International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 14s, 2025
https://www.theaspd.com/ijes.php

Biocontrol Potential of Bacillus subtilis AM-7 (RSP2) against Sheath Blight of Rice (Oryza sativa) caused by Rhizoctonia solani.

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Abstract:
Rice sheath blight, a serious global threat to rice production, is caused by the soil-borne fungal pathogen Rhizoctonia solani. A major focus in sustainable agriculture is the use of beneficial microorganisms to enhance plant health and suppress disease. This study aims to identify a potent biocontrol agent against R. solani while simultaneously promoting plant growth. After isolating 85 rhizobacterial strains from the rhizospheric soil of Bhandara district associated with Jayshreerm, Parmal, and Wada varieties of Oryza sativa (rice), they were screened against Rhizoctonia solani using the dual culture method. Among them, strain AM-7 (RSP2) emerged as the most effective, exhibiting a maximum inhibition zone of 42 mm and an inhibition rate of 40%. Twenty potential isolates were selected based on their biochemical characteristics. These isolates were further evaluated for their plant growth-promoting rhizobacteria (PGPR) activities, including the production of hydrogen cyanide (HCN), ammonia, siderophores, indole-3-acetic acid (IAA), phosphate, zinc, and potassium solubilization, nitrogen fixation, and chitinase activity. In vitro screening against Rhizoctonia solani was conducted using the dual culture method. Among these, isolate AM-7 was identified as Bacillus subtilis strain RSP2 through 16S rRNA gene sequencing. Volatile organic compounds (VOCs) produced by B. subtilis were found to be effective in suppressing Rhizoctonia solani in vitro. Fourier-transform infrared (FTIR) analysis of the crude extract of isolate AM-7 (RSP2) revealed the presence of functional groups such as an alkyne group (3308.31 cm ⁻¹), amine salt (2942.79 cm ⁻¹), carboxylic acid group (2831.73 cm ⁻¹), and secondary alcohol (1114.95 cm ⁻¹). All isolates tested positive for indole-3-acetic acid (IAA) production; however, only isolate AM-7 was found to produce chitinase. Upon optimization, the maximum IAA production by isolate AM-7 was recorded as 175 µg/ml after 48 hours of incubation at 37 °C. Sheath blight control is challenging due to the pathogen's adaptability, absence of resistant rice varieties and genes, and limited farmer awareness.

Key words: *Sheath blight, Oryza sativa, Plant Growth Promoting Rhizobacteria, Bacillus subtilis.*

1. INTRODUCTION:

Significant plant loss and decreased output are common in susceptible rice cultivars because of the wide range of diseases and serious illnesses (Xiaojia H, et.al. 2019; Yang S, et.al. 2018). Among the most devastating fungi-related ailments is Rhizoctonia solani, which thrives as a sclerosis in both cultured and uncultured soils without producing asexual spores. (Cao L et al. 2004). The most typical infection brought on rice plants by R. solani is sheath blight (Mausa TA, 2002). Fungicide application is costly, dangerous for human and environmental health, and upsets the equilibrium of good microorganisms in the soil for treating soil-borne diseases. When it comes to the biological management of soil-borne plant diseases, treating seeds with antagonists is a helpful substitute for employing synthetic pesticides. (Ester S et al. 2015; Singh RP et al. 2019). The most prevalent bacterial genus in the soil rhizosphere is Bacillus, as typical bacteria from the soil aggressively colonise roots of plants and assist crops by promoting proliferation. It is a facultative anaerobic, gram-positive, endospore-forming bacterium. I. *R. solani*, the rice pathogen that causes sheath blight. The isolates were also described in terms of their ability to withstand abiotic stress, specific actions that are advantageous to plants (phosphate solubilization and IAA synthesis), as well as activities that promote plant development in greenhouse environments.

Soil bacteria are the primary source of nutrients for host plants, as over 95% of bacteria are found in plant roots. Since bioinoculants are intended to enhance agricultural plant growth and yield, this should be the most abundant source to investigate for possible PGPR. Utilising plant growth-promoting bacteria (PGPRBs), a type of naturally occurring microbes that boost the availability of nutrients to plants, can be advantageous for biocontrol agents, phyto-stimulating agents, and biofertilization (Bloemberg and Lugtenberg, 2001; Gwyn, 2006). According to Glick et al. (1995; 2001), PGPR functions in three ways: it synthesises compounds for the plants, makes it easier for the plants to absorb specific nutrients from the soil, and shields the plants against disease. The

generation of hydrogen cyanide (HCN), solubilization of potassium, zinc, and insoluble phosphate, fixation of atmospheric nitrogen, and a few enzymes are the well-known mechanisms for promoting plant development (Patten and Glick, 2002).

Plant roots contain more than 95% of all bacteria, and soil bacteria provide host plants with a variety of nutrients. Therefore, this ought to be the most abundant resource to investigate to find effective plant growth promoting rhizobacteria that could be helpful for creating biological inoculants that will improve agricultural plant growth and yield. Numerous bacteria's capacity to promote growth might be extremely precise to a a single species, cultivar, or genotype of plant (Bashan, 1998). A wide variety of bacterial species associated with the plant rhizosphere, including those from the genera Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Pseudomonas, Rhizobium, Bradyrhizobium, and Serratia, may have a beneficial effect on plant (Kloepper1989).

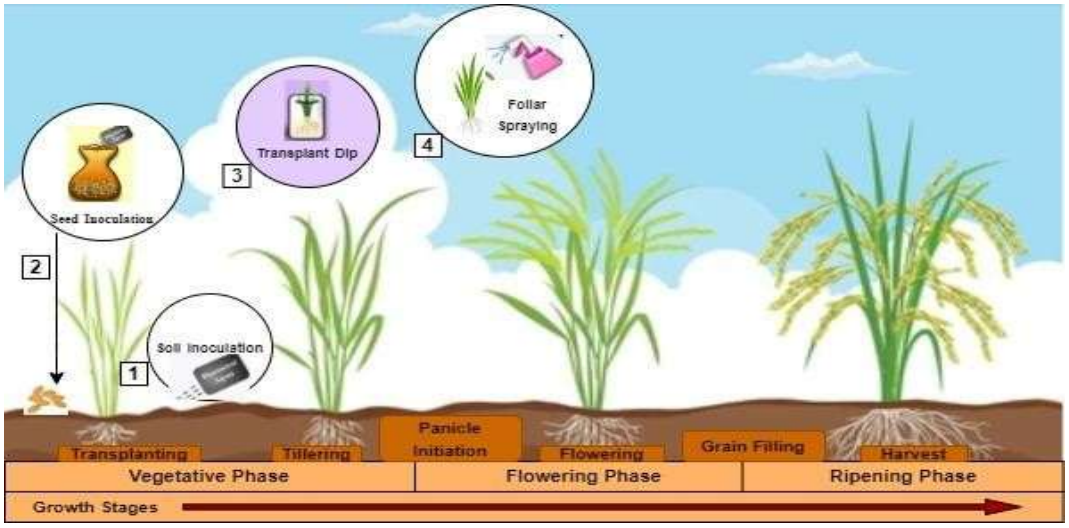


Fig 1: Approaches for the Implementation of PGPR-Based Biocontrol Agents Against Plant Pathogens.

According to Tilak et al. (2005), there are several genera that are linked to the rhizospheric zone of plants & are advantageous to crop development. The most prevalent genera detected in the rhizosphere are Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium, and Serratia. (Manoharachary and Mukerji *et al.*,2006) studied rhizosphere biology and reported that rhizosphere of soil harbour diverse group of microorganisms and they differ qualitatively and quantitatively with climatic, edaphic and biotic factors.

(Arul and Paneerselvam 2011) recovered several strains of Pseudomonas within the the root zone of sugar cane, rice, and groundnut. Aspergillus niger, Aspergillus flavus, Fusarium sp., Curvularia species, and Trichoderma sp. were among the crop pathogenic bacteria that the strains were tested for their antifungal capabilities. The results showed that all tested pathogens were inhibited in their growth. (Hu and Xu 2011) identified Bacillus subtilis QM3, a siderophore-producing bacteria, and demonstrated its capacity for biocontrol.

Crop inoculants are always in need of development because field and vitro environments differ greatly. Therefore, it is imperative to cultivate a rhizobacterial population with a large potential for positive feedback loops (PGPR) in order to enhance agricultural practises and crop productivity. The isolates with numerous PGPR properties from the rice plant's rhizosphere were the primary focus of the current investigation.

2. MATERIALS AND METHODS:

2.1 Study Site & Location: Total 85 rhizobacterial isolates were obtained from ten rhizospheric soil samples at three phases viz vegetative, flowering, and ripening phases of life cycle of three rice varieties viz Jayshreeram, Parmal and Wada in Bhandara district namely Gadegaon Kinhi (21º 09′51″65‴N; 79 º 80′43″33‴W; 265E); Manegaon (20 º 82′46″57‴N; 79 º 87′65″69‴W; 238E) and Khairi (21 º 07′96″9‴N; 79 º 90′37″14‴W; 245E) of Maharashtra. The three locations were only ten km apart from each other, yet the soil textures were distinct.

Plants were carefully uprooted, and excess dirt was shaken off the plant firmly before taking aseptic soil samples. The soil that adhered was accumulate using sterilised polypropylene bags. Every soil sample was separated in two halves; the first half was allowed to air dry subsequently employed to examine the soil's characteristics, while the second half was preserved cold to evaluate the bacterial population. The soils' physicochemical characteristics were assessed through the use of established procedures. The pH, electrical conductivity (EC), organic carbon (C), nitrogen (N), phosphorus (P), and potassium (K) contents of each sample were measured and examined.

2.2 Isolation, Screening of rhizobacteria & culture condition: As stated by Collins and Lyne (2004), the serial dilution approach was used to isolate the bacteria, and then the spread plate technique was used. The inoculation was spread out on sterile nutrient agar in 0.1 ml aliquots of 10-7 dilution, and the samples were then allowed to incubate up to 24 to 48 hours at 37^0C. Representative variety of colonies were pointed out and transferred on nutrient agar slants, incubated at room temperature, and then preserved as isolates. These isolates were then used to study different characteristics and screened on different types of agars viz., Actinomycete isolation agar, Azotobacter isolation agar, ISP-2 medium, Nutrient agar, Oatmeal agar and Pseudomonas isolation agar incubated for 24 - 48 hrs at 37^OC. Colony-forming units (CFUs) per gramme of soil were used to calculate the overall viable count. Furthermore, for regular research and long-term glycerol preservation, bacterial colonies went through purification and preserved at 4 °C & 20 °C, respectively. The National Fungal Culture Collection of India Agharkar Research Institute, Pune is the source of the fungal pathogen R. solani (NFCCI-188).

2.3 Microscopy of fungal pathogen: Upon incubation on potato dextrose agar (PDA) plates for a week, the morphological changes induced the phytopathogenic Rhizoctonia solani mycelia by the antagonistic bacteria Bacillus subtilis (AM-7) strain RSP2 was promptly examined using phase-contrast microscopy (Metzer Ltd.) and scanning electron microscopy (JEOL Ltd).

2.4 Morphological Characterization: The representative isolated bacteria were inoculated into plates of nutrient agar and left for incubation up to 24 to 48 hours at room temperature to examine their morphological properties. Following incubation, the produced colonies were examined for cultural traits such as height, opacity, consistency, size, shape, margin, and colour or pigment. Gram staining of each isolate was performed by Hucker and Conn's modification to observe the morphological feature and Gram nature. Motility of each isolate was performed by hanging drop technique and ability was recorded as described by Cappuccino and Sherman.

2.5 Biochemical Characterization: The hydrolysis capacity of the investigated strains was examined using various sugars as food sources (Karen R, 2012). In short, enough beef extract and peptone were employed, along with test tubes containing different sugars (fructose, lactose, maltose, galactose, glucose, mannitol, sucrose). To find the formation of acid, six of which were control tubes and six of which were inoculated with isolated culture of bacteria were incubated at 37°C for overnight. The pH was then determined using phenol red. The study isolates' capacity for temperature tolerance was assessed in nutrient broth medium that had been inoculated with a 5 µl log-phase culture, after 24-72 hours, a spectrophotometer was used to measure the growth of the inoculated flasks following incubation at 45, 50, 55, and 60 °C. The assay for Hayward catalase was performed. Briefly said, a few drops of 30% Hydrogen peroxide were introduced to pellets of log phase culture of rhizobacteria that were placed onto a glass slide. Catalase activity was tracked by the presence and size of gas bubbles. Spotted onto trypticase soya agar plates, the isolates being studied were incubated for 24 hours at 28.2 °C to perform the oxidase experiment. After incubation, the test organism's surface was treated with tetramethylethylenediamine (TEMED), and a colour shift to deep red was interpreted as an oxide positive. By stabbing the colony into Christensen's urea agar medium, urease synthesis was demonstrated. After 24 hours at 30 °C, urea breakdown was detected by a change in the colour of the tubes.

2.6 Antagonistic effect:
Using the dual culture assay technique established by Rabha et al., isolates were screened using the antagonist test against R. solani. After 72 hours of incubation, the pathogen's mycelial development and the inhibitory zone

were examined to determine the level of antagonism. On days seven and fourteen of the experiment, the percentage inhibition of mycelial growth was derived using the equation (Riungu et al. 2008).

$$\text{Percentage of inhibition} = \frac{C - T}{C} \times 100,$$

Where, C - is the fungal radial growth in (mm) on control plates.

T- represents the fungal radial growth (mm) on a plate that has been inoculated with each antagonist

2.7 Study of Plant Growth Promoting activity: -

A range of plant growth promoting (PGPR) activities were assessed for the 20 selected bacterial isolates in the following list: Phosphate, Zinc, and Potassium solubilization; Production of Siderophores, HCN, Ammonia, and Chitinase; Nitrogen Fixation:

On Kings B agar medium plates, 24-hour-old bacterial cultures were spot-inoculated. Whatman filter paper No. 1 was immersed in a solution of 2% sodium carbonate and 0.5% picric acid and applied on top of plates that were afterwards sealed with parafilm then, the plates were incubated for four days at 30°C. The transition of yellow to orange coloration suggested the production of HCN (Lock, 1948). Every isolate was tested for siderophore synthesis using Chrome azurol S agar medium. After being spot-inoculated onto individual Chrome azurol S agar media plates, 24-hour-old bacterial cultures were incubated for 48 to 72 hours at 30 ^{0}C. Schwyn claimed that the formation of a yellow-orange halo around the growth was favourable for siderophore production. Brick et al. (1991) reported a modified approach for detecting IAA generation. 48 hours were spent at 30^{0}C incubating 24-hour-old cultures of bacterial isolates that had been inoculated into 5ml of nutritional broth with a separate 1 mg/ml concentration of tryptophan. The soup was centrifuged for 30 minutes at 3000 rpm after incubation. The synthesis of IAA was detected using the cell-free supernatant that was collected. IAA was quantitatively analysed utilising the Loper and Scroth (1986) approach. The tubes were incubated with 4 ml of Salkowski reagent (2 ml of 0.5 M FeCl3 + 98 ml of 35% HClO4) added to 2 ml of supernatant. The production of IAA was indicated by the appearance of a pink colour. A colorimeter was used to measure the pink colour at 535 nm after 30 minutes. (Weber and Gordon, 1951) The IAA production was calculated using the standard graph, and the result was represented as µg/ml over control. On Pikovskaya's agar plates, the solubility of phosphate was assessed in bacterial isolates. On Pikovskaya's agar plates, 24-hour-old bacterial cultures were spot-inoculated, followed by a 4-day incubation period at 30°C. PO4 solubilization was demonstrated by bacterial colonies exhibiting a distinct zone of solubilization surrounding growth (Pikovskaya, 1948). All isolates were inoculated using a modified Pikovskaya's medium containing 1% insoluble zinc compound (ZNO). The plates underwent a 48-hour incubation period at 30^{0}C. (Pikovskaya R.E., 1948). After measuring the halo zone surrounding the colony, zinc solubilization was shown to be present. Plates were incubated for three days at 30^{0}C, after inoculation of all isolates onto modified Aleksandrov media (Hu et al., 2006). It was discovered that potassium solubilization occurred after observing the halo zone encircling the colony. It was investigated whether any of the bacterial isolates could create ammonia in peptone water. 24-hour-old cultures were inoculated with 10 millilitres of peptone water in separate tubes, and they were then incubated at 30^{0}C for 48–72 hours. Next, 0.5 millilitres of Nesseler's reagent were introduced into every tube. According to Cappuccino and Sherman (1992), a positive test for ammonia production is shown by the colour turning from brown to yellow. Five days of 30^{0}C incubation were required for the semisolid nitrogen-free bacterial isolates. The formation of pellets was considered an indication that nitrogen fixing was taking place. To check the ultimate level of nitrogen fixation activity, Fresh semisolid medium free of nitrogen was used to further inoculate the culture. (Dobereiner J., 1989). Renwick et al. 1991's approach was used to study chitinase activity. The ability of bacterial isolates to synthesize chitinase was examined. After inoculating the bacterial cultures on separate plates with 1 g/l of colloidal chitin, the plates were left to incubate at 28±2 °C for three to four days. The hyaline zone encircling the bacterial growth was considered a positive test.

2.8 Pot Assay:

A pot experiment, as previously reported by Singh et al., was conducted in a greenhouse chamber to assess the PGPR characteristics and bio-control potential of bacterial isolates of *B. subtilis* AM-7(RSP2). Briefly, *B. subtilis* (AM-7) were used to bacterize pre-surface-sterilized rice seeds (variety WADA) before being put to pots that had already been filled with sterilised sand. Sand pot seedlings treated with *R. solani*, as well as non-inoculated seed, were used as controls. Along with these measurements, root length, shoot length, fresh weight, dry weight, and grain number were also noted. The procedure outlined by Ferjani et al. was adhered to figure out the amount of chlorophyll present in the experimental plants' leaves. In a greenhouse, triplicates of each experiment were carried out.

2.9 Molecular Characterization:

The process of extracting genomic DNA (gDNA) was explained by Pospiech and Neumann. The 16S rRNA genome was amplified from extracted genomic DNA using polymerase chain reaction (PCR) and universal generic primers PA and PH, as per prior reported. UV transillumination and ethidium bromide staining were utilised to visualise the products of polymerase chain reaction that were detected & yielded utilising 1% agarose (wt/vol) gel electrophoresis. The amplifiable 16S rRNA gene transcript was then sequenced by Bangalore Geni (India). Following their sequences were identification and annotation, sequences were submitted in GenBank using BLASTN search conducted against the National Centre for Biotechnology Information (NCBI) database. To align gene sequences with the acquired datasets, the Clustal W algorithm in MEGA version 5.0 software was utilised (Subhashini DV et.al. 2017). The reliability of the topology of the tree and phylogenetic analysis were computed with the use of bootstrap analysis through 1000 replications of the sequences for neighbour joining.

3. RESULTS:

3.1 Physiochemical analysis of soil:

It is apparent from the table values that, pH of soil sample was within the range 6 to 6.8; E.C vicinity of 0.16(S/m), organic carbon 0.61 to 1.28 %. It was also reported that values of N_2, P, K, Ca, Mg and Na were in the range 427 to 896,1 to132, 228 to 1895, 4.32 to 21.64, 2.28 to 38.08 and 0.39 to 3.83 (Kg/ha) respectively. Soil sample viz PM-F45 was found rich in P, K and Na and the values were reported maximum as 132, 1895 and 3.83 (Kg/ha) respectively. Conversely, maximum values of N_2, Ca & Mg were found in soil samples PM –V20, WD-F55, WD –V20 respectively and the values were 896, 21.64 & 38.08 Kg/ha respectively. In the similar way lowest values of Nitrogen (427 Kg/ha), Phosphorus (1.00 Kg/ha), Potassium (228 Kg/ha), Calcium (4.32 Kg/ha), Magnesium (2.28 Kg/ha) and Sodium (0.39 Kg/ha) were reported in soil samples viz JS-R115, WD-F55, JS-V 25, PM-V 20, CL& PM- 70 respectively (Table 1).

Table 1: -Physiochemical analysis of rhizospheric soil of different locations of Bhandara District.

Sr. No.	Name of soil sample	pH	E.C. (S/m)	O.C. (%)	Aval. N2 (Kg/ha)	P (Kg/ha)	K (Kg/ha)	Ca (Kg/ha)	Mg (Kg/ha)	Na (Kg/ha)
1.	CL	6.10	0.16	0.65	455	17.00	309	17.01	2.28	1.15
2.	JS-V25	6.70	0.16	0.86	602	46.00	228	9.90	17.70	0.78
3.	PM-V20	6.80	0.18	1.28	896	123.00	1532	4.32	11.07	0.61
4.	WD-V20	6.50	0.15	0.91	636	66.00	242	8.55	38.08	0.62
5.	JS-F75	6.50	0.15	0.99	693	18.00	336	10.26	10.36	0.86
6.	PM-F45	6.00	0.15	1.20	840	132.00	1895	11.83	8.42	3.83
7.	WD-F55	6.00	0.15	0.65	455	1.00	282	21.64	22.77	0.54
8.	JS-R115	6.00	0.15	0.61	427	74.00	363	8.91	8.39	1.28
9.	PM-F70	6.20	0.15	1.02	714	28.00	1102	11.34	3.16	0.39
10.	WD-F85	6.00	0.15	0.91	627	17.00	444	17.01	5.97	3.33

Physiochemical analysis of soil: EC, electrical conductivity; P, phosphorus; N, Available Nitrogen; K, potassium; O.C., organic carbon; Ca, Calcium; Mg, Magnesium; Na, sodium.

3.2 Morphological and Biochemical characteristics:

Table 2 outlines the colony characteristics of strain AM-7 (RSP2). The colonies of AM-7 were opaque, cream-colored, with umbonate elevation, uneven margins, and a rough surface. The strain was aerobic and motile. Both AM-7 and AM-37 tested positive for several biochemical traits, including urease, starch hydrolysis, sucrose fermentation, methyl red (MR), indole production, galactose, dextrose, mannitol, fructose utilization, endospore formation, amylase, catalase, chitinase activity, and Voges-Proskauer (VP) test. However, they tested negative for other enzymatic activities not listed here.

Sr. No.	Test	Result
Morphological Characteristics		
1.	Gram staining	+
2.	Endospore	+
3.	Motility	+
4.	Cell shape	Rod
5.	Colony colour	Creamy white
6.	Colony shape	Circular, Irregular, Rough, Opaque
7.	Aerobic/Anaerobic	Aerobic
Biochemical Characteristics		
8.	Indole test	–
9.	Methyl red test	–
10.	Voges-Proskauer test	+
11.	Citrate Utilization test	+
12.	Dextrose	+
13.	Mannitol	+
14.	Lactose	+
15.	Fructose	+
16.	Sucrose	++
17.	Galactose	+
18.	Maltose	+
19.	Xylose	+
20.	Arabinose	+
21.	Starch	+
Enzymatic Characteristics		
22.	Catalase	+
23.	Oxidase	+
24.	Urease	–
25.	Amylase	+
26.	Gelatinase	+
27.	Chitinase	+
PGPR Characteristics		
28.	Phosphate solubilization	+
29	Potassium solubilization	+
30.	Zinc Solubilization	+
31.	Ammonia Production	+
32.	HCN production	+
33.	IAA production	175ug/ml
34.	Nitogen fixation	+
35.	Siderophore production	+

3.3 Microscopy of fungal mycelia:

Bacillus subtilis AM-7 (RSP2) inhibited fungal growth in a dual culture, as seen by dual culture method. The study employed phase-contrast microscopy to examine the relationship between the fungal pathogen *R. solani* and the highly promising strain AM-7 (RSP2) (Fig. 2b). A dual culture plate with AM-7 clearly demonstrated the reduction of apical hyphae growth in the presence of bacterial antagonist (Fig. 2g-h) Micrographs of the control treatment revealed undamaged mycelia (Fig. 2a).

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 14s, 2025
https://www.theaspd.com/ijes.php

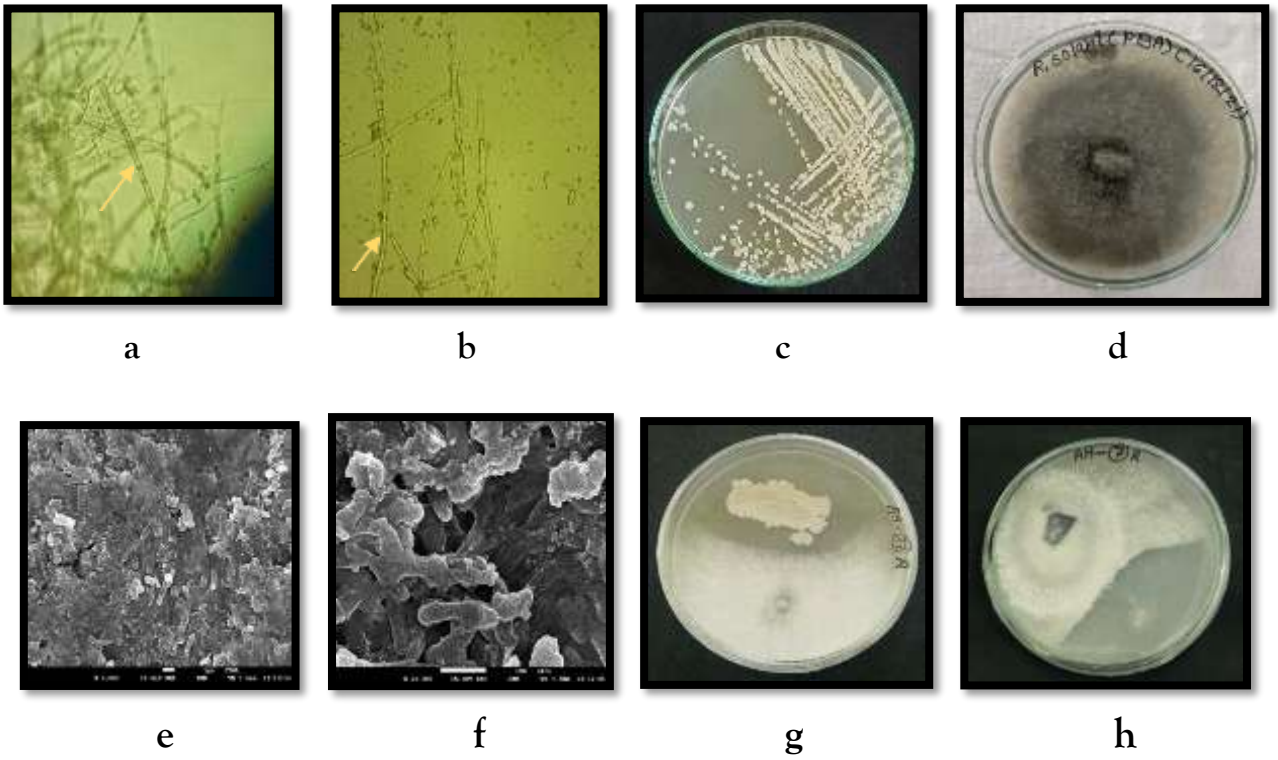


Fig 2: ***B****acillus subtilis* inhibits fungal growth using phase contrast microscopy in antagonism approach. **a,** phase contrast microscopy of control culture of *R. solani* **b** Hyphal deformation of *R. solani* was indicated by the yellow arrow. **c** Control culture plate of *B. subtilis* RSP2 (AM-7). **d** Control culture plate of *R. solani*. **e f** SEM images of *B. subtilis* RSP2 (AM-7). **g** & **h** inhibition effect of AM-37 & AM-7 against fungal growth.

3.4 Screening, Characterization & Identification of antagonistic isolates:

The ten soil samples reported total viable counts ranging from 5.92410 to 7.80183 log CFU/g soil in different ecological niches. The rice rhizospheric soil samples yielded a total of 85 rhizobacterial strains. Antagonism in vitro was tested for in all strains. 30 of the 85 isolates exhibited inhibitory action towards *R. solani*. A spectrum of antagonistic efficiency from medium to good was illustrated by the potent antifungal activity of the bacterial isolates with inhibition zones >20 mm. A potential new resource for the application and isolation of this efficient isolate for sheath-related bio-control has been suggested by the powerful antagonistic impact of *Bacillus subtilis* strain AM -7 (RSP2) against *R. solani*, which showed a 40% inhibition and the biggest inhibition zone of 42 mm.

The identity of strain AM-7 (RSP2) was confirmed as *Bacillus subtilis* through NCBI BLAST search and 16S rRNA gene annotation. Phylogenetic grouping of strain AM-7 (RSP2) and its closest *Bacillus* lineages was performed using the neighbour-joining method (Figure 3). The phylogenetic tree, constructed using the partial 16S rRNA sequence of AM-7 (RSP2), clustered it within a clade comprising *B. tequilensis* strain PNP_1 (OP048818), *B. subtilis* strain C16 (MH424579), *B. subtilis* BAB-2936 (KF853121), and *B. subtilis* strain RSP2 (MN733189). The 16S rRNA gene sequence of *B. subtilis* strain AM-7 (RSP2) was submitted to the NCBI database under accession number OQ726228. The phylogenetic tree based on the 16S rRNA sequences confirms the close evolutionary relationship of strain AM-7 (RSP2) with other *B. subtilis* strains.

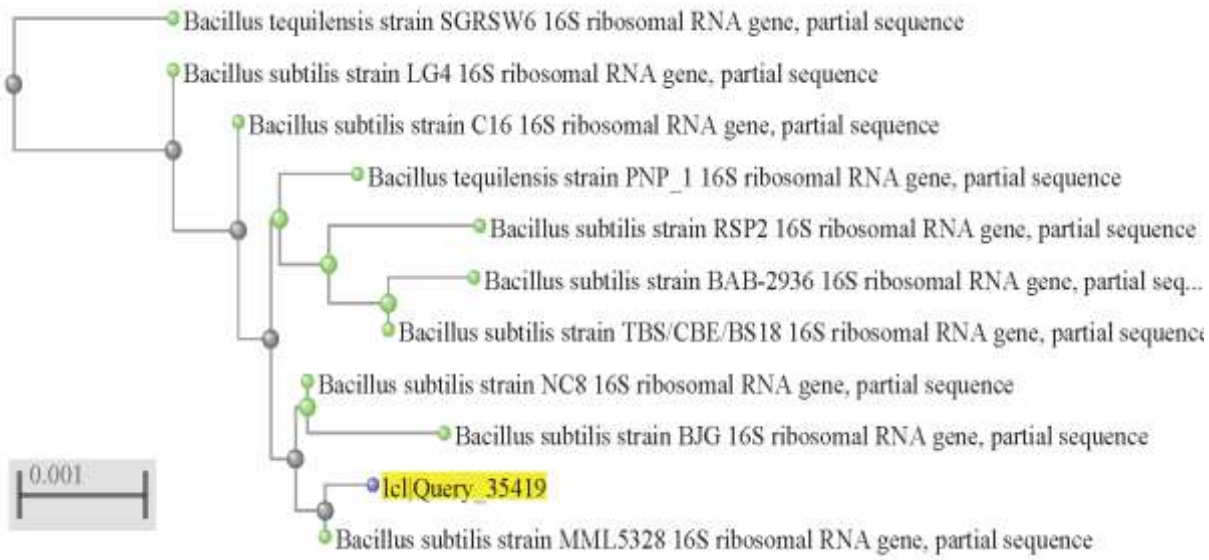


Fig 3: Phylogenetic tree based on 16s rRNA sequences of *B. subtilis* strain (RSP2).

3.5 PGPR activity by potential rhizobacterial isolates:

All isolates except AM-18,27,51,53, and 56 produced HCN; all isolates except AM-18,22,30,42,48,49,51,53, and 56 produced siderophore (fig 4). All the isolates were shown to only be capable of producing indole acetic acid. Out of 20 isolates tested, 8 isolates for P and 9 isolates each for Z and K solubilisation were found positive. NH3 production was reported in 11 isolates; nitrogen fixation in maximum 17 isolates; while chitinase was found produced by only two isolates. When data was interpreted isolate wise, it is evident from the table that out of nine activities, seven activities were reported positive in AM- 1& 4 (except K & chitinase); AM-35 (except P & chitinase); six activities were reported positive in AM-9 (except P, Z and chitinase); AM-22 (except siderophore, K & chitinase); AM -30 & 42 (except siderophore, NH3 & chitinase). However, isolates AM 7 and 37 were found to be positive for every activity. Out of the nine activities examined, isolates AM - 51, 53, and 56 were found to be positive for only two of them.

3.5.1 Optimisation of Indole acetic acid:

The optimization findings clearly indicate that the range of IAA production was 2.36 ± 0.033 to 93 ± 0.0471 µg at 26 °C, 4.5 ± 0.289 to 175 ± 1.155 µg at 37 °C, and 3.66 ± 0.088 to 96.2 ± 0.122 µg at 45 °C. Therefore, 37 °C was identified as the optimum temperature for maximum IAA production. Additionally, the IAA production ranged from 4.5 ± 0.289 to 175 ± 1.155 µg after 48 hours, 3.3 ± 0.333 to 94.8 ± 0.601 µg after 96 hours, and 9.03 ± 0.186 to 81.5 ± 0.252 µg after 144 hours of incubation. The highest IAA production (175 ± 1.155 µg) was recorded by isolate AM-7 after 48 hours, followed by 133.8 ± 0.601 µg by isolate AM-37. Thus, 48 hours was confirmed as the optimum incubation period for maximum IAA production. According to Table 4, isolate AM-7 demonstrated the highest IAA production at the optimal temperature of 37 °C and incubation time of 48 hours.

3.5.2 Percentage wise PGPR activities by Rhizobacterial isolates:

The results clearly indicate that 100% of the isolated strains tested positive for indole-3-acetic acid (IAA) production, 85% for nitrogen fixation, and 75% for hydrogen cyanide (HCN) synthesis (Table 5). However, only a few isolates (10%) exhibited positive chitinase activity (Figure 6). Among them, isolate AM-7 was found to be the most effective in promoting plant growth. This isolate was subsequently identified as *Bacillus subtilis*.

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 14s, 2025
https://www.theaspd.com/ijes.php

Table 4: Optimization of Indole Acetic Acid production by isolates.

Isolates	Conc. Of IAA (µg/ml) Incubation period for			Conc. Of IAA (µg/ml) Temperature at		
	48hrs	96hrs	144hrs	26°C	37°C	45°C
AM 1	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 4	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 7	175±1.155	94.8±0.601	81.5±0.252	93 ±0.0471	175±1.155	96.2 ±0.122
AM 9	126.6±0.882	70.5±0.289	38.2±0.153	22.1 ±0.115	126.6±0.882	20.1 ±0.088
AM 10	6.7 ±0.115	33.2±0.145	22.1 ±0.115	9.23 ±0.119	6.7 ±0.115	8.13 ±0.088
AM 11	4.5 ±0.289	3.3±0.186	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 15	7.03 ±0.088	5.1±0.153	38.16±0.12	2.36 ±0.033	7.03 ±0.088	3.66 ±0.088
AM 18	6.7 ±0.115	33.4±0.208	19.3±0.153	5.5 ±0.115	6.7 ±0.115	3.66±0.088
AM 22	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 25	7.03 ±0.088	7.1±0.153	38.16±0.12	2.36 ±0.033	7.03 ±0.088	3.66 ±0.088
AM 27	126.6±0.882	70.5±0.289	38.2±0.153	22.1 ±0.115	126.6±0.882	20.1 ±0.088
AM 30	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 35	101 ±0.577	49.2±0.145	38.2±0.145	76.2±0.145	101 ±0.577	41.2±0.12
AM 37	133.8±0.601	66.2±0.145	41.2±0.12	81.5±0.252	133.8±0.601	57.6±0.882
AM 42	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 48	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 49	4.5 ±0.289	3.3±0.333	9.03±0.186	3.23 ±0.145	4.5 ±0.289	7.2 ±0.057
AM 51	6.7 ±0.115	33.4±0.208	19.3±0.153	5.5 ±0.115	6.7 ±0.115	3.66 ±0.088
AM 53	66.2±0.145	43.3±0.203	40.2±0.12	5.63 ±0.066	66.2±0.145	24.3 ±0.173
AM 56	66.2±0.145	43.3±0.203	40.2±0.12	5.63 ±0.066	66.2±0.145	24.3 ±0.173

Table 5: Percentage wise PGPR activities by Rhizobacterial isolates.

Sr No.	Activity	No of isolates tested	No of isolates showing positive results	% of positive isolates	No of isolates showing negative results	% of negative isolates
1	HCN Production	20	15	75	05	25
2	Siderophore Production	20	11	55	09	45
3	IAA Production	20	20	100	-	-
4	P Solubilisation	20	8	40	12	60
5	Z Solubilisation	20	9	45	11	55
6	K Solubilisation	20	9	45	11	55
7	NH_3	20	11	55	09	45
8	N_2 Fixation	20	17	85	03	15
9	Chitinase	20	02	10	18	90

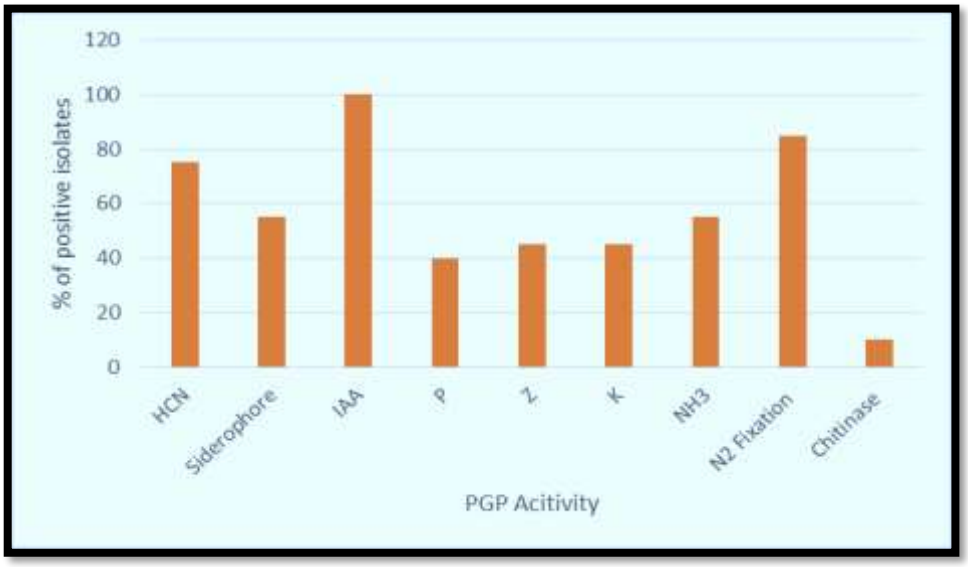


Fig 6: Percentage of PGPR activities by rhizobacterial isolates.

3.6 FTIR Analysis:

The crude extract of the isolated strain AM-7 (RSP2) exhibited prominent absorption bands in the FTIR spectrum at 3308.31, 2942.79, 2831.73, and 1114.95 cm^{-1} (Figure 7). These bands correspond to functional groups such as alkyne, amine salt, carboxylic acid, and secondary alcohol, respectively.

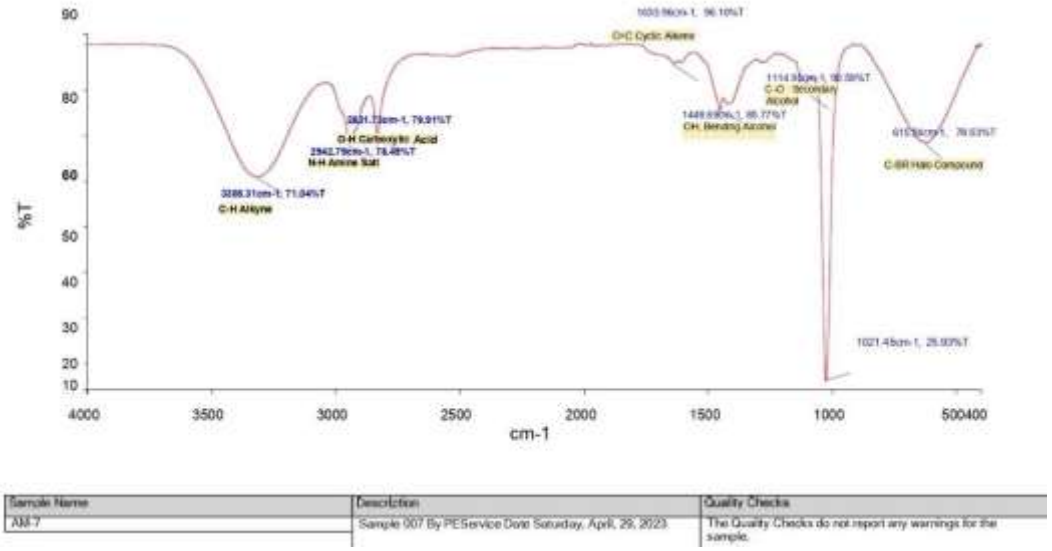


Fig 7: Fourier Transform Infrared Spectroscopy spectrum of Bacillus subtilis AM-7 (RSP2) crude extract.

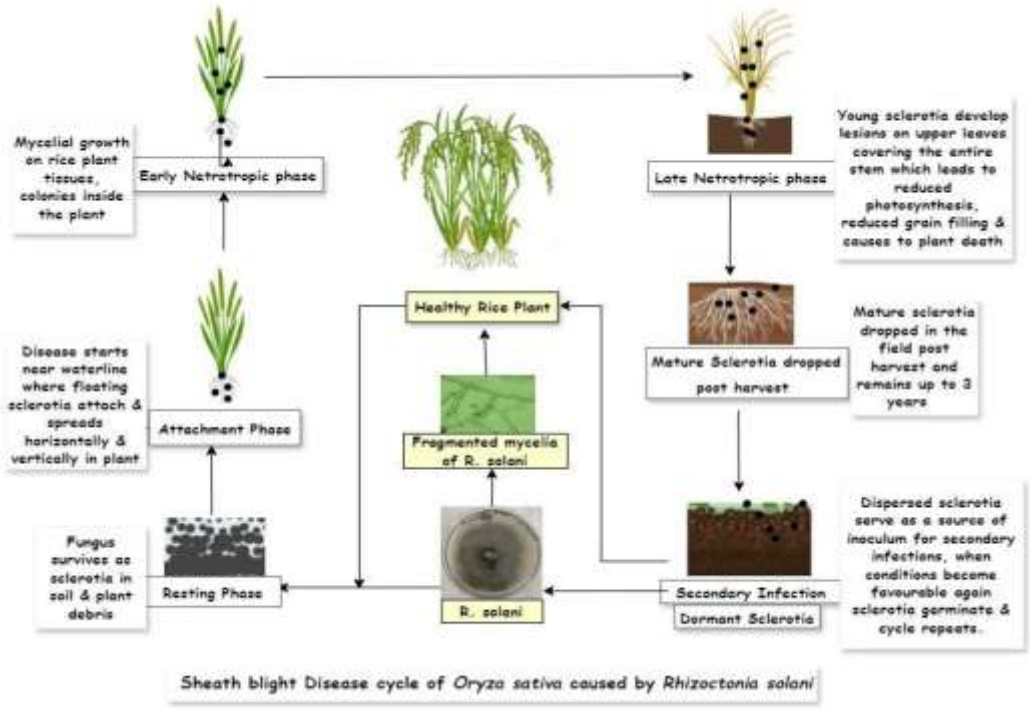
3.7 Pot assay: The outcome of the pot trial indicated the fact that AM-7 (RSP2) was the best strain to inoculate rice plants with in terms of PGP, as shown in Table 6 and Figure 8. shoot length, Grain count, fresh weight, root length, dry weight, and total chlorophyll content were all markedly raised by this strain. R. solani inoculation effectively regressed all growth parameters in rice seeds, but AM-7 (RSP2) inoculation increased plant growth (17.02 ± 0.0145) & (16.03 ± 0.0173) respectively and provided protection against them (**Fig. 8**). Following therapy, there was a notable rise in the number of grains, reaching (71.66 ± 0.6667). The estimation of overall chlorophyll concluded the significantly highest concentrations (1.2448 ± 0.0153 mg/g leaf fr. wt) and (1.2567 ± 0.0154 mg/g leaf fr. wt) in relation to the control & R. solani-treated seed, respectively (Table 6).

Fig 8: Pot assay demonstrated AM-7 (RSP2)'s effectiveness in alleviating the extent of the *R. solani* caused infections. Effect of *Bacillus subtilis* AM-7 (RSP2) treatment at 100 spores/ml on plant growth promotion in rice plants, with distilled water used as a control. An illustration of the shoot and root structure and general health of rice plants impacted by inoculation of bacterial strain AM-7 (RSP2) of seed plants, eighty days immediately following planting.

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 14s, 2025
https://www.theaspd.com/ijes.php

Treatment	SL (cm)	RL (cm)	FW (g/plant)	DW (g/plant)	TC (mg/g fresh weight)	Grain Number
Control	$26^{c} \pm 0.5777$	$14.73^{d} \pm 0.3711$	$12.81^{d} \pm 0.0548$	$4.32^{c} \pm 0.0284$	$0.748^{c} \pm 0.0189$	$41^{d} \pm 0.5773$
AM-7	$36.80^{a} \pm 0.4163$	$30.6^{a} \pm 0.3055$	$21.02^{a} \pm 0.0548$	$9.83^{a} \pm 0.0375$	$2.0869^{a} \pm 0.0119$	$70.33^{a} \pm 0.3333$
RS + AM- 7	$33.66^{ab} \pm 0.6666$	$25.33^{b} \pm 0.2027$	$17.02^{b} \pm 0.0145$	$7.88^{ab} \pm 0.0606$	$1.2448^{b} \pm 0.0153$	$71.66^{a} \pm 0.6667$
RS	$18.48^{e} \pm 0.2962$	$15.06^{d} \pm 0.667$	$9.54^{e} \pm 0.0208$	$1.65^{d} \pm 0.2875$	$0.2002^{e} \pm 0.0121$	$31.66^{e} \pm 0.3333$

Table 6: Shoot length (SL), root length (RL), fresh weight (FW), dry weight (DW), total chlorophyll (TC), grain number (GN) and *Rhizoctonia solani* (RS) are some examples of plant terminology. Every value represents the triple mean ± standard error (SE) of each value. Repeatedly occurring letters after a mean do not significantly alter at $p < 0.05$.



4. DISCUSSION:

Numerous isolated *Bacillus* strains exhibit anti-phytopathogenic fungal activity, making them promising candidates for biocontrol (Pospiech A, Neumann B, 1995). Previous studies on biocontrol of rice sheath blight were conducted by (Boukaew et al. 2013). Through their investigation, they found that Streptomyces philanthi and a commercially available formulation of *Bacillus subtilis* were physiologically potent over rice sheath blight when combined with conventional chemical fungicides. According to (Angel Jenifer et al. 2013), isolates from tomato and paddy rhizospheric soil samples were screened and identified as such when *Azotobacter sp*., *Bacillus sp*. and *Pseudomonas sp*. were assessed for their antifungal properties against the fungal isolates. They stated that they have 90.5% inhibition against *Rhizoctonia spp*. In the present study, strain *B. subtilis* AM-7(RSP2) produces phosphate, ammonia, hydrogen cyanide, nitrogen-fixing activity, chitinase, IAA, and siderophore, it has strong antifungal activity (42 mm zone of inhibition) against the fungal pathogen *R. solani* in the current study. Using a suitable screening technique is crucial for the identification of biocontrol agents. The synthesis of structurally varied antibiotics with a broad spectrum of antifungal action and the capacity to alter other microbes' adhesion to different surfaces is one of the most compelling features of *Bacillus spp*. that contribute to the biocontrol mechanism.

The mVOC 2.0 database now has information on over 2,000 chemicals produced by almost 1,000 different kinds of microbes (Lemfack et al., 2018). This database indicates that chemicals containing sulphur and nitrogen make up the majority of documented Bacillus volatile organic compounds (VOCs), with fatty acid derivatives (alcohols,

ketones, alkanes, aldehydes, alkynes, and acids) accounting for about 70% of the total. While the precise mechanism of action remains unclear at this time, the FTIR analysis findings showed the synthesis of hydrolytic and antibacterial enzymes. Crude compound FTIR examination identified functional groups such secondary alcohol, alkynes, and carboxylic acid. Meanwhile, (Liu et al. 2008) reported strong antifungal activity for carboxylic acid group-containing propanoic acid and octadecanoic acid, while (Raza et al. 2016) reported strong antifungal activity for oleic acid; (Liu et al. 2008) also reported strong antifungal activity for volatile metabolites such as secondary alcohol 3,4-dimethyl-5-hexen-3-Heptanol and Alkynes 1H-indine-1 methylene (Caulier S et. al. 2019).

With a wide range of antagonistic activity against the pathogenic fungi and PGPR features, AM-7 (RSP2) stood out among the 85 rice rhizosphere isolates studied in this study as having the most potential in vitro. Numerous isolated *Bacillus* strains have demonstrated efficacy against phytopathogenic fungi, rendering them promising candidates for biocontrol applications. The synthesis of ammonia, hydrogen cyanide, phosphate, nitrogen-fixing activity, chitinase, IAA, and siderophore by strain *B. subtilis* AM-7 (RSP2) is primarily responsible for the investigations remarkable antifungal effect (42 mm zone of inhibition). To identify biocontrol agents, a proper screening system must be used. Among the most intriguing features of *Bacillus spp*. that support the biocontrol mechanism is their ability to produce structurally varied antibiotics with a wide range of antifungal activity and the capacity to alter other organisms' adherence to various surfaces.

Antagonistic bacteria are the foremost significant biological control agents for crop diseases. Eleven bacterial isolates from rice were previously isolated by Ester et al. These isolates were chosen based on their antagonistic activity and characteristics that promoted crop development. A total of 31 endophytic bacterial species were isolated by Dalal and Kulkarni from various parts of the soybean (cv. JS-353) at several development phases, including the roots, stem, leaves, and root nodules. The bacteria were then screened for their ability to promote (PGPR) activity and their antagonistic action against isolated soil-borne fungal pathogens.

Since all eight isolates of *Bacillus cereus*, two isolates of *B. subtilis*, & *B. mojavensis* were found to synthesize HCN, our results of HCN production are consistent with those of Alemu (2016). Therefore, 75% of the tested *Bacillus* isolates were found to produce HCN. According to Gupta et al. (2015) and Mahalaxmi & Reetha (2009), our results for Bacillus' phosphate solubilizing capacity are consistent with their findings. Our findings show that zinc solubilization has been seen in most *Bacillus sp*. isolates, indicating a 45% total isolate percentage compared to earlier research on rice rhizobacteria.

According to earlier studies, using KSB as biofertilizers to enhance agricultural activities may lower the usage of agrochemicals & promote the production of ecologically sustainable crops (Archana et al., 2013; Prajapati et al., 2013). Comparatively speaking to earlier research, we have documented KSB from *Bacillus*, *Enterobacter*, and *Lysinibacillus*. We have also isolated N2 fixing and NH3 generating bacteria from the rice rhizosphere. These outcomes are equivalent with those findings reported by Chaiharn et al. (2008) & Ahmed et al. (2014), as we were only able to obtain chitinase activity in 10% of the isolates, and both isolates, AM - 7 and AM -37, were tested to be positive with respect to the solubilization of phosphate and chitinase activity. While Shabana Ehsan et al. (2022) reported siderophore activity in 30% of isolates, our data shows that siderophore manufacturing ability is present in 55% of isolates.

Bacillus antagonistic activity is corelated with the production of several distinct peptides with antimicrobial properties (Falardeau J, et al. 2013; Kim PI et al. 2010), extracellular secreted enzymes (Baysal O, et al. 2013), proteins components (Tan S, et al. 2013), & volatile organic compounds (VOCs) (Baysal O et al. 2013; Choudhary DK, et al. 2009). The FTIR investigation findings revealed the creation of hydrolytic and antibacterial enzymes, albeit the specific framework of procedure is remains unclear. Conversely, experiments accomplished by Cheng et al. (2008) and Ahmadzadeh et al. (2006) stated strong antifungal activity against volatile metabolites such hydrogen cyanide and α-methylcinnamic acid, or cinnamon aldehyde, which has an aldehyde group.

5. CONCLUSIONS:

Even though the variety of plant growth promoting activities differ from one rhizobacteria to another, IAA production is a common activity shown by all rhizobacteria while chitinase production is attributed to very

selected few organisms. The current study indicated that the isolate, AM-7 (RSP2), are highly effective in producing metabolites that promote plant growth. It was discovered that B. subtilis's crude supernatant had potent antifungal activity against R. solani, the significantly prevalent plant fungal pathogen that inflict root rot & sheath blight on rice plants. After further research, it was revealed that the volatile chemical substances released by Bacillus subtilis had strong inhibitory effects on Rhizoctonia solani, suggesting that it could be utilised as a chemical pesticide substitute. According to the overall findings, B. subtilis AM-7 (RSP2) is the most effective antagonistic strain that produces the greatest outcomes. Since it is safer for the environment and less expensive for farmers to employ than synthetic herbicides, the AM-7 (RSP2) strain has several essential features that stimulate plant growth and might be used as a crucial component in the regulation of root rot & sheath blight infection in rice. It also poses no health risks to humans. Nevertheless, the effectiveness of biological practises for managing plant infections and promoting plant development hinges on how well they function in actual plant environments. This well-known antagonistic bacterium is the preferred selection for additional research into the biological control of the pathogens R. solani.

ACKNOWLEDGEMENT:
I am extremely grateful to the Principal, Dr. Vinay Chavhan, and the Head of the Microbiology Department, Dr. Alok Rai, of the Seth Kesarimal Porwal College of Arts, Science, and Commerce, Kamptee, Nagpur, for providing the laboratory and library facilities as well as their insightful guidance during this research project.

CONFLICT OF INTEREST:
In accordance with the authors, they have no conflicts of interest.

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