth of treatments compared to the control was calculated as follows:

$$\text{PIRG} = R_c - R_t / R_c \times 100\%$$

Where; R_c is radial growth of *S. rolfsii* in the control plate and R_t is radial growth of *S. rolfsii* toward the antagonist in a dual culture plate.

Growth promotion assay by roll towel method

Effect of bacterial endophytes on seed germination was assessed by standard roll towel method (ISTA, 1993). Bacteria cultured in a LB broth for 48 h were centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was mixed with sterile water. Groundnut seeds were surface sterilized by 0.1% mercuric chloride for 5 min and rinsed with sterile distilled water. The surface-sterilized seeds were blot dried and soaked in the bacterial suspension for 4 h and sterile water was served as a control. Then, the seeds were placed in wet blotters and incubated at room temperature. Each treatment was replicated 3 times and at the 10th day, germination percentage, root length and shoot length was measured randomly (Agrawal and Agrawal, 2013). Plant growth promotion of groundnut seedlings was assessed using the vigour index (VI).

$$VI = \frac{\text{Germination\%} \times \text{mean total length of seedling}}{\text{root length} + \text{shoot length}}$$

Further, total root length, root tips, segment and forks were assessed using a rhizoscanner instrument available at DARS, Chettinad, TNAU, Coimbatore.

Filter paper disc assay

Antimicrobial activity of crude extracts was examined using the disc diffusion method. Sterile filter paper discs of 6 mm diameter were soaked in crude extract and placed on the solidified PDA medium at the 4 corners of the plates at equal distance. Mycelial discs (9 mm) of *S. rolfsii* were collected from the 4-day-old culture and placed at the centre of the petriplate and incubated for 7 days at room temperature (Ramyabharathi and Ragu-chander, 2014). Diameters of the growth inhibition areas were measured along with control plate.

Morphological and biochemical characterizations

Seed endophytic bacteria were morphologically characterized based on colony formation, margin, elevation, texture, colour, surface and opacity. Effective strains were classified as gram positive or negative based on Gram staining. The antagonistic strains were identified to the species level based on the biochemical tests namely KOH, citrate utilization, indole tryptone, catalase, methyl red and vokes proskauer test, urease and gelatin liquefaction test (Bhoonobong et al., 2012).

Molecular characterization

Genomic DNA extraction of the selected bacterial isolates was done using cetyltrimethylammoniumbromide (CTAB) method. The 16S rRNA gene was amplified using the universal primers Fd1 (5'AGAGTTTGATCCTGGCTCAG-3') and Rd1 (5'AAGGAGGTGATCCAGCC-3') (Dhouib

et al., 2019) and the primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India. The concentration of DNA was measured in NanoDrop followed by loading in 0.8% agarose gel electrophoresis and its concentration was observed using UV transilluminator (Inderiati and Franco, 2008). Thermocycler was used to perform PCR, using cycling conditions; initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, extension at 72 °C for 2 min and with a final extension at 72 °C for 7 min consisted of 30 cycles. Final products of PCR were analysed by 1.5% agarose gel electrophoresis. Based on the analysis, the amplified 16S rRNA PCR product of endophytes groundnut (EGN) 1 and EGN 4 was sent for custom sequencing (Barcode Bio-Sciences, India). The obtained sequences were then compared for homology with those present in National Center for Biotechnology Information (NCBI), using Basic Local Alignment Search Tool (BLAST) analysis. The phylogenetic trees were generated using the neighbour-joining method by using MEGA 7.0 software.

Phyostimulation activities

IAA production Salkowski reagent was used for IAA detection and quantification. The bacterial strains were grown in 100 ml nutrient broth with and without 0.5 g/l tryptophan (precursor of IAA) in darkness (Gordon and Paleg, 1957) for 3 days at 28 °C at 110 rpm. Bacterial culture suspensions were centrifuged (30 min at 8000 rpm) and 0.2 ml of the supernatant was mixed with 1 ml Salkowski's reagent (50 ml 35% HClO₄, 1 ml 0.5 M FeCl₃). After 30 min, a pink colour was developed, which indicate IAA production. The absorbance of pink colour was read at 530 nm in spectrophotometer.

Siderophore production Siderophore production of the endophytic bacterial strains was examined, using Chrome azurol S (CAS) agar medium following the protocol of Khamna et al. (2009). Five days post incubation at room temperature (28 ± 2 °C), orange halo around the colony indicated siderophore production. The experiment was carried out with 6 replications and repeated twice.

Phosphate solubilization Pikovskayas medium was employed for detecting solubilization of tricalcium phosphate [Ca₃(PO₄)₂] activity by antagonistic endophytic bacterial isolates. After 5 days of incubation at (28 ± 2 °C), phosphate-solubilizing activity (P-solubilizing positive) was observed by the development of a clear zone around the colonies (Pikovskaya, 1948). The experiment was carried out with 6 replications and repeated twice.

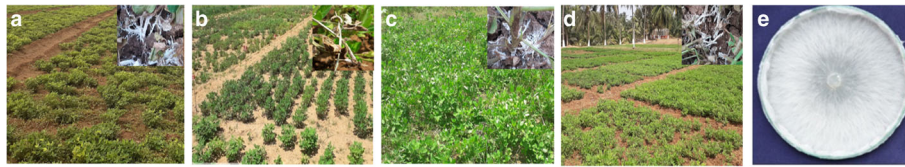


Fig. 1 Stem rot infected fields in (a). Coimbatore (b). Sivagangai (c). Madurai (d). Aliyar (e). Sclerotium in PDA

Protease production Qualitative proteolytic activity of the selected endophytic bacterial strains was determined on skim milk agar medium (El-Deeb et al., 2012). Four days post incubation at ($28 \pm 2^\circ\text{C}$), proteolytic activity was observed by the development of a halo clear zone around the colony of endophytic bacterial isolates. Six replicate plates were conducted and the experiment was repeated twice.

Cellulase production Selected bacterial endophytes were spot inoculated on plates containing carboxy methyl cellulose (CMC) agar (0.05% K_2HPO_4 , 0.025% MgSO_4 , 0.188% CMC sodium salt, 0.02% Congo red, 1.5% agar and 0.2% gelatin). The plates were incubated at 28°C for 48 h. After incubation, CMC plates were observed for the presence of clear zone around the colonies (Kasana et al., 2008).

Culture filtrate test Based on the in vitro test, selected endophytic bacterial isolates with high antagonistic

Table 1 Stem rot incidence in different groundnut cultivars

Isolates	Percent disease incidence
COG 0549	43.47 (41.24) ^e
VRI 7	30.00 (33.21) ^g
COG 0539	14.28 (22.20) ^j
TMV 7	52.00 (46.14) ^d
TMV 2	86.36 (68.33) ^a
CO 6	7.00 (15.34) ^m
TAG 24	56.81 (48.91) ^c
CG 20	40.00 (39.23) ^f
AK 303	62.50 (52.24) ^b
TG 37	20.00 (26.56) ^h
K6	42.00 (40.39) ^e
COG 0537	15.00 (22.78) ^{ij}
Dharani	6.66 (14.95) ^m
TMV 14	10.00 (18.43) ^k
BSR 2	15.62 (23.28) ⁱ
CO 7	15.00 (22.76) ^{ij}
VRI 8	8.82 (17.27) ^l

Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = 0.05$. Values in parentheses are arcsine transformed values

activity were further investigated for their ability to produce inhibitory metabolites. The test was conducted using the method described by Intana et al. (2008). Erlenmeyer flask 250 ml containing 100 ml Luria-Bertani broth (LB) were inoculated with the selected bacterial isolates and incubated at $25 \pm 2^\circ\text{C}$ on a rotary shaker (150 rpm) for 72 h. It was centrifuged at 10,000 rpm for 10 min and the pellets were discarded and the supernatant was filtrated with germ filter ($d = 0.25 \mu\text{m}$). The filtrate from each isolate was incorporated into sterilized PDA in ratio 2:1, and 25 ml of the amended agar was poured into each Petri plate and allowed to solidify. Mycelial disc of *S. rolfsii* (9 mm) was centrally inoculated on each plate. Sterilized water was used as the control. The diameter of the mycelial growth of *S. rolfsii* was measured over 7 days in comparison to the control plate (Alsultan et al., 2019).

Production of thermostable antifungal compounds

Protocol described by Ferraz et al. (2016) was used to assess the production of thermostable antifungal compounds. A loopful of 72-h-old culture inoculum was transferred to 250 ml Erlenmeyer flasks containing 100 ml PDA medium, followed by incubation at 150 rpm for 72 h. From that, 10 ml aliquots of each isolate were transferred to Erlenmeyer flasks containing 90 ml PDA medium. Then, it was subjected to autoclaving at 121°C for 30 min. After sterilization, it was poured into a Petri plate and following solidification, a 9-mm culture disc of *S. rolfsii* was transferred to the centre of each plate. *S. rolfsii* that was grown on PDA medium containing sterile water instead of the metabolites is kept as a control. The plates were incubated in a BOD chamber at $25 \pm 1^\circ\text{C}$ and constant light for 10 days. From two perpendicular directions, mycelial growth of *S. rolfsii* was measured in comparison to the control plate.

Effect of metabolites on the growth of *Sclerotium rolfsii*

Agar well diffusion assay modified by Islam et al. (2012) was used to determine the antagonistic activity of metabolites. Twenty millilitres of PDA medium was poured into sterilized Petri plates and after solidification, mycelia disc (9 mm) of test pathogen was placed at the centre. A well of 7 mm diameter was made by punching with a sterile cork borer on the solidified agar plate on the

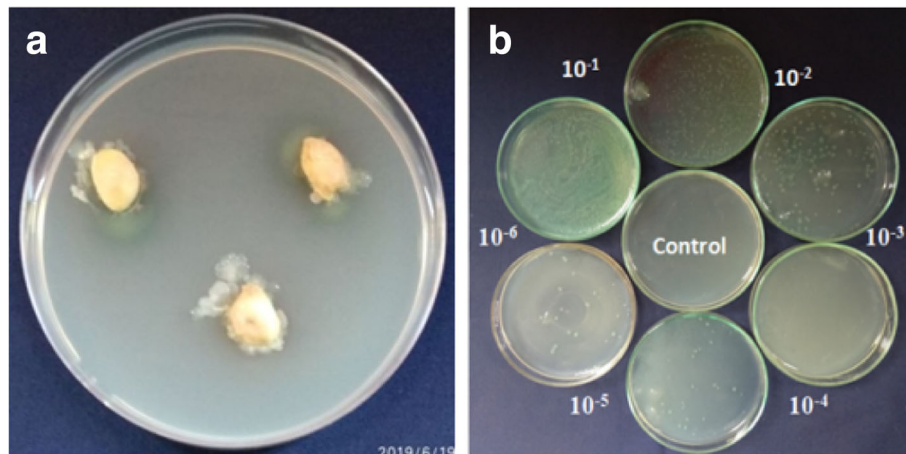


Fig. 2 Isolation of seed bacterial endophytes by (a) split and (b) serial dilution technique

corner in 4 places at an equal distance. Then, the extracted metabolites were poured into the agar wells separately (100 ul) and incubated for 72 h at $28 \pm 2^\circ\text{C}$. After incubation, the inhibitory activity was assessed by measuring the radial growth of *S. rolfsii* in the treated plate compared to control.

TLC and GCMS analysis

Bacterial strains were cultured on a LB broth and ethyl acetate was used for the extraction of crude antibiotics. In TLC analysis, methanol was used to dissolve the dried sample and the plates were developed with isopropanol: ammonia: water (8:1:1). Five microlitres of sample were spotted onto silica gel plate and the plate was visualized using UV transilluminator at short wave length (254 nm). The R_f values were calculated for specific antibiotics (Prabhukarthikeyan and Raguchander, 2016). Gas chromatography/mass spectrometry (GCMS) analysis was used for the selected endophytic bacterial isolates with high antifungal effects for the production of volatile antifungal metabolites as described by Hassi et al. (2012). The compounds were identified based on a comparison of their mass spectra data and retention time as well as with the existing analytical data of specially synthesized reference compounds database.

Statistical analysis

Statistical analyses were done using IRRISTAT version 92 developed by the International Rice Research Institute Biometrics Unit, the Philippines (Gomez and Gomez, 1984), and the percentage values of the disease index were arcsine transformed. Data were subjected to ANOVA at significant level ($P < 0.05$) and means were compared by Duncan's multiple range test (DMRT).

Results and discussion

Survey and isolation of the pathogen

The survey of the pathogen revealed that the stem rot incidence of *A. hypogaea* was 86.6% in Coimbatore, 32% in Aliyar, 26% in Madurai and 21% in Sivaganga districts (Fig. 1 and Table 1). Also, 7 cultivars (COG 0539, TMV 14, CO 7, VRI 8, COG 0537, CO 6, Dharani) showed high resistance to the pathogen, 3 cultivars (VRI 7, BSR 2, TK 37) moderate resistance, 3 cultivars (COG 0549, CG 20, K6) moderately susceptible and 4 cultivars (TMV 7, TMV 2, TAG 24, AK 303) were highly susceptible under field conditions. Five isolates, obtained from 4 different regions of Coimbatore, Aliyar, Madurai and Sivaganga and the *Sclerotium* were isolated from the infected plant samples and the abundant whitish mycelium was observed in a distinctive fan-like pattern. It produced mustard-sized sclerotia of 0.1–0.3 mm after 3–4 days of incubation. Similarly, Rakh et al. (2011) reported that isolation of *S. rolfsii* from groundnut was carried out and maintained on PDA slants incubated at 28°C .

Table 2 Results of various biochemical tests

Isolates	1	2	3	4	5	6	7	8
EGN 1	-	+	+	+	-	+	+	+
EGN 2	-	-	+	+	+	+	+	+
EGN 3	-	+	+	+	-	+	-	-
EGN 4	+	-	+	+	-	+	+	+
EGN 5	+	+	+	+	-	+	+	+
EGN 6	-	+	+	+	+	+	+	-
EGN 7	-	+	+	+	-	+	-	-
TNAU PF 1	-	-	+	+	-	+	+	+
EPC 5	+	-	+	+	-	+	+	+

1 KOH test, 2 citrate utilization test, 3 indole tryptone test, 4 gelatin liquefaction test, 5 methyl red test, 6 urease test, 7 hydrogen peroxide test, 8 VP test

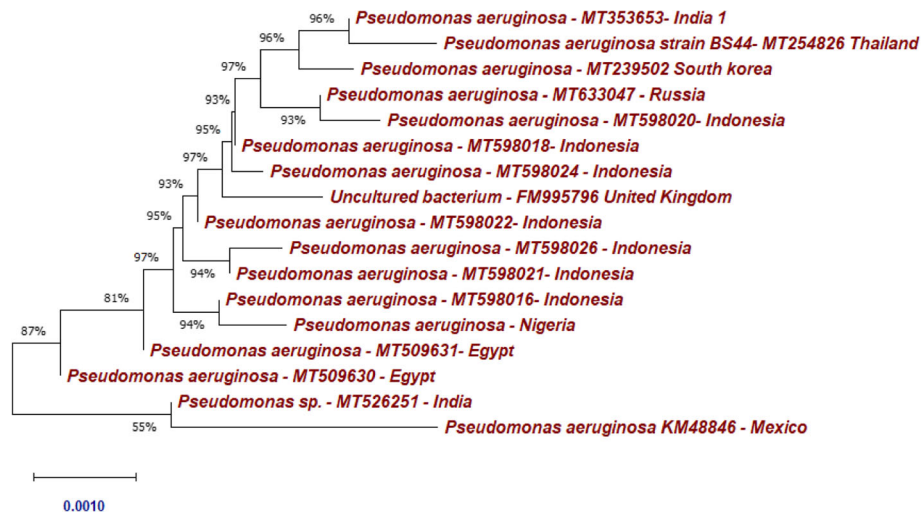


Fig. 3 Phylogenetic tree of the 16S rRNA sequences of *Pseudomonas* spp.

Isolation of seed bacterial endophytes

For the management of stem rot disease in groundnut, bacterial endophytes isolated from the inner tissues of seeds were used as an alternative eco-friendly strategy. Bacterial endophytes isolated from leaves, roots and stems have been reported by many authors (Saini et al., 2015 and Upreti et al., 2015) but endophytes isolated from the seeds have not been studied. Hence, the present study aimed to isolate seed bacterial endophytes and exploiting for the management of stem rot disease in groundnut. In this context, culture-dependent studies by split plot and serial dilution technique (Fig. 2) were undertaken on 17 groundnut cultivars VRI 2, JL 24, CO 6, VRI 8, TAG 24, CO 1, TMV 1, TG 37, CO 4, TMV 2, K 6, TMV 7, TMV 14, CO 7, ALR 3, BSR 9 and TMV 10 for bacterial endophytes isolation and the isolates namely EGN 1 to EGN 16 were isolated from seeds along with standards TNAU-Pf 1 and EPC 5 were used. Similarly, Sobolev et al. (2013) isolated the endophytic bacteria from groundnut seeds by split technique. Xu

et al. (2014) identified 84 endophytic bacteria from the seeds of tomato by serial dilution technique.

Morphological, biochemical and molecular characterization

The colony of seed bacterial endophytes varied from circular to irregular with raised, flat and convex margin was observed and their texture varied from brittle to dry with rough, smooth and glistening surfaces. Based on morphological observation, the majority of colony colour ranged from whitish yellow to dull white and characterized biochemically through various biochemical tests (Table 2). The effective isolates namely EGN 1 and EGN 4 showed positive results for indole tryptone, gelatin liquefaction, urease, hydrogen peroxide and VP test and gram staining is also carried out for effective isolates along with standard EPC 5. The effective isolates showed negative reaction whereas standard EPC 5 showed positive. Similarly, Rajendran et al. (2007) characterized the endophytic species as *Bacillus* in cotton by various biochemical tests like Gram staining, KOH test, utilization

Table 3 Antagonistic potential of seed bacterial endophytes against *Sclerotium rolfsii* through dual culture technique

Isolates	% inhibition over control	Isolates	% inhibition over control
EGN 1	47.77 (43.68) ^b	EGN 10	12.22 (20.43) ^k
EGN 2	25.55 (30.33) ^{ij}	EGN 11	31.11 (33.89) ^f
EGN 3	41.11 (39.87) ^c	EGN 12	0.00 (0.28) ⁱ
EGN 4	60.00 (50.77) ^a	EGN 13	27.77 (31.75) ^{gh}
EGN 5	28.88 (32.45) ^g	EGN 14	33.33 (35.24) ^e
EGN 6	35.55 (36.57) ^d	EGN 15	31.11 (33.89) ^f
EGN 7	46.66 (43.05) ^b	EGN 16	0.000 (0.286) ⁱ
EGN 8	24.40 (29.59) ^j	TNAU Pf 1	40.00 (39.229) ^c
EGN 9	26.60 (31.04) ^{hi}	EPC 5	27.77 (31.758) ^{gh}

Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = 0.05$. Values in parentheses are arcsine transformed values

Table 4 Growth promotion test by roll towel method

Isolates	Vigour index	Germination (%)	Root length (cm)	Shoot length (cm)
EGN 1	4286.70 ^a	86.6 (68.6) ^a	25.0 (29.9) ^b	24.5 (29.66) ^a
EGN 4	2895.35 ^c	73.3 (58.9) ^c	19.5 (29.9) ^c	21.0 (26.56) ^c
TNAU Pf 1	4040.00 ^b	80.0 (63.4) ^b	27.0 (31.3) ^a	23.5 (28.99) ^b
EPC 5	2599.74 ^d	66.66 (54.7) ^d	18.0 (25.10) ^d	20.0 (27.27) ^d
Control	1466.57 ^e	53.33 (46.9) ^e	11.5 (19.8) ^e	16.0 (23.58) ^e

Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = 0.05$. Values in parentheses are arcsine transformed values

of citrate, catalase test, starch hydrolysis, gelatin hydrolysis and methyl red test. Molecular characterization of effective isolates was carried out by extracting the DNA using CTAB method and the concentration of genomic DNA was measured, using NanoDrop. In a molecular approach, the genomic DNA of strains EGN 1 to EGN

8, TNAU-Pf 1 and EPC 5 were used to amplify a fragment coding its 16S rRNA and the isolates were amplified at 1.2 kb (approx). Erjaee et al. (2020) also reported that the isolates M11, TR1, TR12, TR13, TR14 and A11 obtained from herbal plants were 100% identical to *B. pumilus*. After amplification, the best isolate EGN 1

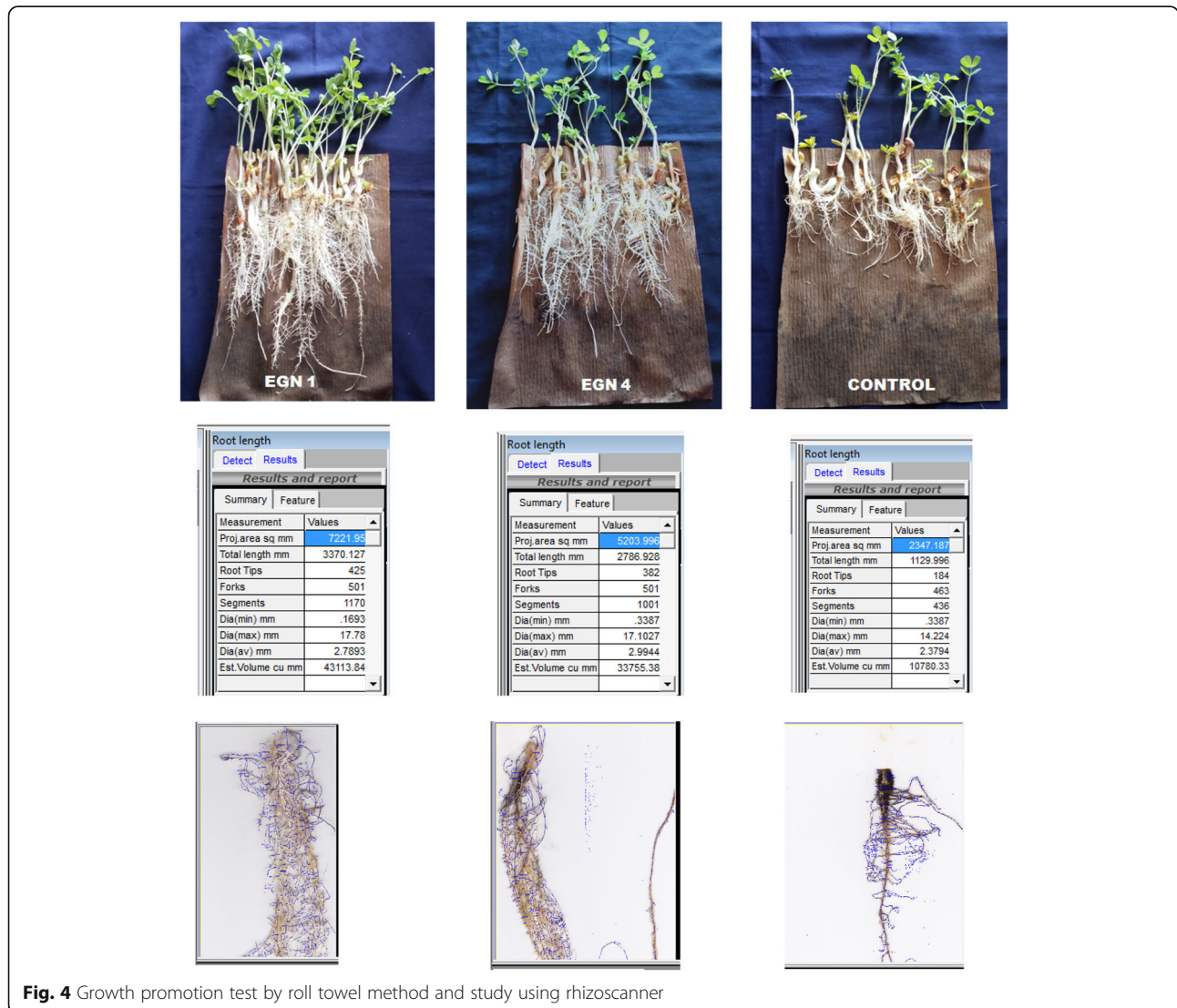


Table 5 Screening of seed bacterial endophytes against *Sclerotium rolfii* by filter paper disc assay

Isolates	% inhibition over control
EGN 1	68.23 (55.69) ^b
EGN 2	11.47 (19.79) ^f
EGN 3	0.00 (0.286) ^g
EGN 4	94.10 (76.12) ^a
EGN 5	0.00 (0.28) ^g
EGN 6	0.00 (0.28) ^g
EGN 7	52.94 (46.68) ^d
EGN 8	57.60 (49.37) ^c
EGN 9	40.20 (39.34) ^e

Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = 0.05$. Values in parentheses are arcsine transformed values

along with EGN 4 was sequenced and phylogenetic tree was generated in MEGA 7.0 software for 16S rRNA gene sequences for EGN 1 and the results revealed that *Pseudomonas* spp. EGN 1 isolate (MT526251) showed 55% similarity with *Pseudomonas aeruginosa* Mexico strain (Fig. 3). Whereas the sequence results of EGN 4

matched with *Acinetobacter* spp. Similarly, *Staphylococcus* is a plant-associated microbe, but recently various studies have reported it as endophytic bacteria (Haidar et al., 2018).

In vitro study

The antagonistic effect of seed bacterial endophytes against *S. rolfii* was assessed on dual culture technique. The results showed that seed bacterial endophytes recorded 25–60.1% of inhibition against *S. rolfii* in vitro (Table 3). Further, triangle method of streaking showed EGN 1, EGN 2, EGN 3, EGN 4, EGN 5 and TNAU-Pf 1 and EPC 5 were maximum inhibition of 100%, whereas isolates EGN 6 and EGN 7 did not show any inhibition. These seed endophytes showed antagonistic potential against *S. rolfii* through dual culture and filter paper disc assay. Correspondingly, Sahu et al. (2019) screened for the antagonistic potential of bacterial endophytes obtained from tomato against *S. rolfii*, 2 endophytic isolates inhibited the radial growth of *S. rolfii* by more than 50%. Kefi et al. (2015) also reported that the cell-free supernatant of the 4 strains inhibited the growth of *Botrytis cinerea* versus control through disc diffusion

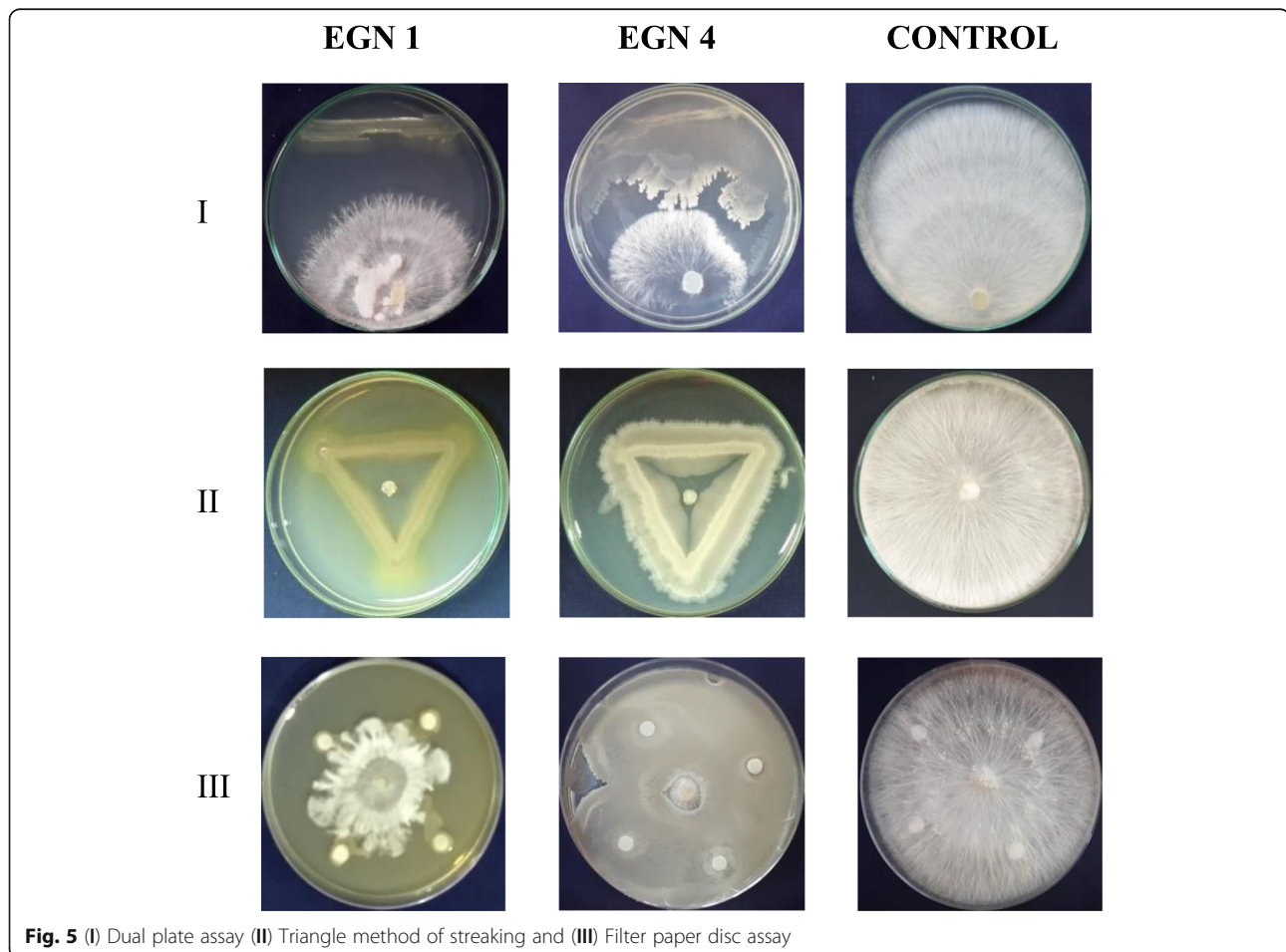
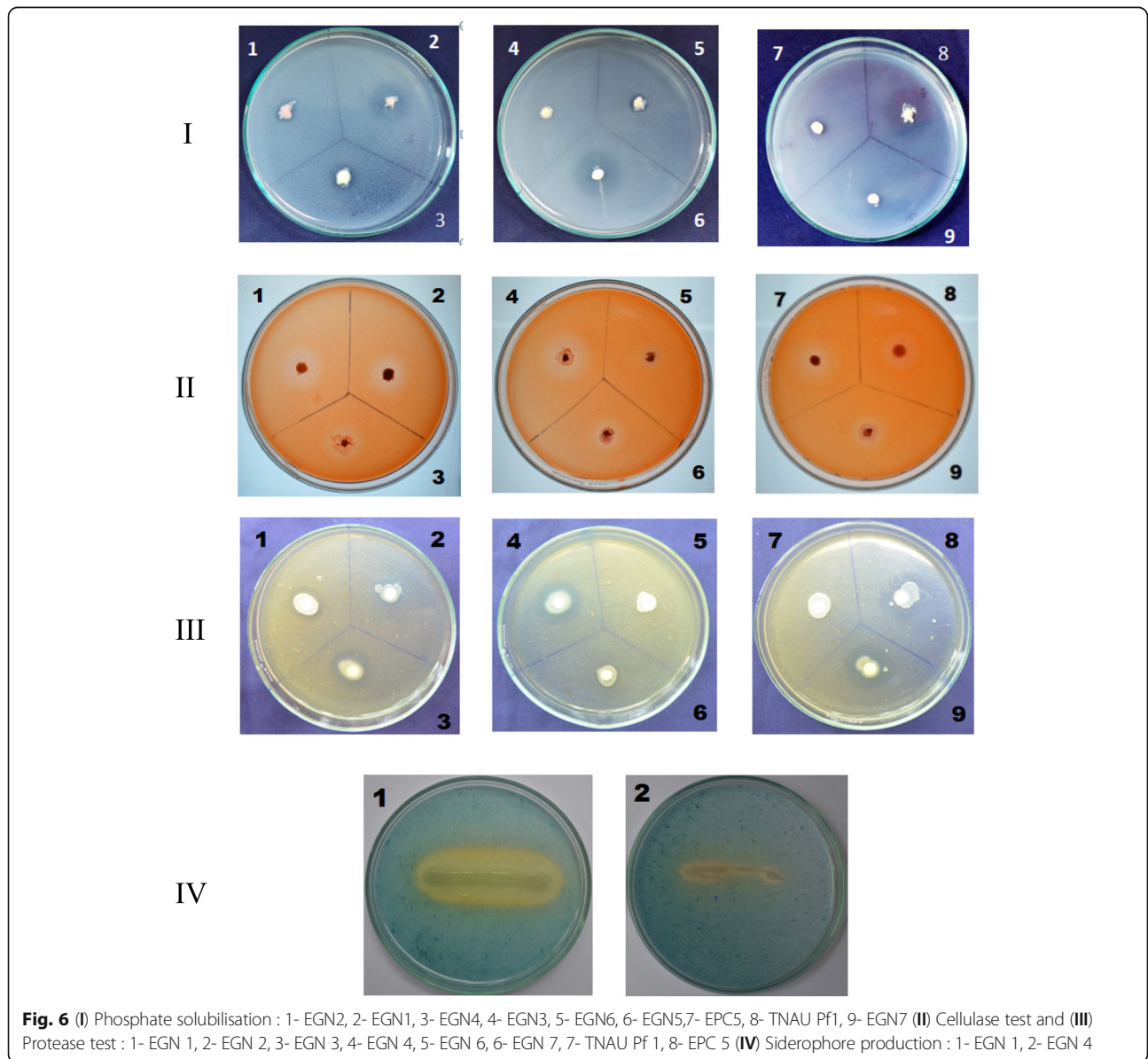


Fig. 5 (I) Dual plate assay (II) Triangle method of streaking and (III) Filter paper disc assay

Table 6 Phytostimulation activities

Isolates	Siderophore production	IAA test	Phosphate solubilization	Cellulase test	Protease test
EGN 1	+++	++	+++	+++	++
EGN 2	+	-	++	+++	++
EGN 3	+++	+++	+	++	+++
EGN 4	++	++	++	+++	+++
EGN 5	+	-	+++	+	-
EGN 6	++	-	++	++	++
EGN 7	++	-	++	++	-
TNAU Pf1	+++	-	+++	+++	+++
EPC 5	++	++	+	++	+++

"+++", good producers; "++", medium producers; "+", low producers; "-", negative reaction



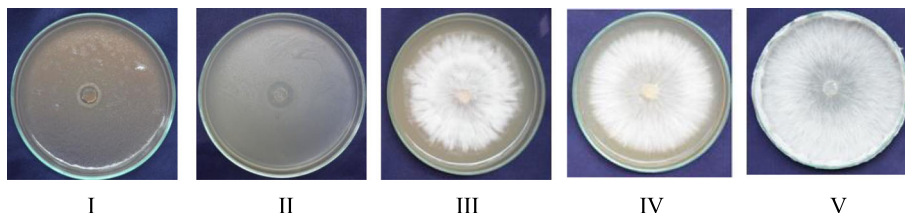


Fig. 7 Culture filtrate test – (I) (EGN 1) and (II) (EGN 4), Production of thermostable compounds - (III) (EGN 1) and (IV) (EGN 4), (V)- Control

method and the strongest activity at a crude extract concentration of 7.81 g ml⁻¹ was exhibited by BF11 strain.

Growth promotion by roll towel method

Results of growth promotion studies showed that EGN 1 increased the vigour index of groundnut seedlings up to 4286.7, followed by TNAU-Pf 1 (4040.0), EPC 5 (2599.74) and EGN 4 (2895.35) through roll towel method. The isolate EGN 1 also recorded high germination (86.67%) with increase root length (25.0 cm) and shoot length (24.5 cm). In the rhizoscanner study, total root length, root tips, segment and forks were higher in EGN 1 (3370 mm, 425 root tips, 501 forks) and EGN4 (2786 mm, 382 root tips, 501 forks) than the control (1129 mm, 184 root tips, 463 forks) (Table 4 and Fig. 4). Similarly, Sundaramoorthy et al. (2012) reported that Pf1 + EPCO16 considerably increased the vigour index of tomato seedlings through roll towel method up to 3840.70, followed by Pf1 (3143.4), Py 15 (2947.05), FP 7 (2989.30), EPC 5 (2814.3) and EPCO 16 (3225.7), respectively, when compared to control.

Filter paper disc assay

In this assay, EGN 4 showed the maximum inhibition (94.10), followed by EGN 1 (68.23%), TNAU-Pf 1 (57.60%), EGN 7 (52.94%), EPC 5 (40.20%) and EGN 2 (11.47%). The isolates, namely EGN 3, EGN 5 and EGN 6 did not show any antagonistic activity against *S. rolf sii*

(Table 5). Antagonistic activity of effective seed bacterial endophytes (EGN 1 and EGN 4) against *S. rolf sii* through (I) dual plate assay, (II) triangle method of streaking and (III) filter paper disc assay is shown in Fig. 5. Similarly, Alsultan et al. (2019) reported that *P. aeruginosa* and *C. proteolyticum* were able to produce cellulase, lipase, protease and pectinase at varying levels and these enzymes participate in the antagonistic activity against *Phytophthora palmivora* causing black pod of cocoa.

Phytostimulation activities

The isolates namely EGN 1, EGN 3 and TNAU-Pf1 were high siderophore producers, whereas EGN 4, EGN 6, EGN 7 and EPC 5 were moderate producers and EGN 2 and EGN 5 were low producers. Four seed endophytic isolates EGN 1, EGN 3, EGN 4 and EPC 5 produced IAA (the hormone responsible of root growth), which was indicated by the production of pink to reddish pink colour in the broth after addition of Salkowski reagent. In case of phosphate solubilization, it was indicated by the formation of a clear halo zone. The isolates EGN 1 and TNAU-Pf 1 had the highest ability to solubilize the phosphate followed by EGN 2, EGN 4, EGN 6 and EGN 7. For the production of hydrolytic enzymes, cellulase and protease test was undertaken for the effective isolates. The results showed that 7 isolates had the ability to produce protease enzyme and 9 showed cellulase production, which indicated the

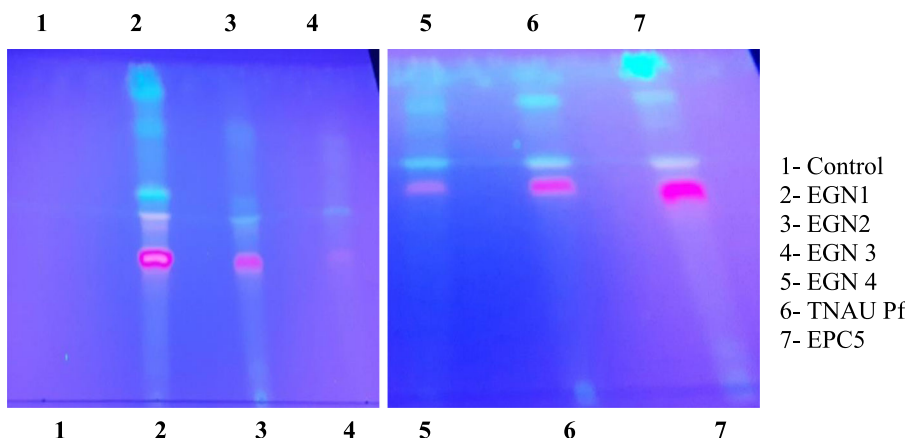


Fig. 8 Thin-layer chromatography (TLC) analysis

hydrolytic enzyme production by endophytes (Table 6 and Fig. 6). Allu et al. (2014) also reported the siderophore production by the bacterial endophytes in chilli, while Wang et al. (2013) reported the growth promoting ability of endophytic bacteria, i.e. 48 isolates were able to produce IAA and 3 isolates showed phosphate solubilization in peanut, nitrogen-fixing endophytic bacteria and their association in sugarcane (Cavalcante et al., 2007).

Culture filtrate assay

The culture filtrate assays showed that the isolates EGN 1, EGN 2, EGN 3, EGN 4, EGN 5, EGN 6, EGN 7, TNAU Pf1 and EPC 5 had a maximum inhibition rate of 100% through amendment of cell-free supernatants to agar plates. They exhibited the effectiveness of culture filtrate on stem rot pathogen (*S. rolfsii*) (Fig. 7).

Thermostable compounds and secondary metabolite production

The effective isolates EGN 1, EGN 4 and EPC 5 were analysed for their presence of thermo stable compounds after autoclaving at 121 °C for 30 min. The mycelial growth was measured in 2 perpendicular directions. It showed that the isolate EGN 1 and EGN 4 had the presence of thermo stable compounds (Fig. 7). Similarly, Kupper et al. (2020) reported that 8 isolates (ACB-AP3, ACB-83, ACB-76, ACB-70, ACB-84, ACB-82, ACB-72 and ACB-69) of

Bacillus spp. produced thermo-resistant metabolites produced inhibitions ranging from 25 to 42%. The secondary metabolite production of effective isolates was assessed through agar well diffusion technique. The isolate EGN 1 showed 97.7% inhibition, followed by EPC 5 (94.4%), EGN 4 (87.7%), TNAU Pf1 (84.4%), EGN 2 (82.2%) and EGN 3 (76.6%). Similarly, Prabhukarthikeyan and Raguchander (2016) tested the antimicrobial activity of *Pseudomonas fluorescens* against *Pythium* and reported that crude antibiotics of *P. fluorescens* FP 7 inhibited the mycelial growth of 40 mm accounted for 55.55% followed by *P. fluorescens* Pf1 (52.22% inhibition).

TLC and GC-MS analysis

Thin-layer chromatography (TLC) was carried out for effective isolates and it showed different bands (Fig. 8). The R_f values were as follows: EGN 1 (0.33, 0.44, 0.52 and 0.72), EGN 3 (0.29, 0.53), EGN 4 (0.46, 0.53), TNAU-Pf 1 (0.46, 0.53) and EPC 5 (0.46, 0.53 and 0.73). The compounds which were identified through GC-MS analysis in EGN 1 were octadecanal, pyrrol, hexadecanoic acid, oleic acid, cis-vaccenic acid, harmine and pyrrolo compounds and EPC 5 had Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), pentadecanoic acid, hexadecanoic acid, harmine and pyrrol (Fig. 9). Alike, Pratiwi et al. (2017) determined metabolite compound of *P. aeruginosa* through TLC analysis, the 11th

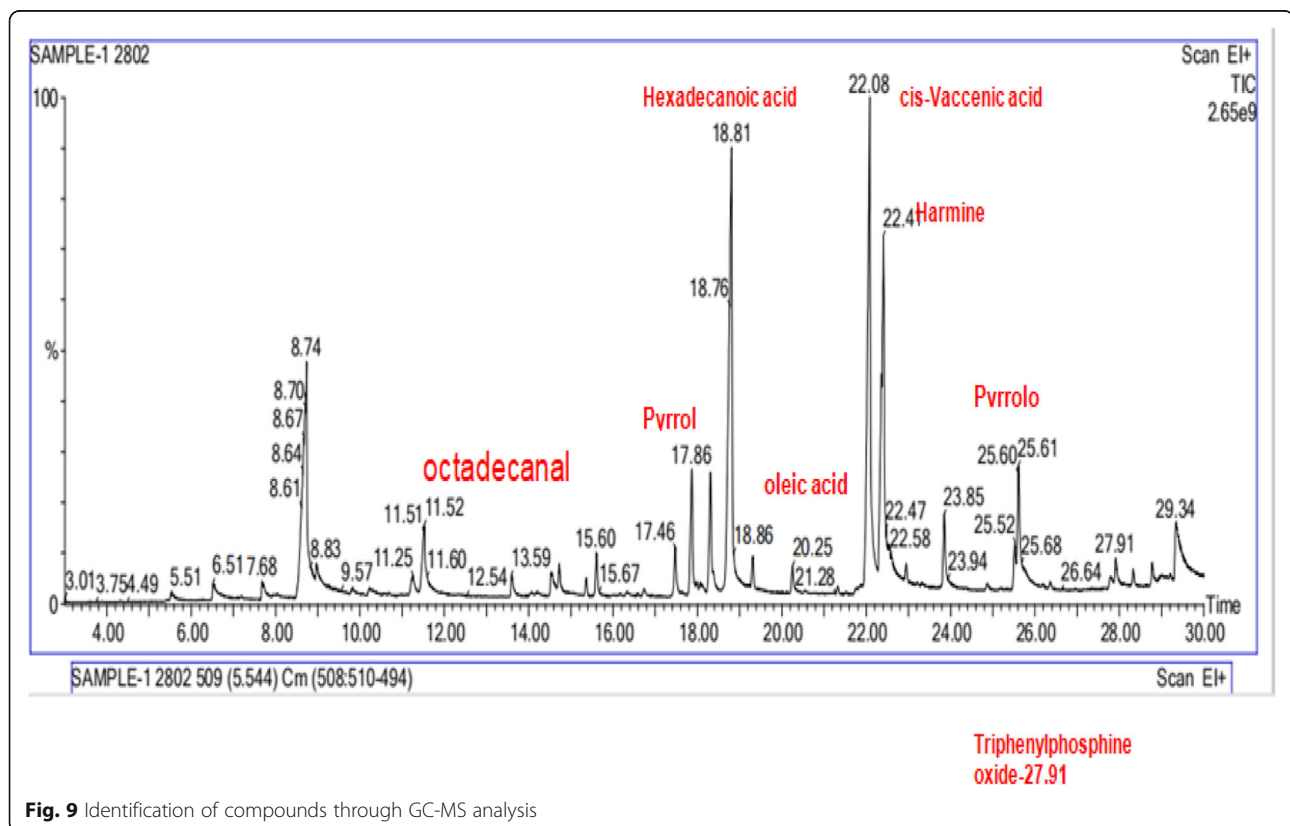


Fig. 9 Identification of compounds through GC-MS analysis

fraction showed antibacterial activity with the R_f value of 0.53.

Conclusion

From the culture-dependent analysis, the effective isolate *Pseudomonas* spp. EGN 1 proved to be the most promising bio agent for the management of the stem rot (*Sclerotium rolfsii*) in groundnut.

Abbreviations

PDI: Percent disease incidence; PDA: Potato dextrose agar; NA: Nutrient agar; VI: Vigour index; CTAB: Cetyltrimethylammoniumbromide; NCBI: National Center for Biotechnology Information; EGN: Endophytes groundnut

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Authors' contributions

LR, SKM and GK designed the experiment. TA, LR and SKVP conducted the experiment and wrote the article and performed the statistical analysis. LR and GK revised the article and MP did the growth promotion, rhizoscanner study and its interpretation. All authors approved the final article after reading.

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Competing interests

The authors declare that they have no competing interests.

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