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STUDIES ON THE MANAGEMENT OF BROWN SPOT IN RICE BY USING BACTERIAL BIO-FUNGICIDE

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ABSTRACT

The present studies were undertaken to investigate the effect of bacterial bio-fungicide against brown spot in rice. The bacterial isolates of *Bacillus subtilis* were able to be isolated from rice rhizosphere in five different localities of Villupuram District, Tamil Nadu. Among the bacterial isolates, the isolate *B. subtilis* BS 3 showed a significantly inhibited the growth of brown spot pathogen *Bipolaris oryzae* when compared to control treatment. The conidial germination of *B. oryzae* was found to be decreased with an increase in the concentration of culture filtrates of bacterial isolate *B. subtilis*. A maximum conidial germination was inhibited by culture filtrate of *B. subtilis* at 20% against *B. oryzae* under cavity slide method and Poisoned food technique. In carrier formulation of bacterial antagonistic agents, talc powder was found to be significantly more effective in its maintenance of a bacterial antagonistic population than any other carrier material. It was followed by Lignite powder, Peat soil, lignite fly ash and kaolin carrier materials in the decreasing order of merit. Among the various dosages of *B. subtilis* tested for seed treatment, rice seeds treated @10 g/ kg of seeds showed a maximum germination percentage, root length, shoot length and vigour index and reduce the incidence of brown spot in rice var. ADT 43. Also, a maximum bio-efficacy was observed on foliar application of *B. subtilis* @ 2.5 kg/ha. The increasing quantity of foliar spray build up the phyllosphere population of *B. subtilis* and the highest number of bacterial colonies were observed in 2.5 kg/ha.

Key words*: *Rice, Brown spot, Bacillus, Antagonists

Introduction

Rice (*Oryza sativa* L.) is an important staple food for many Asian countries. For more than half of the humanity 'rice is life' (Gnanamanickam, 2009) and hence is called as 'grain of life'. Over 90 per cent of the rice in the world is produced and consumed in Asian countries and rice is serving as the pillar for food security in many developing nations (Channakeshava and Pankaja, 2018). Rice is used in starch and brewing industries the by-products of rice milling, rice husk and bran are used as cattle and poultry feed. Rice provides more than one fifth of calories consumed by the human's worldwide (Jatoi *et al.*, 2018). Besides rice is used as rice flakes, puffed rice, rice wafers and canned rice (Haque *et al.*, 2022). Rice crop is affected by many fungal, viral and bacterial diseases, some may cause little amount of loss but some causes heavy yield losses. A significant rice disease in the world is brown spot of rice caused by *Helmenthosporium oryzae* (teleomorph = *Cochliobolus miyabeanus*), which reduces yield by 26% to 52% (Kumar and Srivastava, 2019). Brown spot is a one kind of fungal disease that can affect certain parts of rice plant such as panicle branches, leaf sheath, leaves, coleoptile, spikelets and glumes. The disease is also mentioned as sesame leaf spot, fungal blight, Helminthosporium blight, Glume blotch, Sesame leaf blight, seedling blight and Helminthosporiose.

Brown spot has been associated with two major

epidemics in India (Chakrabarti, 2001) the first in 1918–19 in the Krishna-Godavari delta and the second, during 1942 i.e. in India (Bengal) which resulted in a yield loss of 50-90%. The later of which has been associated with the Great Bengal Famine and death of 2 million people due to food starvation (Chakrabarti, 2001). Brown spot is soil, seed, and air borne (Laha *et al.*, 2017) disease which causes both quantitative and qualitative losses (Hosagoudar *et al.*, 2019).

At present, predominant mean for management of crop diseases are the use of synthetic chemicals. Fungicides, such as Iprodione, Propiconazole, Azoxystrobin, Trifloxystrobin, and Carbendazim have been reported as an effective means to manage the disease (Gupta *et al.*, 2013). Inspite of the availability of chemicals for the effective management of brown spot disease, continuous, inappropriate and indiscriminate use of chemicals is known to cause undesirable effects such as residual toxicity, development of chemical resistance, environmental pollution, health hazards to humans and animals and increased expenditure for plant protection. Therefore, application of beneficial microbes for the management of plant diseases has emerged as a viable alternative in the recent past.

Biocontrol using antagonists appears to be one of the most promising approaches for eco-friendly and green agriculture to protect crop plants. The BCAs must be effective to give high crop yields and good crop quality and provide an economic incentive to the end-users compared to other disease management approaches (He *et al.*, 2021). Antagonistic bacteria belonging to the genus of *Bacillus* and *Pseudomonas* sp. have been widely used for the management of brown spot of rice. *Bacillus* sp. has also been used successfully as a biocontrol agent against a number of diseases (Kloepper *et al*., 2004). Among all, *Bacillus subtilis* is the most commonly used bacterial biocontrol agent, because of its high adaptability and sustainability in soil under diverse environmental conditions. Furthermore, its possession of multiple biocontrol mechanisms enables it to overcome phytopathogenic defenses (Porto *et al.*, 2022). The present studies were undertaken to investigate the effect of bacterial antagonists against brown spot in rice.

Materials and Methods

Isolation of the Bacterial antagonist

Rhizosphere colonizing bacterial inoculant *Bacillus* were isolated from soil region of rice cultivated in Thennamadevi, Venganthur, Thuravi, Pidaripattu and Devanur in Villupuram district of Tamil Nadu, India. The soil samples from these above areas were tested out and used for the *Bacillus* isolation. A microbial suspension was prepared from each location by shaking one gram of the sample into 10 ml of sterile distilled water fifteen minutes. Subsequently, the samples were subjected to the serial dilution method. Following this, bacterial colonies were isolated using Nutrient Agar medium and an abundance of rough colonies with irregular, waxy edges of bacterial growth was observed. From each petri dish, a single bacterium was selected and streaked onto microbial slants containing Nutrient Agar and subcultures were made. The bacterial isolates underwent biochemical characterization and identification, following the guidelines outlined in Bergey's manual for determinative bacteriology (Breed *et al.*, 1989).

Biochemical characterization

Gram staining:

The process began by smearing a bacterial bio control agent onto a slide measuring 75 × 25 mm. The slide was dried in the air and fixed with heat. Next, the smear was flooded with crystal violet for approximately sixty seconds and washed with distilled water. Afterwards, Lugol's iodine solution was added, and waited for 30 seconds, then the smear was decolorized using ethyl alcohol (95%). The slide was once again rinsed with water and counter stained agent with safranin for thirty seconds. Finally, the slide was washed with distilled water and allowed to dry before being observed under a microscope. Bacterial cells that appeared red were identified as Gram

Plate 1: Rice Brown spot infected leaf.

negative, while those that appeared violet were identified as Gram positive (Cyrabree and Hindshill, 1975).

Motility test:

The designated antagonistic organism inoculated in semisolid agar medium was prepared and incubated under room temperature condition for three days. After this incubation period, the stage of motility of the organism was observed (Ragavan, 2003).

Potassium hydroxide (KOH) test/ String test (Suslow *et al.*, 1982):

A solution containing 3% potassium hydroxide (KOH) was placed on glass slide that had been previously coated with an antagonistic agent. The KOH solution was thoroughly mixed with an inoculation needle. When observing the resulting slide, it was found that gram negative bacteria displayed thick, elongated strands that appeared stringy in nature. Conversely, gram positive bacteria did not exhibit any stringing characteristics.

Starch hydrolysis (Stolpe and Godkeri, 1981):

A suspension of old antagonistic bacteria was made and a filter paper disc was immersed in it. The disc was then carefully placed onto petri plates that were filled with SA medium, also known as Starch Agar and incubated for approximately forty-eight hours. Following this period, the petri plates were flooded with 1% iodine solution. As a result, a translucent halo was observed around the bacterial colonies, indicating their ability to utilize starch, while the rest of the petri plates turned blue due to the presence of the iodine solution.

Gelatin liquefaction (Stolpe and Godkeri, 1981):

An old antagonistic bacterial suspension was prepared and a filter paper disc was immersed into the suspension. These soaked discs were then positioned in a Petri dish containing Nutrient gelatin (Nuteint agar mixed with Gelatin) medium. The Petri dishes were incubated for two days at room temperature. After the incubation period, the Petri dishes were treated with a solution of $AgCl_2$, also known as Silver Chloride, at a concentration of 12.5%. The results showed that the presence of a yellow halo surrounding the bacterial colonies indicated that the gelatin had been utilized by the bacteria.

Plate 2: Grain discolouration.

Methyl Red test (Borkar, 2017):

The MR-VP (Methyl Red and Voges Proskauer) broth was prepared by combining Buffered Peptone – 7.00 g/lit., Glucose – 5.00 g/lit., Dipotassium Peptone – 5.00 g/lit and Distilled water – one lit. This mixture was taken in test tubes and inoculated with the antagonistic bacterial isolates. The tubes were incubated at a temperature of 99.5°F (37.5°C) for 96 hours. After the incubation period, five drops of the methyl red indicator (chemical formula - C15H15N3O2) were added to the test tubes. Positive results were indicated by the appearance of a red color.

Hydrogen sulfide (H_2S) production test (Borkar, 2017):

Pre-sterilized tubes containing SIM (Sulfide Indole Motility) medium were streaked with the test cultures all along the walls of the test tubes. Inoculated tubes were incubated for 48 hrs at 98.6°F (37°C). After incubation, the development of black colour along the line of the stab was noted as positive for the test.

Catalase test (Shankara *et al.*, 2017):

A loopful of 48 hr. slant growth of the test bacterium was smeared on a clean glass slide and was covered with few drops of hydrogen peroxide. If gas bubbles are produced, the reaction will be positive.

Plate 3: Auxenic culture of *B. oryzae*.

Casein hydrolysis (Reynolds, 1979):

Milk powder was added to nutrient agar media. Test bacteria was streaked on the petri plates and incubated for 24-48 hrs. Plates were observed for clear zone around the bacterial colonies.

IAA Production test (Ahmad *et al.*, 2005):

All the test bacterial culture was inoculated in nutrient broth with tryptophan (0.2%) or without tryptophan incubated at 28 ± 2°C for 15 days for 7 days. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). Development of a pink colour indicates IAA production.

HCN Production test:

Production of HCN was determined as per Wei *et al.*, (1996). Bacteria were grown on TSA supplemented with 4.4g/l of glycine, white filter paper strips soaked in picric acid solution (2.5 g of Na_2CO_3 and 1 lit. of water) were placed in the lid of each Petri dishes, sealed with parafilm and incubated for two to three days at 28±2°C. After incubation HCN production was indicated by the presence of a coloured zone around the bacteria.

Urease activity test (Borkar, 2017):

Nutrient agar medium with urea were inoculated with test bacteria. A clear zone around the colonies indicates the positive result.

Carbon sources utilized by antagonistic bacterium (Pranaya *et al.*, 2020)

The antagonistic test bacterium was inoculated into test tubes filled with Nutrient Broth (NB) with various types of carbon sources including Glucose, Sucrose, Maltose and Lactose. Additionally, a small amount of PSP (Phenolsulfonphthalein / Phenol red) was added in the broth. The inoculated test tubes were placed in room temperature for 48 hours. The presence of a positive reaction was indicated by the appearance of a yellow color.

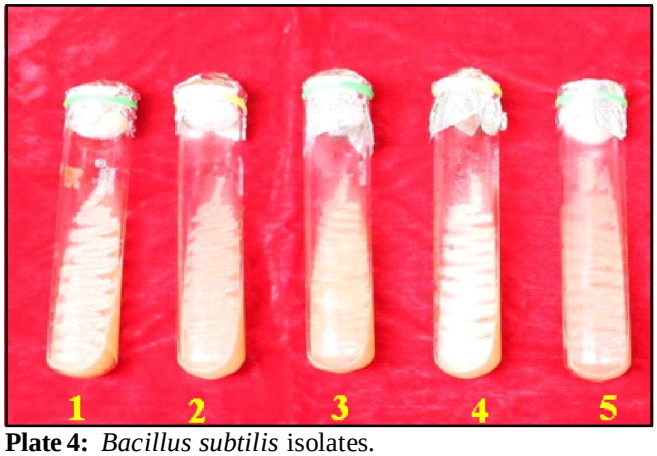

Plate 4: *Bacillus subtilis* isolates.

***In vitro* efficacy of *B. subtilis* against *B. oryzae* (Bo_7) - Dual culture technique (Dennis and Webster, 1971)**

The effectiveness of different strains of *B. subtilis* (named BS 1, BS 2, BS 3, BS 4, BS 5) was tested by streaking them to one side of a petri dish containing PDA medium. At the opposite side, one week-old culture of the most virulent strain of *B. oryzae* (Bo7) was placed. After a week of incubation, the diameter of fungal growth caused by *B. oryzae* was measured in millimeters, and the percentage of inhibition over control was calculated. The per cent inhibition of *B. oryzae* fungal growth was evaluated according to Vincent (1927).

$$PI = CD\text{-}TD / CD \times 100$$

Where,

PI = Inhibition per cent of *B. oryzae*,

CD = Diameter of *B. oryzae* mycelial growth in control and;

TD = Diameter of *B. oryzae* mycelial growth in treatment

Effect of *B. subtilis* on *B. oryzae* (Bo_7) - Poisoned food technique (Grover and Moore, 1962)

Different volumes (5, 10, 15 and 20 ml) of antagonistic culture filtrates of *B. subtilis* were mixed with 100 ml of sterilized PDA medium in order to achieve final concentrations of 5%, 10%, 15% and 20% respectively and poured into separate petri dishes. Control plate without *B. subtilis* culture filtrate was maintained. Inoculated petri dishes were incubated at room temperature until the pathogenic fungal growth in the control treatment covered the entire petri plate. The diameter of the fungal growth

Plate 5: Virulent culture of *B. subtilis*.

of the pathogen was measured for each concentration of antagonistic filtrate.

Effect of *B. subtilis* on *B. oryzae* (Bo_7) - Cavity slide method

Different volumes (5, 10, 15 and 20 ml) of antagonistic culture filtrates from the virulent *B. subtilis* isolate (BS 3) were added to 100 ml of sterile distilled water. A single drop of the *B. subtilis* isolate at each concentration was placed in a cavity slide. The pathogenic culture of *B. oryzae* (Bo7) was placed in the solution. A cover slip was placed on the cavity slide and the treatment slides were incubated under room temperature conditions. The percentage of *B. oryzae* conidiation was measured on 1st, 2nd and 3rd day of incubation (Ann *et al.*, 2015).

Evaluation of plant growth promotion activity of *B. subtilis* (BS 3) isolate by roll towel method (ISTA, 1993)

The effectiveness of a virulent isolate of talc-based formulation *B. subtilis* (BS 3) in promoting plant growth was tested using the ISTA (International Seed Testing Association) roll towel method. This method involves treating rice seeds with varying dosages of the *B. subtilis* formulation (2.5, 5.0, 7.5 and 10.0 g per kg of ADT 43) and assessing the germination percentage. Each dosage was tested using 25 treated rice seeds, which were placed on a pre-soaked seed germination sheet. Another germination sheet was placed on top and gently pressed to keep the seeds equidistant. The sheets along with the rice seeds were rolled up and incubated for two weeks in a growth chamber. Seeds treated only with water served as control. Three replications were maintained for each treatment including control. The percentage of germinated rice seeds, root and shoot length of seedlings were measured and the vigour index was calculated as per the standard formula given by Abdul-Baki and Anderson (1973).

Plate 6: Pot culture treatment.

Table 1: Isolation and morphological characters of *Bacillus subtilis* isolates.

Sr.	Locality	Isolates	Colony Morphology
1.	Venganthur	BS 1	White with serrated margin
2.	Vakkur	BS 2	Whitish grey with serrated margin
3.	Muttathur	BS 3	White with serrated margin
4.	Devanur	BS 4	White with serrated margin
5.	Thumbur	BS 5	Whitish grey with serrated margin

Vigour index of rice seedlings = (Mean root length + Mean shoot length of rice seedlings in cm) × Germination Percentage of rice seeds

Survival of *B. subtilis* (BS 3) in different carrier materials

The most virulent strain of *B. subtilis* (BS 3) was grown on Nutrient Broth (NB). A loopful of this strain was inoculated into sterilized NB and placed in a rotary shaker at room temperature for 48 hours. The viability of *B. subtilis* (BS 3) in various carrier materials including talc, peat soil, lignite, lignite fly and kaolin was assessed using standard protocols. These materials were powdered, sieved and placed in individual polypropylene bags (with a capacity of 200 g per bag) and sealed. The bags containing the carrier materials were autoclaved at 121.6°C and 15 psi for 30 minutes for three consecutive days. The pH of the carrier materials was adjusted to a neutral level using $CaCO_3$. The carrier materials were mixed with a two-day-old inoculum of *B. subtilis* (BS 3) (120 ml with 10^9cfu/ml of bacterial colonies) and thoroughly incubated at room temperature. Samples of the carrier materials were collected at 0, 30, 60 and 90 days after incubation to assess the microbial viability. The quantity of *B. subtilis* (BS 3) bacterial colonies was determined using the serial dilution method.

Results

Isolation, Morphological and biochemical characterization of *Bacillus subtilis*

Five isolates of *B. subtilis* were able to be isolated from rice rhizosphere in different localities of Villupuram District. The colonies of *B. subtilis* isolates appeared white to whitish grey in color with serrated margins. The identified isolates were designated as BS 1 to BS 5 (Table 1). Various tests were conducted to identify the effective native isolates of *B. subtilis*. All the bacterial isolates produced positive result in Gram staining, Starch hydrolysis, Gelatin liquefaction, Casein hydrolysis, IAA production test, HCN production test, Catalase test, Glucose utilization, sucrose utilization. They produce negative result in Pigment production, KOH test, Methyl red test, H2S test, Lactose utilization, Maltose utilization and Urease activity (Table 2). The results of biochemical

Table 2: Biochemical characteristics of *Bacillus subtilis* isolates.

Sr.	Characters	BS 1	BS 2	BS 3	BS 4	BS 5
1.	Gram-strain reaction	+	+	+	+	+
2.	Motility test	+	+	+	+	+
3.	KOH test	-	-	-	-	-
4.	Starch hydrolysis	+	+	+	+	+
5.	Gelatin liquefaction	+	+	+	+	+
6.	Methyl Red test	-	-	-	-	-
7.	H2S test	-	-	-	-	-
8.	Casein hydrolysis	+	+	+	+	+
9.	IAA production	+	+	+	+	+
10.	HCN production	+	+	+	+	+
11.	Catalase test	+	+	+	+	+
12.	Glucose utilization	+	+	+	+	+
13.	Sucrose utilization	+	+	+	+	+
14.	Maltose utilization	-	-	-	-	-
15.	Lactose utilization	-	-	-	-	-
16.	Urease activity test	-	-	-	-	-
+ indicates positive, - indicates negative						

tests compared with Bergey's manual of Determinative Bacteriology.

In vitro efficacy of *B. subtilis* against *B. oryzae* (Bo_7) (Dual culture)

In general, all the native *B. subtilis* tested inhibited the mycelial growth of *B. oryzae* (Table 3). However, among the isolates, the isolate BS 3 showed a maximum inhibition and significantly inhibited the growth of *B. oryzae* (29.48 mm) which was 67.24 per cent reduction on the growth of the pathogen when compared to control. A least growth inhibition of the pathogen (48.63%) was exhibited by the isolate BS 2.

Effect of Culture filtrate of *B. subtilis* on conidial germination of *B. oryzae* (Cavity slide method)

The effect of culture filtrate of *B. subtilis* on conidial germination of *B. oryzae* were examined and the results

Table 3: *In vitro* efficacy of *B. subtilis* against *B. oryzae* (Bo_7) (Dual culture).

Sr.	Isolates	Colony Diameter (mm)	Percent Inhibition Over Control (%)
1.	BS 1	33.65	62.61^{b} (52.31)
2.	BS 2	46.23	48.63^{e} (44.21)
3.	BS 3	29.48	67.24^{a} (55.08)
4.	BS 4	41.12	54.31^{d} (47.47)
5.	BS 5	37.78	58.02^{c} (49.61)
6.	Control	90.00	–
* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)			

Table 4: Effect of culture filtrate of *B. subtilis* on *B. oryzae* (Bo_7) conidial germination (Cavity slide method).

Sr.	CCF	Conidial germination (%) *		
		1st day	2nd day	3rd day
1	5	26^{d} (30.65)	34^{d} (35.66)	39^{d} (38.64)
2	10	21^{c} (27.27)	24^{c} (29.33)	27^{c} (31.30)
3	15	12^{b} (20.26)	15^{b} (22.78)	18^{b} (25.10)
4	20	8^{a} (16.42)	10^{a} (18.43)	12^{a} (20.26)
5	Control	58^{e} (49.60)	70^{e} (56.78)	88^{e} (69.73)
CCF: Conc. of culture filtrate; * Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)				

are summarized in Table 4. The conidial germination of *B. oryzae* was found to be decreased with an increase in the concentration of culture filtrates of *B. subtilis*. A maximum conidial germination was inhibited by culture filtrate of *B. subtilis* at 20%. It was followed by 15% conc. of the culture filtrate of *B. subtilis.* The 5% conc. of the culture filtrate was found to be the least effective.

Effect of culture filtrate of *B. subtilis* (BS 3) on fungal growth of *B. oryzae* (Bo_7) (Poisoned food technique)

The results of the *in vitro* studies conducted to find out the effect of culture filtrate of *B. subtilis* (BS 3) on the mycelial growth of *B. oryzae* are compiled in Table 5. The mycelial growth of *B. oryzae* was found to be decreased with an increase in the conc. of culture filtrates of *B. subtilis*. Culture filtrate of *B. subtilis* at 20% inhibited the mycelial growth of *B. oryzae* upto a greater extent.

Survival of *B. subtilis* (BS 3) in different carrier materials

From the first day to three months, the survival capacity of the antagonist in talc powder, lignite, peat

Table 5: Effect of culture filtrate of *B. subtilis* (BS 3) on fungal growth of *B. oryzae* (Bo_7) (Poisoned food technique).

Sr.	*B. subtilis* conc. of culture filtrate (%)	Mycelial growth (mm) *	Percent inhibition over control
1	5	61.25^{d}	31.94 (40.11)
2	10	39.90^{c}	55.66 (49.85)
3	15	17.44^{b}	80.62 (64.55)
4	20	4.51^{a}	94.98 (77.05)
5	Control	90.00^{e}	—
* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)			

Table 6: Survival of *B. subtilis* (BS 3) in different carrier materials.

Sr.	Carrier materials	Population (× 10^6 cfu/g) *			
		0 days	30 days	60 days	90 days
1	Talc powder	73.1[a]	65.7[a]	57.6[a]	42.8[a]
2	Lignite powder	70.5[b]	58.3[b]	51.2[b]	36.1[b]
3	Peat soil	67.9[c]	55.1[c]	44.1[c]	31.5[c]
4	Lignite fly ash	64.7[d]	49.5[d]	39.4[d]	24.9[d]
5	Kaolin	50.3[e]	31.0[e]	35.1[e]	20.4[e]

* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

soil, lignite fly ash and kaolin has been determined. In particular, talc powder was found to be significantly more effective in its maintenance of a bacterial antagonistic population of 42.8 × 10^6 g^{-1} than any other carrier material (Table 6). It was followed by Lignite powder, Peat soil, lignite fly ash and kaolin carrier materials in the decreasing order of merit. A minimum *B. subtilis* (BS 3) antagonistic microbial population was noted in Kaolin carrier material.

Evaluation of rice crop growth promotion activity of antagonistic agent by roll towel method

The results depicted in Table 7 stated that when compared to the control treatment, all dosages of antagonistic bio control agent *B. subtilis* (BS 3) treated seeds increased the germination percentage and improved plant growth. Among the various dosages of *B. subtilis* tested, rice seeds treated @10 g/ kg of seeds showed a maximum germination percentage (92.41%), root length (16.87 cm), shoot length (12.24 cm) and vigour index (2690.05) of rice var. ADT 43. It was followed by *B. subtilis* treated at 7.5 g / kg of seeds showed germination percentage (81.73%), root length (15.25 cm), shoot length (11.87 cm) and the vigour index (2216.51). The least level of germination percentage, root, shoot length and vigour index was noticed at control treatment.

Table 7: Influence of different doses of talc-based *B. subtilis* (BS 3) inoculum on plant growth promotion (Roll towel method) of rice var. ADT 43.

Sr.	DTI	GP	RL	SL	VI
1	2.5	69.14[d] (56.25)	13.14[d]	10.47[d]	1632.39[d]
2	5.0	74.32[c] (59.55)	14.65[c]	11.23[c]	1923.40[c]
3	7.5	81.73[b] (64.69)	15.25[b]	11.87[b]	2216.51[b]
4	10.0	92.41[a] (74.00)	16.87[a]	12.24[a]	2690.05[a]
5	Control	66.15[e] (54.42)	11.31[e]	08.56[e]	1314.40[e]

DTI: Dosage of talc-based inoculum(g/kg of seed); GP: Germination percentage (%) ; RL: Root length (cm) *; SL: Shoot length (cm)*; VI: Vigour index *
* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Effect of different doses of talc-based formulation treated to rice seeds on the brown spot incidence (Pot culture)

The data depicted in Table 8 revealed that treating rice seeds with different doses of talc-based *B. subtilis* (BS 3) provide better control of *B. oryzae* when compared to control. Among them, a maximum effect was noticed in rice seeds treated with *B. subtilis* at a rate of 10 g/kg. A minimum effect was observed when treated with *B. subtilis* at 2.5 g/kg.

Table 8: Effect of different doses of talc-based *B. subtilis* (BS 3) treated rice seeds on the management of brown spot (Pot culture).

Sr.	DTI	DI	PIC
1	2.5	60.81[d] (51.24)	30.24 (33.36)
2	5.0	54.65[c] (47.66)	42.46 (40.66)
3	7.5	41.47[b] (40.08)	61.84 (51.84)
4	10.0	29.54[a] (32.92)	70.36 (57.01)
5	Control	75.42[e] (60.27)	—

DTI: Dosage of talc-based inoculum(g/kg of seed); DI: Disease incidence (%) *; PIC: Percent increase over control
* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

Effect of foliar application of talc-based inoculum of B. subtilis (BS 3) on the management of rice brown spot (Pot culture)

The results of the present study showed that rice brown spot can be managed by foliar application of *B. subtilis* (BS 3). A maximum effectivity was observed on foliar application of *B. subtilis* @ 2.5 kg/ha. The increasing quantity of foliar spray build up the phyllosphere population of *B. subtilis* (Table 9) and the highest number of bacterial colonies were observed in 2.5 kg/ha. The highest incidence of *B. oryzae* was observed in pathogen

Table 9: Effect of foliar application of talc-based inoculum of *B. subtilis* (BS 3) on the management of rice brown spot (Pot culture experiment).

Sr.	DTI	DI	PP
1	*B. subtilis* @ 1.0	45.65[d] (42.50)	1.2[d]
2	*B. subtilis* @ 1.5	39.12[c] (38.71)	1.6[c]
3	*B. subtilis* @ 2.0	34.69[b] (36.08)	2.0[b]
4	*B. subtilis* @ 2.5	30.53[a] (33.54)	2.6[a]
5	Control (Uninoculated)	56.24[e] (48.58)	0.0[e]
6	Control (Inoculated)	85.31[f] (67.46)	0.0[e]

DTI: Dosage of talc-based inoculum (kg/ha); DI: Disease incidence (%) *; PP: Phyllosphere population of BS 4(10^4) *
* Mean of three replications; * In a column, means followed by a common letter are not significantly differ at 5% level by Duncan's multiple range test (DMRT)

inoculated control treatment (85.31%) and it was followed by uninoculated control treatment (56.24%).

Discussion

Biochemical characterization of *B. subtilis* isolates

The differences in the gram reaction and biochemical characteristics of *B. subtilis* isolates under varying soil and climatic conditions have already discussed by previous researchers (Karan, 2021; Kai, 2020; Seenivasan *et al.,* 2012). All the isolates of *B. subtilis* were capable to produce Gelatin liquefaction and starch hydrolysis under *in vitro* conditions, the phenomenon, which is the main characters of *Bacillus*. The production of catalase test by *B. subtilis* was already reported by several workers (Karan, 2021; Tariq *et al.,* 2016). The efficiency of *B. subtilis* on the negative reaction of Methyl red test was confirmed by Seenivasan *et al.,* (2012).

Antagonistic activity of *B. subtilis* isolate (Dual culture)

All the five isolates of *B. subtilis* tested under Dual culture showed different antagonistic effect against *B. oryzae* (Bo7). Among them, the isolate BS 3 show maximum inhibition against the growth of rice brown spot pathogen. The important criteria for various bio-efficacy of *B. subtilis* isolate in the suppression of fungal plant pathogens exhibited competition for nutrition between pathogen and their antagonistic organism, production of antimycotic constituents. Similar observations were made by earlier researchers (Deng *et al.,* 2019; Hashem *et al.,* 2019).

Bacillus produce antimycotic constituents which can be used as a substitute in place of fungicides or as a supplement to the use of bio control agents for controlling fungal plant pathogens (Ongena *et al.,* 2005). The present study assured that the inhibition of *B. oryzae* was due to production of harmful secondary metabolites by the bio-protectant. Several workers have also reported that suppression of fungal growth was due to the production of antifungal metabolites by *B. subtilis* (Aprilia *et al.,* 2021). Similarly, Rajasekar *et al.* (2019) reported the antifungal activity of Rice Associated Phyllosphere (RAP) communities with twenty phylloplane bacterial isolates tested against *B. oryzae*. Among the twenty isolates *B. subtilis* (PI5) showed a maximum inhibition (52.96%) followed by *P. fluorescens* (PI1) with 52.59 % inhibition over control.

Effect of antagonistic culture filtrate of *B. subtilis* on *B. oryzae* pathogenic fungal growth and conidial germination

In the present *in vitro* experiment, antagonistic filtrate of *B. subtilis* (BS 3) at twenty percent arrested the fungal growth and conidial germination of *B. oryzae*. Carissimi *et al.,* (2009) mentioned that *Bacillus* sp. (E164) culture filtrate arrested the conidial germination, germ tube and mycelial growth. Similarly, *B. subtilis* DL76 culture filtrate inhibited the mycelial growth, conidiation, appressorium formation and multiple stress response on *M. grisea* causing rice blast disease (Kgosi *et al.,* 2022). Several people have also demonstrated the inhibition of fungal growth and conidial germination of plant pathogens by different antagonistic isolates of *B. subtilis* filtrates (Xie *et al.,* 2020). The above conidial and pathogenic growth inhibition by *B. subtilis* reports is in agreement with the present findings.

Survival of antagonistic agent in different carrier materials

A maximum number of bacterial colonies was noticed when talc powder was used as a carrier material. This was obtained after storage period of three months while in comparison with various carrier materials. These results corroborate with the findings of Álvarez *et al.,* (2016) who reported a safe storage period of more than three months for sustaining the viability of *B. cereus* strain B25 in talc powder formulation. The success of any formulation designed to control plant pathogens or enhance plant growth depends on the quantity and longevity of the bio control agents present. This is because the shelf life of the microbial formulation greatly affects the commercialization of the bioprotectants. Additionally, the materials used to carry and support the antagonistic agents should not only extend their shelf life for commercial purposes but also not hinder their ability to effectively combat pathogens over time (Shaikh and Sayyed, 2015). Talc powder has served as a carrier substance for antagonistic bacterial formulations. Remarkably, talc-based carrier materials can support the longevity of bio- pesticidal microbial populations for as long as six months (Vidhyasekaran and Muthamilan, 1995). Our reports are consistent with previous scientific results. Similarly, Jayasudha *et al.,* (2017) also reported vermiculite and talc based bioformulation of *Bacillus subtilis* strain KK-9A recorded the highest number of colonies forming unit examined at fifteen days interval up to three months of storage. These antagonistic carrier formulation reports add support to the present findings.

Evaluation of plant growth promotion activity of *B. subtilis* (BS 3)

In the present experiment, seed treatments with *B. subtilis* (BS 3) improved the germination percentage, root, shoot length and vigour index when compared to control treatment. These research outputs are in promise with

Sumathra (2023) who reported that improvement in plant growth, shoot, root height and vigour index of rice by treatment with *B. subtilis*. PGPR directly increases the crop attributes, by improving phytonutrients through the effect of growth regulators, siderophores, enzymes, secretion of volatile organic compounds involved in nitrogen fixation and solubilisation of Phosphorus (Kumar *et al.,* 2012). Similar reports have described improved growth promotion and germination percentage of seeds in inoculated with *B. subtilis* (Doolotkeldieva and Bobusheva, 2022; Konappa *et al.,* 2020).

Effect of Seed treatment with talc-based formulation of *B. subtilis* for the management of rice brown spot

The effectiveness of using *B. subtilis* as a seed treatment for reducing brown spot disease in rice was found to decrease as the dosage of *B. subtilis* increased. A maximum reduction in disease incidence was observed when rice seeds were treated with 10 g/kg of rice seeds. Similar results were obtained by Chiangsin *et al.,* (2018) in rice for the management of brown spot. Earlier also, an increase in disease management through seed treatment with *B. subtilis* has been well documented (Zhu *et al.,* 2021; Palupi *et al.,* 2017). In the present pot culture experiment, *B. subtilis* was found to reduce brown spot of rice under polyhouse condition. *Bacillus* could act as strong elicitor of plant innate immunity (Pršic and Ongena, 2020). Recent results indicate that the application of antagonistic organisms to treat the seeds has been proven to enhance the strength of plant cell walls, leading to a significant decrease in the occurrence of pathogen infection. The bio-protectant *B. subtilis* GB519 used for the coating of rice seeds can inhibit the mycelial growth of *M. grisea* and increased the germination percentage and root, shoot fresh weight with higher level of IAA and phosphorus (Zhu *et al*., 2021). Induced Systemic Resistance by PGPR involves biochemical changes in the host cell that leads to the synthesis of antioxidant enzymes (Viswanathan *et al.,* 2003; Viswanathan and Samiyappan 1999). The present research therefore, indicated that Seed treatment with *B. subtilis* (BS 3) promises to be an effective seed coating practice against brown spot of rice.

Effect of foliar spray of *B. subtilis* (BS 3) on the management of brown spot in rice

When *B. subtilis* is used as a foliar spray, it leads to an increase in the phyllosphere microbial population of antagonistic agent and effective in controlling the brown spot disease in rice. The minimum disease incidence was observed when the bacterial antagonist was applied as a foliar spray at a rate of 2.5 kg per hectare. There are various methods available for delivering antagonistic microbes to protect the soil rhizosphere and phyllosphere of crops from invasion by harmful pathogens. This approach has shown great potential in managing the plant health (Nakkeeran *et al.,* 2005). The application of both broadcasting and foliar spraying of *B. megaterium* was found to effectively decrease the occurrence of rice Sheath Blight in both pot culture experiments and field trials (Kanjanamaneesathian *et al.,* 2007). There are numerous scientific studies available in the literature that provide evidence on the successful use of *B. subtilis* as a foliar spray in managing rice diseases. Some of the rice diseases managed with foliar application of *B. subtilis* are Blast (Murunde *et al.,* 2022), Brown spot (Karan, 2021), Bacterial Blight (Akhtar *et al.,* 2020) and Sheath blight (Jayaraj *et al.,* 2004). The above foliar spray of *Bacillus* reports lend support to the present results.

Conclusion

Five isolates of *B. subtilis* were able to be isolated from rice rhizosphere in different localities of Villupuram District. All the isolated exhibited *Bacillus* bacterial reaction compared with Bergey's manual of Determinative Bacteriology. Among the bacterial isolates, the isolate BS 3 showed a maximum inhibition and significantly inhibited the growth of *B. oryzae* when compared to control treatment. The conidial germination of *B. oryzae* was found to be decreased with an increase in the concentration of culture filtrates of *B. subtilis*. A maximum conidial germination was inhibited by culture filtrate of *B. subtilis* at 20% under cavity slide method and Poisoned food technique. In carrier formulation of bacterial antagonistic agents, talc powder was found to be significantly more effective in its maintenance of a bacterial antagonistic population than any other carrier material. It was followed by Lignite powder, Peat soil, lignite fly ash and kaolin carrier materials in the decreasing order of merit. Among the various dosages of *B. subtilis* tested for seed treatment, rice seeds treated @10 g/ kg of seeds showed a maximum germination percentage, root length, shoot length and vigour index and reduce the incidence of brown spot in rice var. ADT 43. Also, a maximum bio-efficacy was observed on foliar application of *B. subtilis* @ 2.5 kg/ha. The increasing quantity of foliar spray build up the phyllosphere population of *B. subtilis* and the highest number of bacterial colonies were observed in 2.5 kg/ha.

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