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RESEARCH ARTICLE

Stem rot (*Sclerotium rolfsii*) suppression and yield enhancement potential of native PGPR of peanut (*Arachis hypogaea*)

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Abstract

Stem rot of peanut, caused by the fungus *S. rolfsii*, is a significant disease occurring worldwide and leads to considerable yield losses. Due to the adverse effects of chemical fungicides, biological control methods have gained importance in recent years. The efficacy of biocontrol can be enhanced by applying indigenous strains that effectively suppress and compete with in situ pathogenic microorganisms. In this study, native PGPR isolates from the genera *Bacillus* and *Pseudomonas* were isolated and tested for their antagonistic activity against *S. rolfsii*, growth-promoting ability and yield enhancement in peanuts under both *in vitro* and *in vivo* conditions. The potential PGPR isolates were subjected for partial sequencing of the 16S rRNA gene and identified as *B. subtilis* PB(20d), *B. amyloliquefaciens* PB(40d), *B. subtilis* PB(50d), *Paenibacillus campinasensis* PB(60d) and *Pseudomonas aeruginosa* PP(30d). The phylogenetic tree organized these strains into their clades alongside reference strains from the NCBI database. Results from pot culture and field experiments revealed that the treatment with the combination of identified PGPR strains exhibited a lower incidence of stem rot and a higher yield of peanuts compared to individual applications and control treatment. Therefore, the combined application of native PGPR strains has proven to be more effective in addressing the variability in the performance of individual biocontrol agents and improving overall efficacy.

Keywords

Bacillus; biocontrol; management; native PGPR; peanut stem rot; *Pseudomonas*

Introduction

The peanut (*Arachis hypogaea* L.) is a herbaceous annual mainly grown for its nutritious and tasty edible seeds. It's most commonly called groundnut as the pods are developed underground level and to a much lesser extent as monkey nut, earthnut and goobers. It's one of the World's most popular and universal crops and is being cultivated in significant parts of the world. India is the World's second-largest producer of peanuts, with an annual production of about 7.1 MT (1). As a legume crop, it enriches the soil by adding nitrogen. The seeds are a significant source of edible oil and protein meals considered highly valuable in human and animal nutrition. Currently, its role in a heart-healthy diet is to address the emerging foremost heart diseases (2). The production of peanuts is decreasing each year due to soil-borne diseases. In particular, stem rot (white mould or southern blight) caused by *Sclerotium rolfsii* (teleomorph: *Athelia rolfsii)* is a significant disease occurring worldwide, leading to considerable yield losses ranging from 25 % to 80 % and posing a serious threat to peanut production (3, 4). The infected plants exhibit wilting and profuse white mycelial growth with

mustard-like sclerotia on the soil surface near the crown. As the fungus *S. rolfsii* infects more than 500 plant species and produces hardy sclerotia that can remain viable in the soil for many years, it is challenging to eradicate the disease (5). Chemical fungicides are commonly used to control soil-borne diseases of groundnuts. However, their harmful environmental and human health effects dragged us to an alternate approach. Recently, biological control of crop diseases has gained importance and has become popular among farmers as a powerful alternative to synthetic pesticides. It is a potential biological and eco-friendly means of plant disease management (6).

In the biocontrol approach, plant growth-promoting rhizobacteria (PGPR) primarily serve as potential biocontrol agents for controlling crop diseases. PGPR is a group of bacteria from the genera Pseudomonas, *Bacillus, Azospirillum, Azotobacter, Burkholderia, Klebsiella* and *Serratia* (7). Among these, *Pseudomonas* and *Bacillus* spp. play a vital role in reducing pathogen inoculum, promoting crop growth and inducing resistance against diseases through various mechanisms of action (8, 9). The biocontrol traits and biofertilizer properties of *Bacillus subtilis, Bacillus amyloliquefaciens* and *Paenibacillus* species have been reported by several researchers (10, 11, 12, 13).

Regarding *Pseudomonas* spp., this genus encompasses hundreds of species (14), including nonpathogenic strains of *P. aeruginosa, P. chlororaphis, P. putida, P. syringae* and *P. fluorescens,* which are recognized as potential biocontrol agents for managing various plant diseases. The gram-negative, rod-shaped *P. aeruginosa* is a flourishing plant symbiont that adapts to a broader range of agroecosystems and exhibits potential antagonistic roles in protecting crops from devastating diseases (15, 16, 17). The production of several bioactive compounds and secondary metabolites with potential antifungal properties enables *P. aeruginosa* to display broad-spectrum antagonistic activity (18, 19, 20). Rapid colonization and optimal population level are prerequisites for PGPR to enhance biocontrol efficacy. This can be achieved by isolating and reintroducing native or indigenous biocontrol agents that effectively suppress and compete with in situ pathogenic microorganisms. Other researchers also emphasized this, noting that strains isolated from the specific locales where they will be applied show enhanced adaptability to local soil conditions, climate and plant species and improved effectiveness in promoting plant growth, pathogen suppression and nutrient uptake (21).

The use of individual biocontrol agent may lead to inconsistent performance in controlling crop diseases. Combining more than one biocontrol agent in a single application might overcome the inconsistency and capable of more extensive colonization of the rhizosphere, more consistent expression of beneficial traits under a wider range of soil conditions and of being antagonistic to a larger number of plant pathogens than they applied individually. Hence, the present study aimed to isolate *Bacillus* and *Pseudomonas* spp. from the peanut rhizosphere and develop consortia of promising isolates for controlling stem rot caused by *S. rolfsii* under both pot culture and field conditions.

Materials and Methods

Isolation, identification, mass production and proving pathogenicity of pathogen

Peanut plants exhibiting typical stem rot symptoms, such as wilting and mustard-like sclerotia over fan-like mycelial mats at the collar region, were identified (Fig. 1), collected and transported to the laboratory. The fungal pathogen, *Sclerotium rolfsii*, was isolated on potato dextrose agar (PDA) using the tissue segment method and purified on fresh PDA (22). Observed visually and under a compound microscope for phenotypic characters. The mycelial lawns were randomly torn with a sharp, sterile knife to induce sclerotial production as a form of stress. Mature sclerotia from each plate were then harvested and stored for further use.

Pots filled with autoclave-sterilized potting mixture (red soil: sand: garden soil) were inoculated in the upper 5 cm of soil with 100 sclerotia mixed with 100 grams of soil (23). Surface-sterilized groundnut seeds of the variety VRI 2 were sown and observed for the reproducibility of stem rot symptoms. The pathogen was re-isolated and examined for the phenotypic characteristics of the original isolate.

Isolation and identification of PGPR isolates from peanut rhizosphere soils

Soil samples collected from the peanut rhizosphere at 20, 30, 40, 50, 60, 70 and 80 days after sowing (DAS) were subjected to *Bacillus* spp. and *Pseudomonas* spp. isolation using the dilution plating method (24). The soil suspensions were diluted to a concentration of 10^{-6} and spread onto nutrient agar and King's B agar plates. The plates were incubated at 37°C for five days. Two representative dominant colonies from each plate were picked and streaked onto fresh agar plates. They were named based on the time of isolation, with designations 'PB' for *Peanut Bacillus* or 'PP' for *Peanut Pseudomonas*, followed by individual isolate numbers.

Fourteen *Bacillus* isolates obtained from peanut rhizosphere were initially identified based on their colony morphology and Gram reaction. Further tested for biochemical activities using a commercial kit (Hi Bacillus TM identification kit (KB013) (Himedia Laboratories Pvt. Ltd, Mumbai), which employs a standardized colourimetric test system. A total of 12 tests were carried out and the results were interpreted based

Fig. 1. Stem rot (*S. rolfsii*) infected peanut plants under field conditions.

on (+) or (-) reactions of reference *Bacillus* species that had already been identified. Ten *Pseudomonas* sp. isolates obtained from peanut rhizosphere were readily identified based on their phenotypic characteristics. They were further evaluated for their reactions to KOH, levan formation and siderophore production. The isolates were cultured for 48 hours. A loopful of each culture was mixed with a drop of 3 % KOH solution on a clear glass slide and observed for the changes confirming Gram-negative bacteria. They were also assessed for levan formation by streaking onto agar supplemented with 5 % sucrose and observing the growth over 3 to 5 days. Additionally, the isolates were streaked onto Chromeazurol S agar plates, incubated at 28 °C and observed for siderophore production (25).

Screening of PGPR isolates against S. rolfsii under in vitro

Bacillus and *Pseudomonas* isolates obtained from peanut rhizosphere were tested for their antagonistic activity against *S. rolfsii* using a dual culture technique *in vitro*. Mycelial discs from an actively growing colony of *S. rolfsii* were placed individually at the centre of each Petri dish containing PDA. After 12 hours of incubation, sterile Whatman No. 40 filter paper discs measuring 5 mm diameter were positioned at four equidistant points around the fungal disc. Cell suspensions (10^{-8} cfu mL^{-1}) obtained from 48-hour-old broth cultures were applied to the filter paper discs at 25 µl in each Petri dish separately for each isolate. Filter paper discs treated with sterile water served as control (26). Four replications were maintained for each isolate. Observations were taken when the pathogen achieved complete growth in the control plate. Percent inhibition over control was calculated using the formula ((C - T) / C) × 100, where C is the mycelial growth of *S. rolfsii* in the control plate and T is the mycelial growth of *S. rolfsii* in the dual culture plate."

Assessing the growth-promoting activity of PGPR isolates on peanut seedlings

Bacillus and *Pseudomonas* isolates obtained from peanut rhizosphere were tested for their growth-promoting ability on peanut seedlings *in vitro.* Each isolate was grown in 250 mL conical flask containing 100 mL of nutrient broth for 48 h on a rotary shaker (150 rev min^{-1}) at 27 ± 2 ºC. Centrifuged at 8000 rpm for 10 min at 4ºC. The resulting pellets were suspended separately in sterile distilled water to obtain the bacterial density of 10^{8} cfu mL^{-1} measured by dilution plating technique. Ten mL of each bacterial suspension was added with 100 mg of carboxymethyl cellulose (CMC). Peanut seeds surface sterilized with 2 % sodium hypochlorite were soaked in each bacterial suspension for two hours and then air-dried under aseptic conditions. Twenty pretreated seeds were sown on pre-soaked germination paper, with four replications maintained. Observations on germination percentage, shoot length and root length were measured after 10 days and the seedling vigour index (VI) was calculated.

Molecular characterization of effective PGPR isolates

Genomic DNA extraction, polymerase chain reaction (PCR) and sequencing : Promising *Bacillus* and *Pseudomonas* isolates were characterized at molecular level. Total DNA of each isolate was extracted by following modified CTAB method (27). Agarose gel electrophoresis was performed to assess the quality of the extracted DNA (28). The genomic DNA of each isolate was subjected to PCR of 16S rRNA (from 27 to 1492) by using primer pairs 27F, 5P-GCGGGATCC / GAGTTTGATCCTGGCTCAG-3P and 1492R, 5P-GCCGTCGAC / GGTTACCT TGTTACGACTT-3P. PCR for each DNA sample was performed in a total volume of 30 µl, containing 50 ng of genomic DNA, 10x Taq buffer, 2.5 mM of each deoxyribonucleotide triphosphate (dNTP) mixture, 2.5 mM of $MgCl_2$, 30 picomoles of each primer and 2 U of Taq DNA polymerase. The following conditions were adopted for PCR with 30 cycles in a thermocycler (Eppendorf Mastercycler, Germany): denaturation at 94ºC for 1 min, annealing at 49ºC for 1 min and extension at 72ºC for 2 min with the final extension at 72ºC for 10 min. The amplified PCR products of ≈ 1.5 kb size from 16S rRNA of each PGPR isolate were sequenced per the manufacturer protocol (Chromgene Biotech Private Limited, Bangalore, Karnataka). The identity of 16S rRNA gene sequences was performed by similarity search using BLAST tool (http:/ blast.ncbi.nlm.nih.gov./BLAST.cgi). The phylogenetic tree was constructed with existing 16S rRNA gene sequences obtained from the NCBI GenBank database by the neighbour-joining method (29) using MEGA 6.0 software (30). The gene sequences have been submitted to the GenBank database and assigned with accession numbers.

Preparation of talc-based formulations of PGPR strains : Talc -based formulations were prepared for *Bacillus* and *Pseudomonas* strains, which were confirmed molecularly. The identified strains include *B. subtilis* strain PB(20d), *B. amyloliquefaciens* strain PB(40d), *B. subtilis strain PB(50d)*, *P. campinasensis* strain PB(60d) and *Pseudomonas aeruginosa* strain PP(30d). A loopful of each bacterial culture was inoculated into the nutrient broth and shaken in a rotary shaker at 150 rpm for 72 h at room temperature (26 ± 2°C). After incubation, 400 mL of each broth culture containing 9 × 10^{-8} cfu mL^{-1}, one kilogram of talc powder, 15 g of calcium carbonate (to adjust pH to neutral) and 5 g of carboxymethyl cellulose (as an adhesive) were mixed under sterile conditions (31). Each bacterial mix was shade-dried for 12 h and then packed in polythene bags. Talc-based bioformulations of each PGPR strain were kept separately at normal room temperature and mixed equally at the time of application.

Evaluation of PGPR strains against stem rot under pot culture conditions (2016-17 and 2017-18) : Pot culture experiments were conducted during December - March of 2016 -17 and 2017-18 to evaluate the efficiency of individual and consortia of PGPR strains against peanut stem rot under glasshouse conditions.

This experiment includes eight treatments with three replications as detailed below.

$T_{1.}$ *B. subtilis* PB(20d)

$T_{2.}$ *P. aeruginosa* PP(30d)

$T_{3.}$ *B. amyloliquefaciens* PB (40d)

$T_{4.}$ *B. subtilis* PB (50d)

T_{5} *P. campinasensis* PB(60d)

$T_{6.}$ *B. subtilis* PB(20d) + *P. aeruginosa* PP(30d) + *B. amyloliquefaciens* PB(40d) + *B. subtilis* PB(50d) + *P. campinasensis* PB(60d)

$T_{7.}$ Difenoconazole (0.05 %)

$T_{8.}$ Control

Twenty-five cm dia pots were filled with autoclaved pot mixture. (Red earth: sand: garden soil). One hundred sclerotial bodies of *S. rolfsii* were mixed with 100g of soil and inoculated on the upper 5 cm of soil. Peanut seeds of variety VRI 2 treated with talc-based bio-formulation of either a single PGPR strain (10g kg^{-1}) or a consortium of PGPR strains (10g kg^{-1}) were sown at 10 seeds per pot. Watering was done once in three days. Germination Percentage was calculated at 10 DAS and the seedling vigour index was calculated at 30 DAS for all the treatments. Soil application of a talc-based bioformulation, either of a single PGPR strain or a consortium of PGPR strains, was performed at 5 g per pot on 30 and 50 DAS. The chemical difenoconazole (0.05 %) was applied at the time of the disease appearance. Symptoms of stem rot were observed regularly and the mean disease incidence was calculated for each treatment. Pod yield per plant was recorded.

Evaluation of PGPR strains against stem rot under field conditions (2016-17 and 2017-18)

A field experiment was conducted at Tamil Nadu Rice Research Institute, Aduthurai, during December -March of 2016 - 17 and 2017 -18 to evaluate the efficiency of individual and consortium of talc-based formulations of PGPR strains against peanut stem rot disease. The trial was laid out with eight treatments and replicated thrice in a randomized block design. Seeds of the peanut variety VRI 2, treated with talc-based formulations of a single PGPR (10g kg^{-1}) and a consortium of PGPR strains (10g kg^{-1}), were sown with a spacing of 30 x 10 cm in the plots of 4 × 3 m size. Soil applications of talc-based formulations of single PGPR strain and consortia of PGPR strains were done at 2.5 kg ha^{-1} on 30 and 50 DAS. The chemical difenoconazole (0.05 %) was applied at the time of the disease appearance. Germination percentage and seedling vigour index were calculated at 10 DAS and 30 DAS, respectively. Stem rot incidence was recorded regularly until harvest. Pod yield per ha was recorded. The mean maximum and minimum temperatures observed throughout the study were 31.4^{O}C and 21.2^{O}C, respectively. The total rainfall observed was 247.3 mm. The soil type in the experimental plot was sandy loam.

Statistical analyses

The present experimental data were analyzed using analysis of variance (ANOVA) by Agres Statistical Software Package Version 3.01 (32) and Duncans' multiple range test at the 5 % significance level.

Results and Discussion

Isolation and characterization of the pathogen (S. rolfsii)

The fungus, *S. rolfsii* isolated from the stem rot-infected peanut tissue exhibited a white, cottony mycelium and its colony grew quickly, eventually covering the entire dish. After ten days, the mature mycelium produced mustard-like sclerotia that were initially white then turned yellow. Finally, chestnut brown at maturity (Fig. 2). Under the microscope, septate hyphae and some cells with clamp connections were observed. Based on its morphology, the pathogen was confirmed as *S. rolfsii.* The stem rot symptoms were reproduced in the inoculated peanut plants, the pathogen was re-isolated and the pathogenicity was proved.

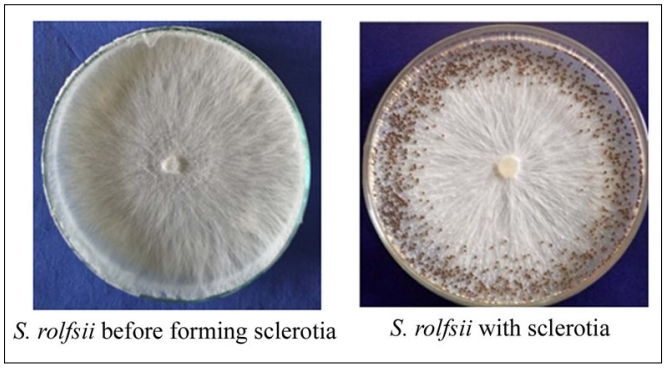

Fig. 2. The fungal pathogen, *S. rolfsii* isolated from stem rot infected peanut plants.

Isolation and characterization of biocontrol agents (PGPR isolates)

In the present study, 14 *Bacillus* sp. isolates were isolated and identified based on their colony morphology, including dry and flat with irregular margins. They showed favourable to Gram reaction. The results from a series of 12 biochemical tests in a commercial kit confirmed that all tested isolates were close to *Bacillus* spp. 10 *Pseudomonas* sp. isolates were identified based on their characteristic yellow-green fluorescence. However, the isolate 30dPP1 exhibited dark green fluorescence. All isolates reacted positively to KOH, forming thin strands of chromosomal threads, confirming their Gram-negative status. On 5 % sucrose-amended nutrient agar, the isolates formed large, convex, white, domed and mucoid colonies called levans. Additionally, they developed a yellow-orange halo around their colonies on Chromeazurol S agar, indicating siderophore production.

Screening PGPR isolates against S. rolfsii under in vitro

Bacillus and *Pseudomonas* isolates were screened for their biocontrol characteristics. Out of 14 *Bacillus* sp. isolates tested against *S. rolfsii* growth using the dual culture technique *in vitro*, maximum inhibition was observed in isolates 60d PB4, 40d PB1, 20d PB2 and 50d PB3 with percentage inhibition values of 74.0 %, 65.1 %, 61.6 % and 55.1 % respectively (Table 1 & Fig. 3). *Bacillus* species are well-known for producing a

Table 1. Evaluation of *Bacillus* sp. isolates for inhibition of *S. rolfsii* in dual culture assays and enhancement of peanut vigour index

S. No	*Bacillus* isolates	Growth of *S. rolfsii* (dia in cm)	Per cent mycelial inhibition over control	Seedling vigour index on peanut	Per cent vigour increase over control
1	20d PB1	2.41	46.4 (42.94)	2032	55.35
2	20d PB2	1.73	61.6 (51.71)	2815	115.21
3	30d PB1	2.63	41.6 (40.17)	2530	93.43
4	30d PB2	3.20	28.9 (32.52)	1964	50.15
5	40d PB1	1.57	65.1 (53.8)	3200	144.65
6	40d PB2	2.46	45.3 (42.31)	2271	73.62
7	50d PB2	2.60	42.2 (40.51)	2297	75.61
8	50d PB3	2.02	55.1 (47.93)	2920	123.24
9	60d PB3	2.63	41.6 (40.17)	2530	93.43
10	60d PB4	1.17	74.0 (59.35)	3383	158.64
11	70d PB2	2.41	46.4 (42.94)	2677	104.66
12	70d PP2	2.46	45.3 (42.31)	2486	90.06
13	80d PB1	2.76	38.7 (38.47)	2560	95.72
14	80dPB3	3.20	28.9 (32.52)	2450	87.31
15	Control	4.50	-	1308	-
	CD (P<0.05)	0.36	-	203.1	-

Values are mean of four replications. Values in parenthesis are arcsine transformed

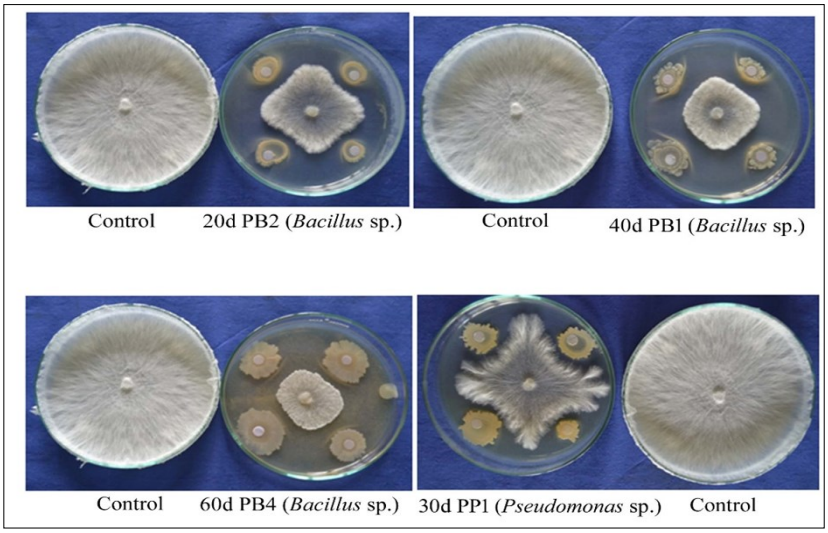

Fig. 3. Antagonistic potential of PGPR isolates against *S. rolfsii.*

variety of antimicrobial compounds, including surfactins, fengycins, bacillomycin-D, bacillaene, macrolactin, difficidin, bacillibactin and bacilysin, which are detrimental to the growth of various plant pathogens (33). They secrete hydrolytic enzymes, such as chitinase, β-glucanase, protease, cellulase and lipase to hydrolyze specific cell wall components *viz*., chitin, glucan, protein, cellulose and lipids respectively that lead to the disintegration of cell wall matrices, loss of protective and functional properties (34). Transmission electron micrographs study clearly distinguished the infected mycelial cells with normal cells of *S. rolfsii*, isolated from peanut when co-cultured with *Bacillus*. It demonstrated that the pyknotic, integrated mitochondria, cleaved nuclear membranes and separated nucleoli on infected mycelial cells as against normal cells that appeared full of intact mitochondria, cell and nuclear membranes and gathered nucleoli (35). Similarly, the cytoplasmic disintegration, leakage, ruptured, swollen and shrivelled hyphae of *Gaeumannomyces tritici* in the presence of *B. subtilis* was already demonstrated (36). Regarding the *Pseudomonas* spp., of the 10 isolates tested against *S. rolfsii*, the isolate 30d PP1 showed maximum inhibition (39.0 %) under *in vitro* (Fig. 3 -4). This isolate was later identified as *P. aeruginosa* in the same study. Antifungal activities of *P. aeruginosa* against a wide range of phytopathogens have been documented (37). The antifungal compounds, such as 2,4-diacetyl phloroglucinol (2,4-DAPG), pyoluteorin and pyrrolnitrin and various other bioactive compounds as well as siderophores, are produced by *P. aeruginosa* (18). The highest growth reduction abilities of *P. aeruginosa* against fungal pathogens such as *Fusarium oxysporum, Botrytis cinerea, Botryosphaeria dothidea* and *F. fujikuroi* were already reported (18). The above-mentioned antimicrobial actions of PGPR isolates might be the reason why they inhibited the mycelial growth of *S. rolfsii* in the present study.

Effect of PGPR isolates on growth of peanut seedlings

The results from the roll towel method of growth promotion study indicated maximum peanut vigor indices of 3383, 3200, 2920 and 2815 for the isolates 60d PB4, 40d PB1, 50d PB3 and 20d PB2, respectively, compared to the control, which exhibited a minimum vigor index of 1307. This demonstrated an increased vigour index of 115 % to 158 % for the best-performing bacterial isolates compared to the control (Table 1). *Bacillus* spp. can directly improve plant growth by producing phytohormones, include indole acetic acid (IAA), cytokinins (CKs), gibberellins (GAs) and abscisic acid (ABA) (38). Significant increases in the growth of peanut plants were observed with *B. subtilis* and *B. amyloliquefaciens* (10,11,39). Additionally, *Paenibacillus* species have the potential to improve plant growth and involved in the production of organic acids, ACC deaminase, indole-3-acetic acid (IAA), siderophores, nitrogen fixation and phosphate solubilization (12). Among tested *Pseudomonas* isolates, 30d PP1 treated peanuts showed a maximum vigor index of 3758 which was 183.4 % increase when compared to control (Fig. 5).The plant growth-promoting attributes, such as IAA production, siderophore production, nitrogen fixation and phosphate solubilization have been well documented for *P. aeruginosa* (40).

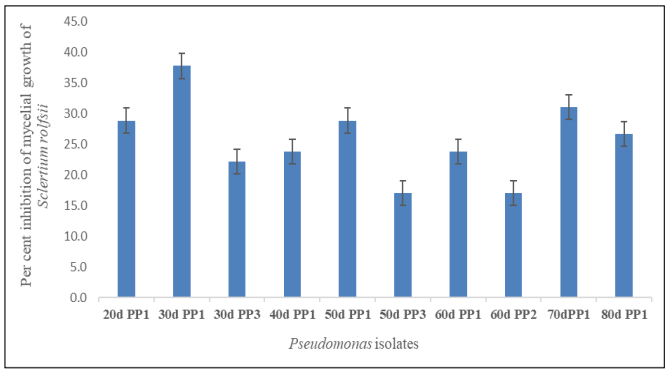

Fig. 4. Evaluation of *Pseudomonas* sp. isolates for inhibition of *S. rolfsii* in dual culture assays.

Genomic characterization of promising PGPR isolates

PGPR isolates demonstrated the best performance in inhibiting the growth of the pathogen *S. rolfsii* and enhancing the vigour of peanut seedlings subjected to molecular identification. The total genomic DNAs isolated from *Bacillus* spp. isolates, such as 20d PB2, 40d PB1, 50d PB3 and 60d PB4 and *Pseudomonas* isolate, 30d PP1 were found to be intact and of good quality. Amplified genomic products of approximately 1.5 kb from 16S rRNA of each isolate were sequenced. Homology searches using BLAST at the National Centre for Biotechnology Information (NCBI), USA, revealed the nucleotide sequence identities of *Bacillus* strains ranging from

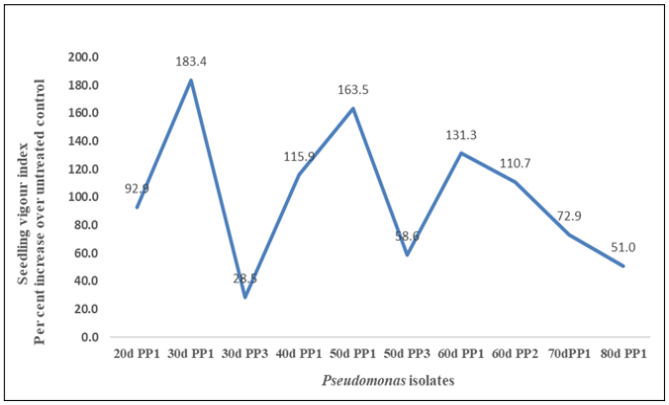

Fig. 5. Growth-promoting ability of *Pseudomonas* sp. isolates on peanut seedlings under *in vitro* (roll towel method).

96 % to 99 % with existing *Bacillus* sequences and of *Pseudomonas* strain 99 % with existing *Pseudomonas* sequences. The partial sequences were submitted to NCBI GenBank and assigned with the accession numbers. The *Bacillus* isolates 20d PB2, 40d PB1, 50d PB3, 60d PB4 were identified as *B. subtilis* strain PB(20d) (MH793439.1), *B. amyloliquefaciens* strain PB(40d) (MH793440.1), *Bacillus subtilis* strain PB(50d) (MH793442.1) and *Paenibacillus campinasensis* strain PB(60d) (MH793446.1) respectively. The *Pseudomonas* isolate, 30d PP1 was identified as *Pseudonomonas aeruginosa* strain PP(30d) (MH793445.1). The phylogenetic relationship was established between PGPR strains based on the maximum livelihood of evolutionary distances of the partial 16S rRNA. The phylogenetic tree organized these strains into clades alongside reference strains from the NCBI database (Fig. 6).

Evaluation of PGPR strains against stem rot under pot culture and field conditions

As an introduced biocontrol agent could affect the indigenous microbes in the soil, native PGPR under the genus *Bacillus* and *Pseudomonas* from peanut rhizosphere were used in this study (41). *Bacillus* species are known for their ability to secrete metabolites that prevent pathogen infection, stimulate plant growth and form endospores to survive adverse environmental conditions, making them long-lived (42). *Pseudomonas* species are effective biological control candidates against devastating fungal and bacterial pathogens that cause plant root and foliar diseases (9).

Talc-based formulations of PGPR strains were tested both individually and in combination for their effectiveness in reducing stem rot disease and promoting growth in groundnuts grown in potted soil inoculated with *S. rolfsii* and in natural field conditions. Results from the pot culture experiment conducted during 2016-2017 revealed that the chemical treatment T_7 significantly reduced the occurrence of stem rot to 11.11 %. Additionally, the treatment with the combination of PGPR, T_6 exhibited a minimum stem rot incidence of 27.8 %, which was significantly lower than their individual applications or the untreated control (T_8) (77.8 %) (Table 2). The same trend was observed in the second pot culture experiment conducted during 2017-18. Next to the T_7 treatment, a minimum stem rot incidence of 22.2 % was observed in the T_6 treatment compared to other treatments and T_8 (72.2 %) (Table 2). The best-performing T_6 treatment significantly reduced the stem rot by 64 % to 69 % compared to the T_8 treatment in the pot culture experiments conducted

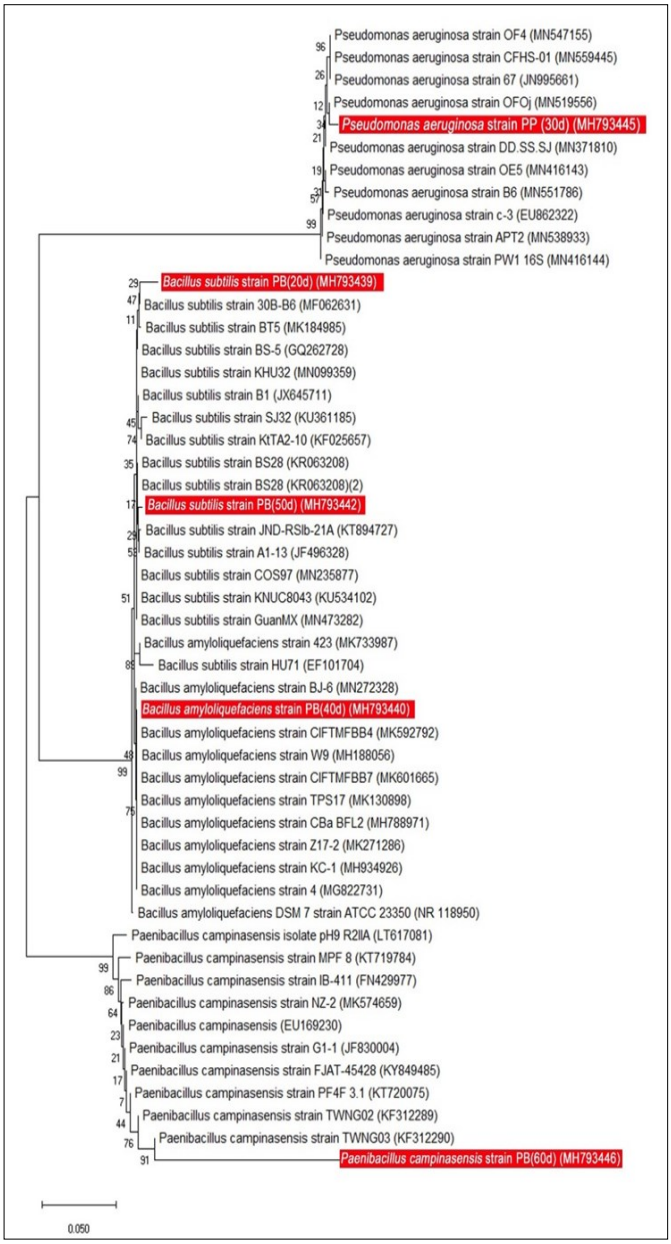

Fig. 6. Neighbour-joining tree of 16S rRNA gene isolated from *Bacillus* and *Pseudomonas* strains. The related strains, strain name and gene accession number are indicated in the tree. Bootstrap values of more than 500 (from 1000 replicates) are shown at the nodes as %.

in two separate trials. The T_7 treatment demonstrated superior effectiveness in controlling stem rot disease (Table 2).

Results from the field experiment conducted during 2016-17 showed that next to the T_7 treatment, less stem rot incidence of 10.7 % was observed in the T_6 treatment than in individual treatments and T_8 (PDI: 33.0 %). This demonstrated a 67.7 % reduction in stem rot in the T_6 treatment compared to T_8 (Table 3). A similar trend was recorded in the field experiment conducted during 2017-18. Results revealed that the treatment receiving chemical (T_7) recorded the least stem rot incidence of 5.97 % followed by a significant reduction was observed in the T_6 treatment (18.7 %) compared to other treatments and T_8 (48.1 %). Hence, the T_6 treatment resulted in a 61.1 % reduction in stem rot compared to T_8 (Table 3).

The production of cyclic lipopeptides is a key antifungal mechanism exhibited by *B. subtilis* (43). They are versatile weapons for the control of various plant diseases. They are non -ribosomally synthesized molecules classified into three major

families: surfactins, iturins and fengicyns, all of which could potentially be involved in biocontrol (44). Meanwhile, *Paenibacillus* species demonstrate biocontrol abilities by stimulating plant resistance through the induction of autophagy. This process helps eliminate invading pathogens and leads to enhanced expression of autophagy-related genes in plants, particularly in their defence against necrotrophic fungi such as *S. rolfsii* (45). They are generally considered an excellent biofilm-forming biocontrol agent, protecting against plant pathogens (46). These *Bacillus* species may compete with *S. rolfsii* for nutrients and space by occupying root sites due to rapid multiplication. They might produce hydrolytic enzymes, secondary metabolites, antibiotics, volatile compounds, or trigger-induced systemic resistance (ISR) (47) to limit *S. rolfsii* growth and significantly reduce stem rot disease in peanuts.

Regarding pod yield, the T_6 treatment recorded an increased pod weight of 20.05 g per plant, compared to other treatments and T_8 (9.91g plant^{-1}) under pot culture conditions conducted during 2016-17. In addition to that, T_6 treatment improved the seedling vigour index to 4540 compared to T_7 and T_8 (2250) (Table 2). The same trend was observed in the pot culture experiment conducted during 2017-18. The results showed a significant increase in pod yield of 23.45 g per plant was observed in the T_6 treatment compared to other treatments and T_8 (12.55 g plant^{-1}). This result was supported by the vigour index test on peanut seedlings, which showed a higher vigour index of 4490 in the T_6 treatment than other treatments, T_7 and T_8 (2232) (Table 2). It led to an increase in the seedling vigour index by 101 % and a yield increase ranging from 86 % to 102 % compared to T_8, as observed in pot culture conditions conducted in two separate trials. Results from the field experiments conducted during 2016-17 showed that the T_6 treatment significantly enhanced the seedling vigour index to 4308 at 30 DAS and increased the pod yield to 1778 kg ha^{-1} compared to other treatments and T_8 (vigour index: 2618; yield: 1004 kg ha^{-1}). This indicates that the T_6 treatment improved the seedling vigour index and peanut yield by 64.6 % and 77.09 %, respectively, compared to T_8 (Table 3). Similarly, results of field experiments conducted during 2017-18 revealed that the T_6 treatment significantly improved the seedling vigour index to 3606 than T_8 (2277) and other treatments at 30 DAS. In the case of pod yield, a maximum of 1530 kg ha^{-1} was observed in the treatment, T_6 than T_8 (880 kg ha^{-1}) and other treatments. Additionally, increases of 58 % and 73 % in seedling vigour index and yield, respectively, were observed in the treatment, T_6 compared to T_8 (Table 3).

The *B. subtilis* strains effectively enhanced chickpeas' plant growth parameters inoculated with *S. rolfsii* (48). *Bacillus* lipopeptides are implicated in the promotion of plant growth as well as in the biocontrol of plant diseases. The extensive root proliferation in peanuts is induced by PGPR (49). Mixed application of *B. subtilis* and *B. amyloliquefaciens* showed significant reduction in diseases caused by *Rhizoctonia solani* and simultaneously enhanced the physiological parameters of the potato plants (50). The combination treatment, T_6, might show synergism in reducing stem rot and improving plant

Table 2. Efficacy of PGPR on growth parameters, yield, and stem rot incidence of peanuts in pot culture conditions (2016 - 17 & 2017-18)

Trt. No.	Per cent seed germination		Vigour index		Per cent increase over control		Pod yield (g/plant)		Per cent increase over control		Per cent disease incidence (PDI)		Per cent decrease over control	
	I year	II year	I year	II year	I year	II year	I year	II year	I year	II year	I year	II year	I year	II year
$T_{1.}$	93.3	93.3	2532	2693	12.54	20.65	12.80	13.74	29.16	9.48	61.10	55.55	21.44	23.07
$T_{2.}$	100.0	100.0	4018	3757	78.58	68.32	17.57	21.79	77.30	73.63	44.44	33.33	42.86	53.84
$T_{3.}$	93.3	93.3	2819	2851	25.29	27.73	12.56	15.94	26.74	27.01	55.55	49.99	28.57	30.77
$T_{4.}$	93.3	93.3	2936	2964	30.49	32.80	11.11	16.05	12.11	27.89	55.55	44.44	28.57	38.46
T_5	93.3	93.3	3615	3065	60.67	37.32	15.22	20.28	53.58	61.59	44.44	44.44	42.86	38.46
$T_{6.}$	100.0	100.0	4540	4490	101.78	101.17	20.05	23.45	102.32	86.85	27.77	22.22	64.29	69.23
$T_{7.}$	93.3	93.33	3769	3530	67.51	58.15	17.26	21.98	74.17	75.14	11.11	11.11	85.71	84.61
$T_{8.}$	80.0	80.00	2250	2232	-	-	9.91	12.55	-	-	77.77	72.21	-	-
CD (P<0.05)	5.5	5.5	361.4	403.11	-	-	1.92	1.08	-	-	6.46	9.4	-	-

T_1: *B. subtilis* PB(20d); T_2: *P. aeruginosa* PP(30d); T_3: *B. amyloliquefaciens* PB(40d); T_4: *B. subtilis* PB(50d); T_5: *P. campinasensis* PB(60d); T_6: T_{1+} T_{2+} T_{3+} T_{4+} T_5; T_7: Difenoconazole (0.05 %); T_8: Control; Values are mean of three replications.

Table 3. Efficacy of PGPR on growth parameters, yield and stem rot incidence of peanut under field conditions (2016 – 17 & 2017-18)

Trt. No.	Seedling vigour index		Per cent increase over control		Per cent disease incidence (PDI) (from 30 DAS to Harvest)		Per cent decrease over control		Pod yield kg ha^{-1}		Per cent increase over control	
	I year	II year	I year	II year	I year	II year	I year	II year	I year	II year	I year	II year
$T_{1.}$	2993	2640	14.32	15.94	25.50	30.11	22.73	37.35	1026	975	2.19	10.80
$T_{2.}$	3469	3019	32.51	32.59	16.16	25.67	51.03	46.59	1534	1270	52.79	44.32
$T_{3.}$	3047	2628	16.39	15.42	29.00	32.84	12.12	31.67	1044	1003	3.98	13.98
$T_{4.}$	3100	2701	18.41	18.62	26.66	29.61	19.21	38.39	1066	1015	6.18	15.34
T_5	3157	2814	20.59	23.58	20.00	27.39	39.39	43.01	1354	1113	34.86	26.48
$T_{6.}$	4308	3606	64.55	58.37	10.66	18.70	67.70	61.09	1778	1530	77.09	73.86
$T_{7.}$	3816	3333	45.76	46.38	2.00	5.97	93.94	87.58	1485	1377	47.91	56.48
$T_{8.}$	2618	2277	-	-	33.00	48.06	-	-	1004	880		-
CD (P<0.05)	**413**	**267**		**-**	**5.6**	**7.97**	**-**	**-**	**158.68**	**122.9**		**-**

T_1: *B. subtilis* PB(20d); T_2: *P. aeruginosa* PP(30d); T_3: *B. amyloliquefaciens* PB(40d); T_4: *B. subtilis* PB(50d); T_5: *P. campinasensis* PB(60d); T_6: T_{1+} T_{2+} T_{3+} T_{4+} T_5; T_7: Difenoconazole (0.05 %); T_8: Control; Values are mean of three replications.

growth and pod yield of peanuts. Hence, the combination of promising PGPR strains could play a significant role in managing stem rot disease in peanuts in an eco-friendly manner.

Conclusion

The results of the present experiment indicated that the combined application of native PGPR strains, specifically *Bacillus subtilis* PB(20d), *Bacillus amyloliquefaciens* PB(40d), *Bacillus subtilis* PB(50d), *Paenibacillus campinasensis* PB(60d) and *Pseudomonas aeruginosa* strain PP(30d) resulted in a lower incidence of stem rot and a higher yield of peanuts compared to individual applications and the untreated control. Therefore, to overcome the inconsistent performance of individual bacterial biocontrol agents and to enhance the biocontrol efficacy in managing the peanut stem rot caused by the fungus *S. rolfsii*, combined applications of native PGPR strains are shown to be more effective.

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Authors' contributions

RT carried out the entire experiment and drafted the manuscript. NR assisted in genomic DNA extraction from PGPR. KK assisted in characterizing PGPR. VK assisted in formulating treatments in pot culture and field. DR statistically analyzed all the data. NR assisted in the interpretation of the results.

Compliance with ethical standards

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

Ethical issues: None

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