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Exploration of the Potential of *Bacillus* spp. as an Antagonist and PGPR against Stem and Pod Rot of Groundnut

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ABSTRACT

Background: Stem and pod rot is an economically important soil borne disease of groundnut caused by *Sclerotium rolfsii* Sacc. resulting in significant yield losses across the globe. *Bacillus* spp. is rod-shaped and gram-positive bacterium which is predominantly found in the soil. Hence, considering the importance of the disease, the present investigation was carried out to evaluate the indigenous *Bacillus* spp. for plant growth promotion and to inhibit the pathogen in groundnut.

Methods: Totally nineteen *Bacillus* spp. isolates from healthy groundnut rhizosphere and their antagonistic potential was recorded. The molecular characterization of five efficient *Bacillus* spp. was carried out using 16S rDNA. Later, morphological and biochemical characterization and plant growth promoting traits were recorded.

Result: All nineteen isolates of *Bacillus* spp. showed antagonistic potential against *S. rolfsii.* Five *Bacillus* spp. isolates which showed higher inhibition of the pathogen were identified as *B. subtilis, B. pacific, B. cereus,* two isolates belonging to *B. tropicus* through molecular technique. The morphological, biochemical characters and growth promoting traits were recorded.

Key words: *Bacillus* spp., Bio-control, Characterization, PGPR.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop and in recent years, stem and pod rot caused by *Sclerotium rolfsii* Sacc. was found to be most devastating causing significant yield losses and results in huge losses. The groundnut growing farmers are facing serious problem of the disease. All states that grow groundnuts are affected by the stem and pod rot disease and it is particularly more in Maharashtra, Gujarat, Tamil Nadu and Karnataka. The yield loss caused by the pathogen is 25 per cent, but sometimes reaches 80-90 per cent (Grichar and Bosweel, 1987).

At present, a number of fungicides have been used for the management of disease. However, chemicals are harmful to human and animal health. Alternatives to fungicides include antagonistic microorganisms. The beneficial organisms known as bioagents reduce the harmful effects of plant diseases and encourage good plant responses. The Biological control agents are also known to promote the plant growth. is the process by which one or more organisms reduce the inoculums or disease-causing activities of a pathogen (Cook and Baker, 1983).

Hence, use of plant growth promoting rhizobacteria provides a useful alternative for growth promotion as well as plant disease management. *Bacillus spp.* is a rod-shaped, Gram positive bacterium, frequently found in soil and reported to produce endospores to resist dry environments and high temperatures. The purpose of the current investigation was to study antagonistic potential, characterization and plant growth promoting traits of indigenous *Bacillus* spp. against *Sclerotium rolfsii.*

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MATERIALS AND METHODS

Isolation and maintenance of the pathogen

Groundnut plants showing typical symptoms of stem and pod rot were collected from groundnut fields of UAS, Raichur during *Kharif*, 2021 cropping season and the pathogen was isolated on Potato Dextrose Agar (PDA) medium by hyphal tip technique. Pure cultures of the pathogen *S. rolfsii* were maintained on PDA by periodical transfers.

Isolation of *Bacillus* spp.

Healthy rhizosphere soil from groundnut fields was collected by sampling the random soil sampling. The nineteen isolates of plant growth promoting rhizobacteria, *Bacillus* spp. Isolates were obtained by serial dilution technique using Hichrome *Bacillus* agar medium from different regions of Karnataka. The *Bacillus* spp isolates were kept under observation daily for the appearance of green colonies.

Antagonistic potential of *Bacillus* spp. isolates against *Sclerotium rolfsii*

Nineteen isolates of *Bacillus* spp. were screened for antagonistic potential against *S. rolfsii* by dual culture technique. The degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. The per cent inhibition over the control was calculated by using the formula (Vincent, 1927).

$$I = \frac{(C-T)}{C} \times 100$$

Where;

I = Per cent inhibition of mycelium.

C = Growth of fungal mycelium in control.

T = Growth of fungal mycelium in treatment.

The efficient isolates of indigenous *Bacillus* spp. which showed more than 50 per cent mycelial inhibition of pathogen were selected for molecular, morphological as well as biochemical characterization and to study plant growth promoting traits.

Molecular characterization of efficient *Bacillus* spp.

The DNA isolation of five efficient isolates was carried out by using standard CTAB method. The universal primers 16s rDNA F (GAG-TTT-GAT-CCT-GGC-TCA) and 16s rDNA R (AGA-AAG-GAG-GTG-ATC-CAG) were synthesized at Eurofins, Bangalore, India for characterization of isolates. Amplification was conducted in a thermal cycler (Veriti, Applied Biosystems, Singapore) and the PCR programming was done as per Yugander *et al*. (2017). Initial denaturation was set for 1 min at 94°C for 1 cycle. Whereas, denaturation time was set for 1 min at 96°C, annealing for 1.5 min at 58°C and extension for 1.5 min at 72°C for 35 cycles. The final extension was set for 8 min at 72°C for 1 cycle.

Morphological characterization of efficient *Bacillus* species

The characteristics of five efficient *Bacillus* species such as shape of cells was recorded under microspcope during Grams staining process, the colour and texture of colony by visual observation.

Biochemical characterization of efficient *Bacillus* species

Gram's staining

The Gram staining of *Bacillus* s*pp.* was carried out as per the standard protocol by using Grams staining kit and subjected for microscopic examination.

KOH test

A loopful of bacterial culture from well grown colony was taken and the test was carried out as per the procedure given by Sallam *et al*. (2013). The formation of strands of viscoid material represents the Gram negative bacteria and Gram positive bacteria do not produce viscoid thread.

Catalase test

A loopful of single well isolated colony was placed on a clean microscope slide and added with 3 per cent H_2O_2. A positive result of catalase production was characterized by the rapid evolution of O_2 which is evidenced by bubble formation (Yunting *et al*., 2013).

Starch hydrolysis

The test culture was spot inoculated on to the starch agar plates and kept for incubation. Later, the petri plates flooded with Lugol's iodine and left few minutes. Formation of clear zone around the colony was taken as positive for the test (Eckford,1927).

Urease test

The young cultures were inoculated to the test tubes containing sterilized ureabroth and incubated. The development of pink color was taken as positive for the test.

Casein hydrolysis

Young cultures of *Bacillus* spp. were spotted on to the skimmed milk agar plates and incubated. The production of clear halo around the colony was taken as positive for the test (Seeley and Vandemark, 1970).

Gelatin liquefaction

The test cultures of *Bacillus* spp. were inoculated to the presterilized nutrient gelatin deep tubes and were incubated at 28±2°C for 24 h and kept in a refrigerator at 4°C for 30 minutes. The tubes with cultures that remained liquefied were taken as positive and those that solidified on refrigeration were taken as negative for the test (Blazevic and Ederer, 1975).

Indole production

The test cultures were inoculated to the pre-sterilized SIM agar tubes and incubated for 48 h. After incubation, each tube was added with 10 drops of Kovac's reagent. The production of cherry red colour was taken as positive for test.

H_2S production

Pre-sterilized tubes containing SIM agar were stabbed with the test cultures all along the walls of the test tubes and incubated for 48 h at 28±2°C. After incubation, the development of black color along the line of the stab was positive for the test (Cappuccino and Sherman, 1992).

Endospore staining

The endospore staining was carried out as per the procedure given by Harley and Prescott (2002) by using Malachite green. Under a light microscope, green colour cells were endospores and pink coloured cells were vegetative cells.

Plant growth promoting traits of *Bacillus* species

HCN production

The rhizobacteria were streaked onto nutrient agar plates amended with Glycine (4.4 g/l), plates were inverted and a

piece of Whatman filter paper no. 1 impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with parafilm and incubated for 96 h. Change in colour of the filter paper from orange to brown was considered as production of HCN. A change of colour from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction, respectively.

IAA production

It was measured by the method described by Patten and Glick (2002) and the detection of IAA was determined by the development of pink color.

Siderophore production

It was assayed by a plate method using the Chromoazurol sulphonate (CAS) agar method (Neilands and Schwayn, 1987).

Volatile compound production

In vitro volatile antimicrobial activity was assessed using paired plate method using NA medium for bacterium and PDA for pathogen along with control and kept for incubation. Later, colony diameter of the fungal pathogen was measured and compared with the control.

RESULTS AND DISCUSSION

Isolation of pathogen

The results indicated that, the white mycelia growth of pathogen was observed within 3 to 4 days. Formation of sclerotial bodies was also after 8-10 days.

Isolation and maintenance of native isolates of *Bacillus* spp.

Nineteen isolates of *Bacillus* spp. were successfully isolated and designated as BS-1 to BS-19. Similarly, *Bacillus* spp. were isolated by using nutrient agar by serial dilution technique (Bharose *et al*., 2017).

Antagonistic potential of *Bacillus* spp. isolates against *Sclerotium rolfsii*

The per cent inhibition of mycelial growth varied greatly among the nineteen isolates. The isolates BS-3 (58.51%), BS-2 (57.03%), BS-6 (56.29%), BS-10 (55.55%) and BS-7 (52.96%) showed more than 50% mycelial inhibition of pathogen (Table 1 and Fig 1). Rajkumar (2016) screened thirty *B. subtilis* isolates against *S. rolfsii* reported that the isolates showed different levels of inhibition of mycelial growth of *S. rolfsii*. Among them, BS16 inhibited maximum mycelial growth 64.04 per cent followed by BS 30 (62.20%) and minimum inhibition of mycelial growth was observed in case of BS17 (11.98%).

Molecular characterization of potential *Bacillus* spp. Isolates

Five efficient *Bacillus* spp. isolates, BS-2, BS-3, BS-6, BS-7 and BS-10 were amplified for 16S rDNA (Fig 2) and the species were identified as *Bacillus tropicus* (ON564730), *Bacillus subtilis* (ON564689), *Bacillus pacificus* (ON564610), *Bacillus cereus* (ON564773) and *Bacillus tropicus* (ON564907), respectively. The accession numbers were deposited in genebank. Phylogram of five *Bacillus* species was constructed to analyze the diversity among them (Fig 3). The results in the dendrogram showed two major clusters. Cluster-I comprised two sub groups. In sub-group I, *B. pacificus* shared 36 per cent similarity with reference organism *B. cereus*. Whereas, *B. cereus* shared 16 per cent similarity between *B. pacificus* and *B. cereus*. *B. tropicus* was out grouped from *B. pacificus*, *B. cereus* and two reference *B. cereus* strain. Sub-group II consists of *B. tropicus* which was out grouped from *B. pacificus* and *B. tropicus* reference strains. Cluster-II consists of *B. subtilis* shared 99 per cent similarity with *B. subtilis* strain (Fig 3). Hernandez *et al*. (2014) reported that *B. subtilis* and *B. cereus* are known to suppress growth of *S. rolfsii.*

Morphological characterization of potential isolates of *Bacillus* spp.

The results (Table 2 and Fig 4) indicated that all species were rod shaped and colony was rough in texture. The colour of the colony was white in *B. pacificus* and creamy inrest of the species. Rajkumar (2016) identified the cultures of *B. subtilis* based on characters such as shape, texture of colony, colony morphology and colour of colony. All the isolates were rod shaped, isolates BS-2, BS-3, BS-4, BS-5,

Table 1: Antagonistic potential of *Bacillus* spp. against *S. rolfsii.*

Bacillus spp. Isolate number	Radial mycelial growth* (mm)	Per cent mycelial inhibition*
BS-1	63.66	29.25 (32.75)
BS-2	38.66	57.03 (49.05)
BS-3	37.33	58.51 (49.9)
BS-4	78.00	13.33 (21.42)
BS-5	68.00	24.44 (29.63)
BS-6	39.33	56.29 (48.62)
BS-7	42.33	52.96 (46.7)
BS-8	53.00	41.11 (39.88)
BS-9	63.00	30.00 (33.21)
BS-10	40.00	55.55 (48.19)
BS-11	78.00	13.33 (21.42)
BS-12	72.00	19.99 (26.57)
BS-13	69.00	23.33 (28.89)
BS-14	88.66	1.48 (6.99)
BS-15	88.33	1.85 (7.82)
BS-16	82.33	8.51 (16.97)
BS-17	86.33	3.51 (10.81)
BS-18	76.66	14.81 (22.64)
BS-19	85.66	4.81 (12.67)
Control	90.00	-
S.Em.±	0.72	
C.D. at 1%	2.76	

*Mean of three replications.

Figure in parenthesis are arcsine values.

BS-11, BS-12, BS-19 and BS-20 were cream and remaining isolates white in colour. Colony morphology was smooth in case of BS-3, BS-6, BS-9, BS-13, BS-17 and BS-18 and remaining isolates were rough. Biochemical characterization of all the isolates showed Gram positive and positive for endospore production, catalase, oxidase, gelatin hydrolysis and starch hydrolysis and negative for KOH test and indole test. Among the 30 different isolates of B. subtilis, the isolates BS-1, BS-5, BS-6, BS-7, BS-9, BS-13, BS-16, BS-20, BS-23, BS-24, BS-25, BS-27, BS-28 and BS-29 showed positive for HCN production. The isolates like, BS-5, BS8, BS-13, BS-20 and BS-20 were negative and all the isolates recorded positive for IAA production except BS-20, BS-23, BS-24, BS-25, BS-27 and BS-2. Wafula *et al*. (2014) reported the

Table 2: Morphological characterization of efficient *Bacillus* species.

Isolates	Shape	Colour	Texture
Bacillus tropicus (ON564730)	Rod	Creamy	Rough
Bacillussubtilis (ON564689)	Rod	Creamy	Rough
Bacillus pacificus (ON564610)	Rod	White	Rough
Bacillus cereus (ON564773)	Rod	Creamy	Rough
Bacillus tropicus (ON564907)	Rod	Creamy	Rough

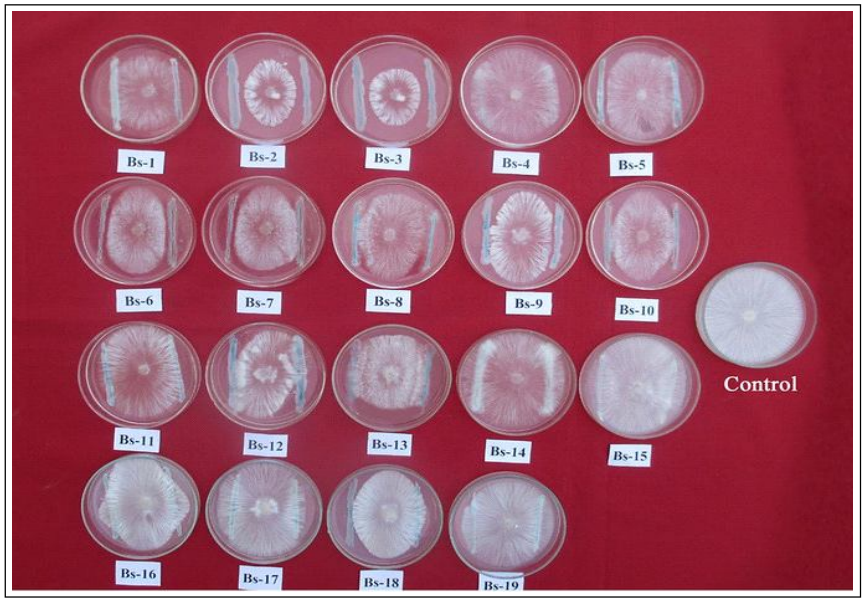

Fig 1: Mycelial inhibition of *S. rolfsii* by *Bacillus* spp isolates.

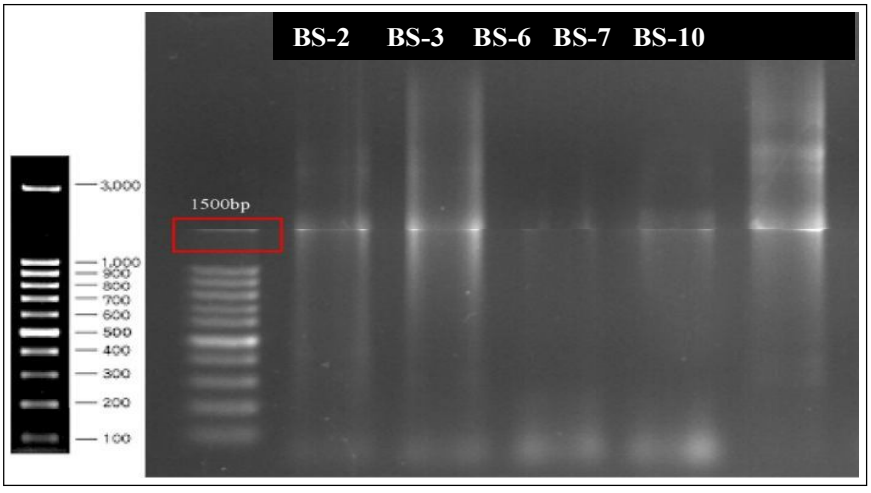

Fig 2: Gel picture showing amplified products of *Bacillus* species.

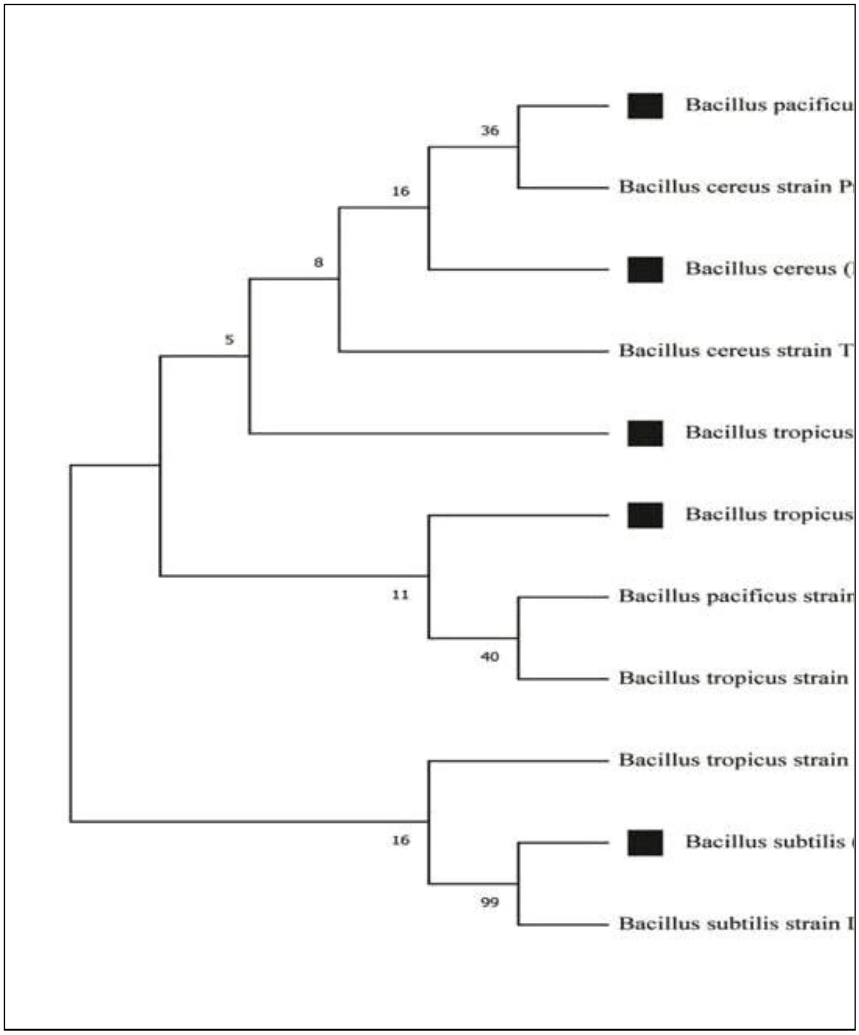

Fig 3: Phylogenetic tree depicting diversity among the different *Bacillus* species.

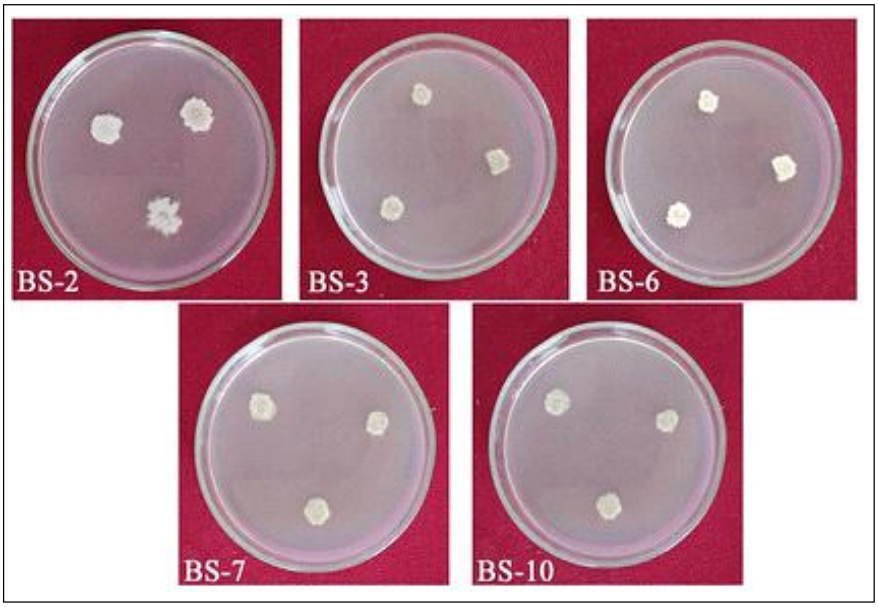

Fig 4: Morphological characters of efficient *Bacillus* species.

morphological characterization of *B. subtilis* like colour, form, shape and elevation of pure colonies. The colony morphology of the isolates ranged from flat and filamentous or branching. They were smooth or rough and the colour ranged from white to creamy.

Biochemical characterization of efficient isolates of *Bacillus* spp.

Gram's staining

All the *Bacillus* species for gram staining indicated that, they were Gram positive showing blue coloured cells by taking primary stain (Fig 5).

KOH test

All five *Bacillus* species showed negative for KOH test without forming any mucoid string (Fig 5).

Catalase test

All five *Bacillus* species showed positive for the catalase test by forming gas bubbles (Fig 5).

Strach hydrolysis

The clear zone around the colony indicates positive for test for all five *Bacillus* species (Fig 5).

Urease test

All five *Bacillus* species developed pink colour indicating positive urease test (Fig 5).

Casein hydrolysis

The formation of halo zone indicated positive test for casein hydrolysis in all the five *Bacillus* species (Fig 5).

Gelatin liquefaction

In this study, all the five *Bacillus* species have shown positive for the test indicated by the tubes remained liquefied even after refrigeration (Fig 5).

Indole test

All five *Bacillus* species showed negative for the indole test wherein there was no formation of cherry red colour (Fig 5).

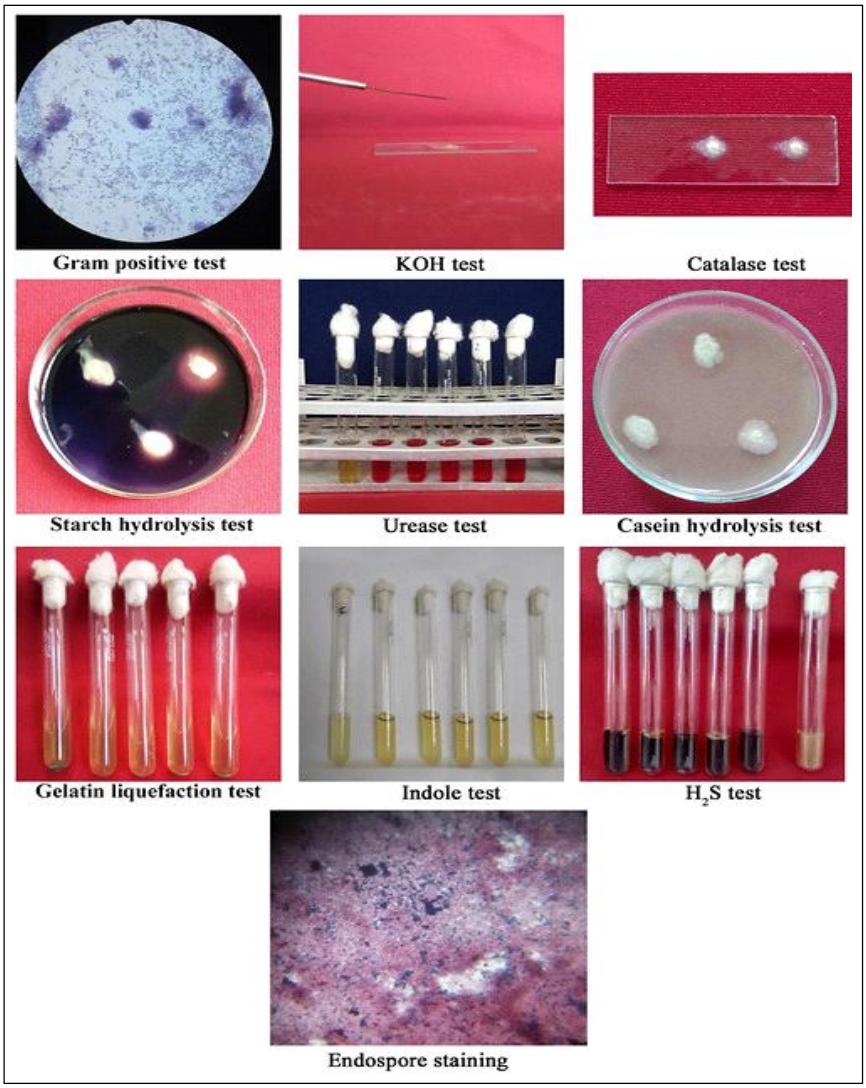

Fig 5: Biochemical tests of efficient *Bacillus* species.

H_2S production

Formation of black colour along the line of stab indicated H_2S production by all five *Bacillus* species (Fig 5).

Endospore staining

All the tested *Bacillus* species produced endospore when stained with malachite green stain (Fig 5).

Rajkumar (2016) reported that all the tested isolates were Gram positive and positive for endospore production, catalase, oxidase, gelatin hydrolysis and starch hydrolysis and negative for KOH test and indole test. Among the 30 different isolates of *B. subtilis*, 25 isolates showed positive for HCN production and all the isolates recorded positive for IAA production. Venant *et al*. (2013) reported that isolates of *B. subtilis* were indole and methyl red negative and positive for citrate, catalase, urease, oxidase, starch hydrolysis, KOH test and gelatin hydrolysis.

Plant growth promoting traits efficient *Bacillus* species

HCN production

Bacillustropicus and *B. subtilis* showed higher level of HCN production and recorded as strong (+++). *B. pacificus* and *B. cereus* recorded moderate scoring (++) and *B. tropicus* weak (+) (Fig 6). Similar type of results was obtained with Ramya bharathi and Raguchander (2014), who reported that, *B. subtilis* was found to be positive for hydrogencyanide production.

IAA production

Five *Bacillus* species were showed positive for the production of IAA (Fig 6). Bharose *et al*. (2017) also observed

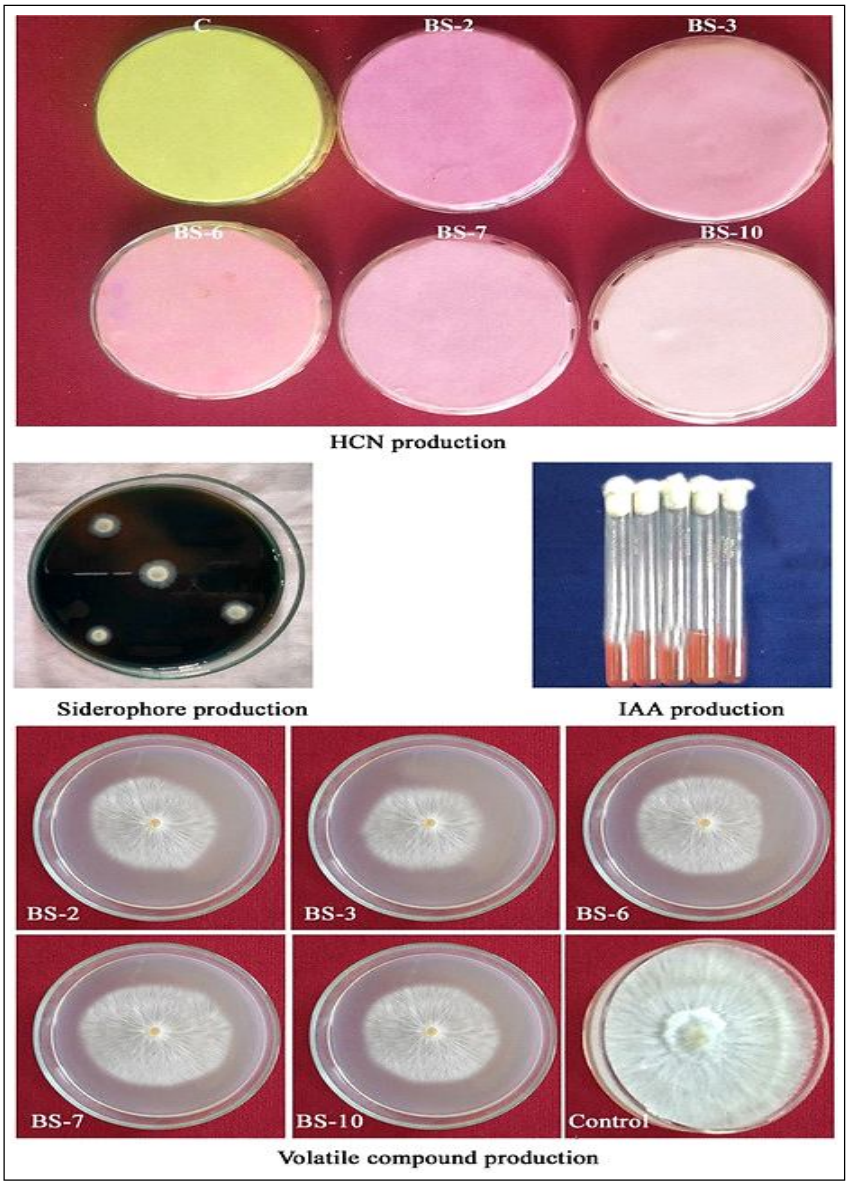

Fig 6: Plant growth promoting traits of efficient *Bacillus* species.

the production of IAA by the isolate JND-KHGn-29-B of *B. subtilis*. Sivasakthi *et al*. (2013) tested 10 isolates of *B. subtilis* for the production of IAA, results revealed that, the isolate BS8 produced maximum amount of IAA *i.e*., 26 µg/ml. Dinesh Singh *et al*. (2012) studied on nine isolates of *B. subtilis* which were isolated from rhizospheric soil of tomato plants. Among them, BS9 exhibited maximum indole acetic acid production (0.139 µg/ml) and ammonia production under *in vitro* conditions.

Siderophore production

All five species of *Bacillus* produced siderophore where the *B. tropicus* (BS-2) and *B. pacificus* produced higher amount of siderophore (4 mm orange colour zone) and *B. subtilis* and *B. cereus* recorded moderate siderophore production (2-4 mm) and *B. tropicus* (BS-10) produced less siderophore (2 mm) (Fig 6). Sivasakthi *et al*. (2013) tested10 isolates of *B. subtilis* for the production of siderophore, results revealed that, the isolate BS8 produced maximum amount of siderophore *i.e*., 9 µg/ml. The bacterial isolateJND-KHGn-29-A was found to have siderophore activity (Bharose *et al*., 2017).

Volatile compounds production

The results revealed that the species produced considerable amount of volatile metabolites which varied with the species. The *B. subtilis* showed maximum per cent inhibition (47.03%) followed by *B. tropicus* (BS-2) (44.07%) and *B. pacificus* (42.22%) (Fig 6). The results obtained in this study are in line with Ramyabharathi and Raguchander (2014) they reported that, *B. subtilis* EPCO16 strain was effectively inhibited the growth of *F. oxysporum* f. sp. *lycopersici* to an extent of 60.78 per cent *in vitro* through the production of volatile compound. The volatile compounds produced bya strain of *B. subtilis* found to be antifungal to *R. solani* and *Pythium ultimum* (Fiddaman and Stephen, 1993).

CONCLUSION

In the present study, out of nineteen Bacillus spp. isolates from healthy groundnut rhizosphere, five isolates showed higher inhibition of the Sclerotium rolfsii and they were identified as *B. subtilis*, *B. pacific*, *B. cereus*, two isolates belonging to *B. tropicus* through molecular technique using 16S rDNA. The morphological and biochemical characters of these five isolates was also studied indicating that they belong to *Bacillus* spp. Apart, these species exhibited higher plant growth promoting traits such as HCN, IAA, sederophore and volatile compound production. These findings open-up the perspective of using these *Bacillus* spp. as PGPMs with their potentiality for plant growth promotion and induction of volatile compound production against pathogen as a sustainable approach.

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Conflict of interest: None.

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