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UNRAVELING THE PROTECTIVE POTENTIAL OF SPERMOSPHERE BACTERIA IN MITIGATING RICE BROWN SPOT (*Bipolaris oryzae* (Breda de Haan) Shoemaker) INFECTION

J. Sheela*,[1], E. G. Ebenezar[2], M. Theradimani[3], S. Ragul[4], M P. Tamilmalar[5], A. Aravinthkumar[6], N. Rajinimala[7], M. Paramasivan[8] and M. Arumugampillai[9]

[1] - Professor and Head, Department of Plant Pathology, VOC Agricultural College and Research Institute, Killikulam, Vallanad - 628252, Thoothukudi District, Tamil Nadu, India.
[2] - Professor, Department of Plant Pathology, VOC Agricultural College and Research Institute, Killikulam, Vallanad - 628252, Thoothukudi District, Tamil Nadu, India. E-mail: ebenezar.eg@tnau.ac.in
[3] - Dean, VOC Agricultural College and Research Institute, Killikulam, Vallanad - 628252, Thoothukudi District, Tamil Nadu, India. E-mail: mtheradi@gmail.com
[4,5] - Ph.D. Scholars, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu, India. E-mail : ragulvivasayam@gmail.com, papujeni876@gmail.com
[6] - Ph.D. Scholar, Division of Plant Pathology, IARI, New Delhi - 110012, India. E-mail: aravinth98a@gmail.com
[7] - Associate Professor (Pl. Pathology), Rice Research Station, Ambasamudram - 627401, Tirunelveli District, Tamil Nadu, India. E-mail: rajinimala@tnau.ac.in
[8] - Associate Professor (Plant Pathology), Regional Research Station, Virudhachalam - 606001, Tamil Nadu, India. E-mail: madathisivan@gmail.com
[9] - Professor (Plant Breeding & Genetics), Department of Genetics and Plant Breeding, VOC Agricultural College and Research Institute, Killikulam, Vallanad - 628252, Thoothukudi District, Tamil Nadu, India. E-mail: mapillai1@hotmail.com
*Corresponding author E-mail: sheela.j@tnau.ac.in

ABSTRACT

Bipolaris oryzae, fungal pathogen causing rice brown spot disease, exerts a significant economic impact by reducing rice yield and quality. The costs associated with the management of this destructive pathogen, including fungicide applications and other preventive measures, increase the cost of cultivation and pave the way for the economic burden faced by rice farmers and the broader agricultural industry. Taking this into consideration, 32 spermosphere bacteria were isolated from various rice landraces and challenged against the virulent isolate of *Bipolaris oryzae* (OM977033) under *in vitro* conditions. Among them, six strains of spermosphere bacteria from different rice seeds exhibited notable inhibitory effects on the tested pathogen. SPKKM 4 isolated from Navara, SPKKM 2 from Mappillaisamba, SPKKM 5 from Navara black, SPKKM 18 from Kavuni, SPKKM 9 from Mallikar and SPKKM 32 from ADT 44 rice seeds exhibited pronounced inhibition of *Bipolaris oryzae* mycelial growth. Furthermore, seedling vigor assessments revealed the remarkable growth-promoting potential of SPKKM 5, SPKKM 4, and SPKKM 2 among the tested bacterial strains. Molecular characterization confirmed their identities as *Bacillus subtilis* (OQ073461), *Acinetobacter schindleri* (OK342196) and *Acidovorax spp.* (OP522279), respectively. Subsequent pot culture experiments demonstrated that *Bacillus subtilis* (SPKKM 5) applied at 10 ml kg^{-1} as seed treatment and foliar spray (0.5%) at flowering and 15 days later, resulted in a remarkable 65.86% reduction in disease incidence compared to the control.

Keywords: Rice brown spot, *Bipolaris oryzae*, spermosphere bacteria, rice landraces, *Bacillus subtilis*

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INTRODUCTION

Rice ranks second position in the World in area and production. For about 50% People in Asia consume rice as the major food crop. Rice crop is highly suitable to areas that have tropical climate with high humidity and lengthy sunshine hours. In the phase of changing climatic patterns, rice cultivation encounters considerable hurdles that substantially affect growth, yield, and quality due to various animate and inanimate causes. Among the animate causes, brown spot of rice, *Cochliobolus miyabeanus* (Anamorph: *Bipolaris oryzae* Shoemaker), is of prime importance, as they significantly influence the quantity and quality of rice. Every year, Millions of hectares of rice are affected (Bag *et al*., 2023). Usually, chemical fungicides have been employed to manage this disease. However, their prolonged use raises concerns related to residual toxicity, pathogen resistance and environmental pollution. The spermosphere represents the microbial community associated with germinating seeds and the nearby soil. The presence of beneficial microorganisms in the spermosphere has the potential to enhance key factors such as percentage of germination, germination speed and seedling vigour (Singh *et al*., 2023). The ability to induce such effects is attributed to bacterial secretion of phytohormones at concentrations adequate to induce structural and functional alterations in seed tissues. Typically, seed harbours a diverse array of microorganisms that can be steeply spread across generations (Ai *et al*., 2023). "Investigating the temporal transition of the microbiome from seed to seedling, as well as its spatial shift from root to shoot and from tissue surface to internal tissue can provide insights into the intricate interactions between the host and the related microbiome. This encompasses the multitude of microorganisms that, through modulation of seed exudates, may influence rhizosphere and phyllosphere establishment" (Trivedi *et al*., 2020). 'The external application of such beneficial microbiota or microbial consortia has proven effective in stimulating plant growth and imparting resistance to many stresses in different crops' (War *et al*., 2023). These applications have been instrumental in augmenting tolerance to diseases, insect pests, and nematodes in diverse cropping systems. In light of these considerations, this study aimed to i) Isolation of spermosphere microbes from the seeds of rice land races ii) Identification of isolated spermosphere microbes and iii) Testing the efficacy of identified spermosphere microbes against brown spot of rice.

MATERIALS AND METHODS

Isolation of brown spot pathogen: Brown spot pathogen infected rice leaves, exhibiting minute oval brown spot symptoms, were collected from various locations *viz*., Killikulam, Tiruchendur, Veera Kerlam Puthur, Alwarthirunagar, Thenthiruperai, Vannimanagaram, Nallur (Thoothukudi district) and Ambasamudram (Tirunelveli districts) of Tamil Nadu, India. Samples were meticulously labelled for accurate identification and analysis. Then the collected leaf samples were washed gently and brown spot pathogen was isolated under aseptic conditions using the direct plating method (Long *et al*., 2023). In this method, the infected samples were cut into 50 mm pieces and surface sterilized with sodium hypochlorite (0.1%) for 30 seconds. Subsequently, washed thrice with sterile distilled water to remove remaining sodium hypochlorite. Autoclaved Potato Dextrose Agar (PDA) medium, added with streptomycin sulphate (100 ppm), was poured and allowed to solidify in the sterilized Petri dishes. The sterilized leaf bits were incubated in Biological Oxygen Demand (BOD – REMI, CIS-24 Plus, India) incubator at 28±2°C until the isolation of pathogen. After that, the leaf bits were aseptically transferred to the PDA poured Petri plates.

Isolation of spermosphere microbes: In 2020, twenty-five distinct rice landraces were obtained from the Genetics and Plant Breeding Department in VOC Agricultural College and Research Institute at Killikulam, Vallanad, Tamil Nadu, India. These landraces were procured to investigate the antagonistic role of bacteria within the spermosphere. In this study, wet land soil was collected and kept in an autoclave to undergo intermittent sterilization for three days at 121°C @ 5 psi pressure for 20 minutes, to eliminate the soil microbes. Then the sterilized soil was filled in paper cups of uniform size and the seeds of rice landraces were sown in each paper cup separately, watered and labelled. After three days of sowing, the germinated seedlings were transferred carefully using sterile forceps to a test tube with 10 ml of sterile water and manually shaken well for 10 minutes. Then the spermosphere sample solution was serially diluted up to 10^{-5} and 10^{-6}, to isolate inhabiting bacteria. One ml of the desired dilutions of 10^{-5} and 10^{-6} were plated on Nutrient Agar (NA) medium separately and incubated in BOD (REMI, CIS-24 Plus, India) at 28℃. By the next day morning, distinctly grown single colonies of bacteria were isolated and purified by streaking method (Aswini *et al*., 2023).

Characterization of the pathogen of interest:

Molecular characterization of *Bipolaris oryzae*: The virulent isolate of *Bipolaris oryzae* was cultured on the broth (Potato Dextrose) and followed by seven days of incubation at 28°C constant shaking in a BOD incubator cum shaker (REMI, CIS-24 Plus) at 100 rpm. After incubation, DNA extraction was carried out with slight modifications in the "Cetyl Trimethyl Ammonium Bromide (CTAB) method" (Doyle and Doyle, 1987; Fazio *et al*., 2023). In the current study, except centrifugation all other steps followed as per the procedure (Doyle and Doyle, 1987; Fazio *et al*.,2023). Centrifugation was done at 14,000 rpm instead of 12,000 rpm. The particle was dried to remove the remaining ethanol after discarding the supernatant solution. The particle was soaked in 50 µl of TE buffer and then evaporated. The DNA sample was preserved at -20°C. DNA quality was assessed using agarose gel electrophoresis. Sequencing and NCBI blast analyses were carried out.

***In vitro* efficacy of spermosphere bacteria against *Bipolaris oryzae*:** The antagonistic ability of thirty-two spermosphere bacterial isolates against *B. oryzae* were tested by dual culture method (Zhou *et al*., 2022). Autoclaved PDA medium was transferred aseptically onto Petri dishes (90 mm). After solidification, from the actively growing culture (7 day old), 5mm diameter mycelial disc was plugged and placed one cm apart from the edge of the Petri plate, using cork borer. Then, spermosphere bacterial isolates were streaked on the contradictory side of the Petri plates and Petri plates with pathogen alone kept as negative control. All plates were placed in BOD (REMI, CIS-24 Plus, India) at 28℃ for further observation. Three replications were maintained and completely randomized design (CRD) method was followed for the experiment. The inhibition percentage was calculated when the fungus in the negative control plate attains its maximum growth of 90 mm (Lal *et al.*, 2022). The percent mycelial growth inhibition was calculated using the formula given below.

Mycelial growth inhibition (%) = C-T/C X 100

Where:

C = Growth in control plate (mm)

T = Growth in treatment plate (mm)

Testing the effect of spermosphere microbiomes on seedling growth: The potential ability of isolated spermosphere bacteria were tested on rice seed for germination, seedling vigour and vigour index by Modified Roll towel method (Parthasarathy *et al*., 2022). The bacterial suspensions ($5x10^8$ cfu ml^{-1}) of six best isolates screened in the dual plate technique *viz.,* SPKKM 2, SPKKM 4, SPKKM 5, SPKKM 9, SPKKM 18 and SPKKM 32 were made. The seeds of ASD 16 rice variety were dried in a blotter paper after being soaked in the respective bacterial suspension for overnight. Sterile water-soaked seeds used as control**.** The seeds were placed between two layers of germination paper and rolled in towels and placed in the germinator in upright position. Observation on percentage of germination, length (cm) of the root and shoot were taken after five days onwards. Final count was taken on 14 days after the study. Vigour index was calculated using the formula specified by Sharma *et al*. (2023).

Vigour Index = Germination (%) × Seedling length {Shoot (cm) + Root (cm)}

Characterization of best identified spermosphere bacteria:

Biochemical characterization: The biochemical assays including gram staining, catalase, casein, starch hydrolysis, citrate utilization, KOH, urease, phosphate, methyl red, HCN, siderophore production were performed following Palaniappan *et al*. (2010) protocol with slight modifications *viz.,* optimizing reagent concentrations and incubation times.

Molecular characterization: Standard CTAB method was used to isolate the genomic DNA from the best three identified spermosphere bacteria *viz.,* SPKKM2, SPKKM4 and SPKKM5, with slight modifications in the method (Doyle and Doyle, 2019). In the present study, except centrifugation all the other steps were followed as per the procedure given by Doyle and Doyle (1987). Centrifugation was done at 14,000 rpm instead of 12,000 rpm. After the removal of supernatant, the particle was dried to remove the excess ethanol. The particle was soaked in 50µl of TE buffer and after evaporation, the DNA sample was stored at -20°C. The quality of isolated DNA was assessed by loading 2.5 µl of DNA into 0.8 % Agarose gel electrophoresis.

Antagonistic potential of spermosphere bacteria against brown spot disease (Pot culture): This experiment was conducted at the glasshouse in the Department of Plant Pathology of VOC Agricultural College and Research Institute located at Killikulam, Vallanad, Thoothukudi District of Tamil Nadu, India with seven treatments and three replications. For the study purpose, the susceptible variety ASD 16 was used. Bacterial cultures of SPKKM 2, SPKKM 4, SPKKM 5 and *Bacillus subtilis* (BS 1) TNAU liquid formulation were used for seed treatment @ 10 ml kg^{-1} (5×10^8 cfu ml^{-1}) and sown in zinc trays. Uniform pots of size 30 x 20 cm diameters were selected and filled with pot mixture. After twenty-one days, seedlings were transplanted in mud pots (3 seedlings per pot). The pots were labelled, regular agronomical practices were followed and no chemical spraying was supplemented. After 25 days of planting, the plants were artificially inoculated by spraying the conidial suspension of *Bipolaris oryzae* (5×10^8 conidia ml^{-1}). Subsequently, pathogen inoculated plants were sprayed with 0.5% of respective bacterial cultures containing 5×10^8 cfu ml^{-1}. The plants sprayed with the conidial suspension of *B. oryzae* alone served as negative control. The plants which were not treated with conidial suspension and bacterial suspension served as positive control. The development of brown spot disease symptom was recorded and scored from 35 days to 70 days after sowing using the IRRI 0-9 'standard disease scale' of IRRI (2002) as shown in Table 1.

Table 1. Score description used for disease evaluation

Score	Description
0	No disease observed
1	Less than 1% infected area in leaf
2	1 - 3% infected area in leaf
3	4 - 5% infected area in leaf
4	6 - 10% infected area in leaf
5	11 - 15% infected area in leaf
6	16 - 25% infected area in leaf
7	26 - 50% infected area in leaf
8	51 - 75% infected area in leaf
9	76 - 100% infected area in leaf

'Per cent Disease Index (PDI)' was calculated by the following formula (Sudhasha *et al*., 2020)

$$\text{PDI} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum disease grade}}$$

GC-MS analysis: Crude antibiotics were extracted from the most effective spermosphere bacterial antagonists i.e., SPKKM 5, following the procedure given by Rajaofera *et al.* (2019) with slight modifications. GCMS analysis using a Shimadzu Gas Chromatography (QP 2020 with Rxi 5 MS Column) equipped with the mass detector gold turbo mass gold and an Elite-1 (100 per cent of Polymerized Dimethyl Siloxane), 30 m 0.215 mm ID column: Helium is the gas utilised as the carrier (1 ml/min). Following a two-minute isothermal at 110°C and a nine-minute isothermal at 280°C, the temperature of the injector was fixed at 250°C. The GC was finished in 45 minutes. The material was injected into the chromatograph in one-ml aliquots. The extracts obtained from SPKKM 5 isolate were subjected for GS-MS analysis and the chemical constituents with different retention period were identified.

RESULTS AND DISCUSSION

Isolation of brown spot pathogen: The incubated Perti plates were observed periodically for the initiation of fungal growth of *Bipolaris oryzae*. The hyphal tips from the advancing mycelium were sub-cultured to obtain pure colonies of *Bipolaris oryzae* (Asif *et al*., 2023). Purified cultures were established and designated as BO1, BO2, BO3, BO4, BO5, BO6, BO7, and BO8, representing the eight respective geographic locations. These purified cultures were maintained in PDA slants at 8°C, for future studies. Initially, the pathogen of interest was characterized based on morphological and cultural attributes such as colony colour, mycelial growth and conidia shape, aligning with the established practices in fungal taxonomy. All the eight isolates of *B. oryzae* were tested for their virulence and the isolate obtained from Vannimanagaram (BO6) proved to be the most virulent isolate (OM977033).

As per the objective of the study, virulent isolate (BO6) was first isolated from the rice brown spot infected sample followed by the isolation of spermosphere bacteria from rice genotypes to be used against *B. oryzae*

Isolation of spermosphere bacteria: A total of 32 spermosphere bacteria were obtained from the twenty-five rice genotypes and named serially as SPKKM 1 to SPKKM 32. Potential antagonists were isolated from peas and French bean seeds using techniques like washing, sonication, and incubation in nutrient broth (Walker *et al*.,1998). Gopalakrishnan and Valluvaparidasan (2006) isolated spermosphere microorganisms like *Bacillus subtilis* and *Pseudomonas fluorescens* from rice seeds. Most of the bacteria associated with seeds are classified under Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes phyla (Johnston-Monje *et al*., 2021). In the transmission of microbial resources between plant generations, the initial inoculum of microbes harbored in the seeds play a vital role (Shade *et al*., 2017). "Seed microbiomes may be a valuable resource for the identification and isolation of PGPB. It may be possible to increase the productivity and viability of crop species that are subjected to increasingly harsh growing environments by introducing single PGPB or an artificial consortium of beneficial bacteria to manipulate the function of seed microbiomes" (Hone *et al*., 2021). In the recent study by Newcombe *et al.* (2023), it was highlighted that "*Bacillus* isolates are prevalent in the seeds of 98 plant species belonging to 39 families, and *Bacillus spp.* from seeds exhibited strong antagonistic effects against both seed and foliar fungi".

Characterization - the pathogen of interest:

Molecular characterization of *Bipolaris oryzae:* Extraction of the genomic DNA of BO6 isolate was done using CTAB method. ITS region of brown spot pathogen was amplified by ITS-1 / ITS-4 primers which produced 600 bp. ITS region sequence analysis revealed that BO6 isolate showed sequence similarity with *Bipolaris oryzae* of more than 98%. The BO6 isolate ITS sequence was deposited NCBI database under accession number OM977033.

In vitro* efficacy of spermosphere bacteria against *Bipolaris oryzae: Among the 32 bacterial isolates originated from the spermosphere, six bacterial isolates {SPKKM-4 (Navara), SPKKM-2 (Mappillaisamba isolate 1), SPKKM-5 (Navara black), SPKKM-18 (Kavuni), SPKKM-9 (Mallikar) and SPKKM-32 (ADT-44)} demonstrated their effectiveness against *B. oryzae* in dual plates analysis (Table 2, Fig. 1). The antifungal activity of these spermosphere bacteria can be credited to the production of siderophores and antifungal compounds released by the antagonistic bacteria. Sha *et al.* (2020) demonstrated that *Bacillus amyloliquefaciens* S170 and *Bacillus pumilus* S9 occupy rice plants to check pathogenic infection caused by *Magnaporthe oryzae.*

Seeds treated with *Bacillus subtilis* BS1 TNAU liquid formulation exhibited the highest seed germination at 83.80%, along with a maximum root length (23.50 cm), shoot length (20.20 cm) and the highest vigour index of 3662.49 (Table 3). They were followed by the isolate SPKKM 5 with 83.35% seed germination, root length (19.00 cm), shoot length (19.80 cm), and a vigour index (3596.40). As a result, both treatments were found to be statistically at par. Output of the study conducted by Arkhipova *et al.* (2005) revealed that *Bacillus spp*. can induce cytokinin, a growth-related hormone that promotes cell division, leading to boosted seedling growth and development. Affirming this, Pérez-Montaño *et al*. (2014) reported the growth-promoting effects of *B. subtilis*. Additionally, Karthik *et al*. (2017) stated the efficacy of endophytic bacteria isolated from banana plants in enhancing the seedling vigour index of tomatoes. Secondary metabolites produced by *B. subtilis* were found to control plant pathogens effectively (Harish *et al*., 2009). "*Bacillus spp*. has biocontrol activities that stimulate the plant's defence system, including the production of PR proteins, phytoalexins and activation of induced systemic resistance (ISR) by synthesizing jasmonic acid, ethylene and NPR-1 regulatory gene expression" (Konappa *et al*., 2020).

Characterization - best identified spermosphere bacteria

Biochemical characterization of spermosphere bacteria: The biochemical tests *viz.,* Gram staining, catalase test, Casein hydrolysis, Starch hydrolysis, citrate utilization, KOH test, urease test, phosphate solubilization, methyl red test, HCN production and Siderophore production were performed for the best three spermosphere bacteria and the results are given in the following table 4 (Fig. 2).

Table 2. *In vitro* evaluation of spermosphere bacterial antagonists against *Bipolaris oryzae* (Dual plate technique)

S.no.	Bacterial Isolates	Source	*Mycelial growth of the pathogen (mm)	*Inhibition (%)
1	SP KKM 1	Swarna	32.00	64.44 (53.38) #
2	SP KKM 2	Mappillaisamba isolate 1	15.00	83.33 (65.89)
3	SP KKM 3	Mappillaisamba isolate 2	44.67	50.37 (45.19)
4	SP KKM 4	Navara	13.00	85.56 (67.65)
5	SP KKM 5	Navara black	17.00	81.11(64.22)
6	SP KKM 6	Abiyan	23.00	74.44 (59.61)
7	SP KKM 7	Thuyamalli isolate 1	61.00	32.22 (34.57)
8	SP KKM 8	Thuyamalli isolate 2	46.00	48.89 (44.35)
9	SP KKM 9	Mallikar	21.00	76.67 (64.10)
10	SP KKM10	Keralakandasala	23.00	74.44 (59.62)
11	SP KKM 11	Swarnamalli	61.67	31.48 (34.11)
12	SP KKM 12	Sivappumalli	29.33	67.41 (55.17)
13	SP KKM 13	Jaya	65.00	27.78 (31.78)
14	SP KKM 14	Srilanka	33.33	62.96 (52.50)
15	SP KKM 15	Uma	24.00	73.33 (58.91)
16	SP KKM 16	Anna 4	39.667	55.93 (48.39)
17	SP KKM 17	Adukkan	41.33	54.07 (47.32)
18	SP KKM 18	Gowni	19.67	78.15 (62.12)
19	SP KKM 19	Jaisreeram	37.00	58.89 (50.10)
20	SP KKM 20	Vanaprabhu	45.00	50.00 (44.98)
21	SP KKM 21	Chithirakar	48.33	46.30 (42.86)
22	SP KKM 22	Kullakar	60.00	33.33 (35.24)
23	SP KKM 23	Poongar	50.00	44.44 (41.79)
24	SP KKM 24	Mattaikar	52.67	41.48 (40.07)
25	SP KKM 25	Kattanur	35.33	60.74 (51.18)
26	SP KKM 26	Swarnamasuri	37.33	58.52 (49.89)
27	SP KKM 27	Vellarai	52.00	42.22 (40.51)
28	SP KKM 28	Virendra	55.33	38.52 (38.34)
29	SP KKM 29	Chenellu	57.00	36.67 (37.25)
30	SP KKM 30	IET 23609	39.33	56.30 (48.60)
31	SP KKM 31	Co 44	30.33	66.30 (54.50)
32	SP KKM 32	ADT 44	20.33	77.41 (61.60)
33	Control		90.00	0 (0)
	C.D. ($P_{0.05}$)			1.734
	SE(m)			0.613
	SE(d)			0.867
	C.V.			2.213

* Mean of three replications; # values in the parenthesis are angular / arc sin transformed values

Growth promotion of rice seedlings:
Seed germination, vigour index, root and shoot length

Fig 1. Antagonistic activity of 32 spermosphere isolates against *Bipolaris oryzae (in vitro* conditions).

Table 3. Growth promotion by spermosphere bacteria in rice.

S.No.	Spermosphere microbes	Germination percentage (%)	Root length (cm)	Shoot length (cm)	Vigour index
1	SPKKM-2	80.67^{b}	18.50^{bc}	13.20^{b}	2557.83^{b}
2	SPKKM-4	77.94^{c}	19.00^{b}	13.20^{b}	2510.22^{bc}
3	SPKKM-5	83.35^{a}	23.30^{a}	19.80^{a}	3596.40^{a}
4	SPKKM-9	75.50^{cd}	17.40^{bc}	13.20^{b}	2310.23^{bcd}
5	SPKKM-18	73.67^{d}	17.50^{bc}	13.50^{b}	2285.07^{cd}
6	SPKKM- 32	72.94^{d}	17.40^{bc}	13.40^{b}	2246.55^{cd}
7	*Bacillus subtilis* BS1 (TNAU)	83.80^{a}	23.50^{a}	20.20^{a}	3662.49^{a}
8	Control	70.50^{e}	17.00^{c}	12.50^{c}	2080.45^{d}
	CD (($P_{0.05}$))	**2.68**	**1.99**	**0.49**	**271.13**

*Mean of three replications
The treatment means are compared using the Duncan Multiple Range Test (DMRT).
In a column, means followed by a common letter (s) are not significantly different ($P_{0.05}$)

Table 4. Biochemical characterization of spermosphere bacterial isolates.

Biochemical characteristics	SPKKM 2	SPKKM 4	SPKKM 5
Gram Staining	-	-	+
Catalase	+	+	+
Casein hydrolysis	+	+	+
Starch hydrolysis	-	+	+
Citrate utilization	+	+	+
KOH	-	-	+
Urease	+	+	+
Phosphate solubilisation	+	+	+
Methyl red	+	+	+
HCN	-	-	+
Siderophore	+	+	+

* Positive (+) Negative (-)

It is clear from the table 3, that among the three spermosphere bacteria, SPKKM 5 was found to be positive for the all biochemical assay performed, while rest two isolates lack or show negative to some biochemical tests.

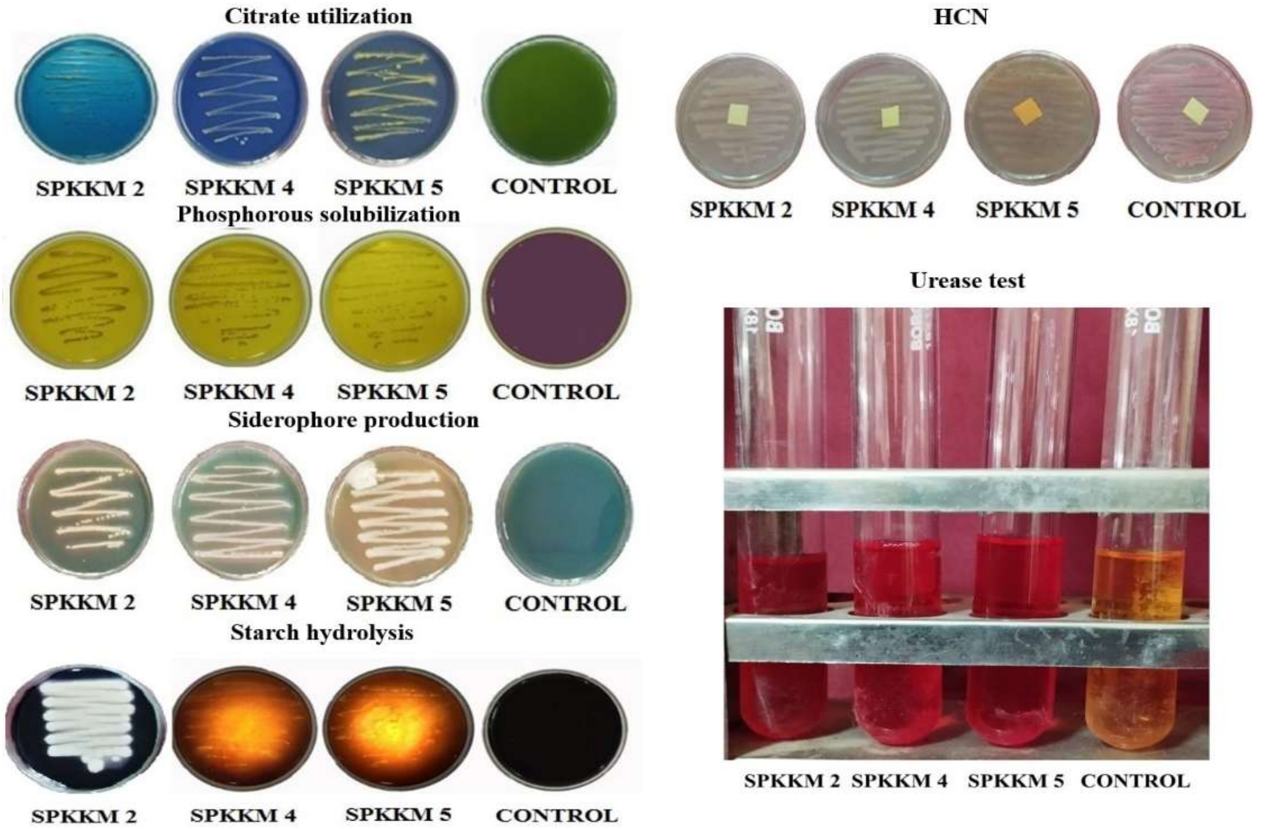


Fig 2. Results of biochemical assay for SPKKM 2, SPKKM 4 and SPKKM 5 isolates

Molecular characterization of spermosphere bacteria: The bacterial isolates from the spermosphere namely, SPKKM-5 (Navara Black), SPKKM-2 (Mappillaisamba isolate 1) and SPKKM-4 (Navara) were molecularly characterized and identified as *Bacillus subtilis* (OQ073461), *Acinetobacter schindleri* (OK342196) and *Acidovorax* sp. (OP522279), respectively. Abushady *et al.* (2005) extracted the genomic DNA of *Bacillus subtilis* strain AB01335-1 and strain AB02238-1 isolated from soil. The 16S rDNA region of *B. subtilis* was amplified by PCR resulted in an amplicon of 1450 bp. The amplified product was sequenced and nucleotide sequence of 16S rDNA region was BLAST searched and identified as *B. subtilis.* Phylogenetic tree of the potential spermosphere bacteria *viz., Bacillus subtilis* (OQ073461) was constructed using the Mega 11.0 software (Fig 3).

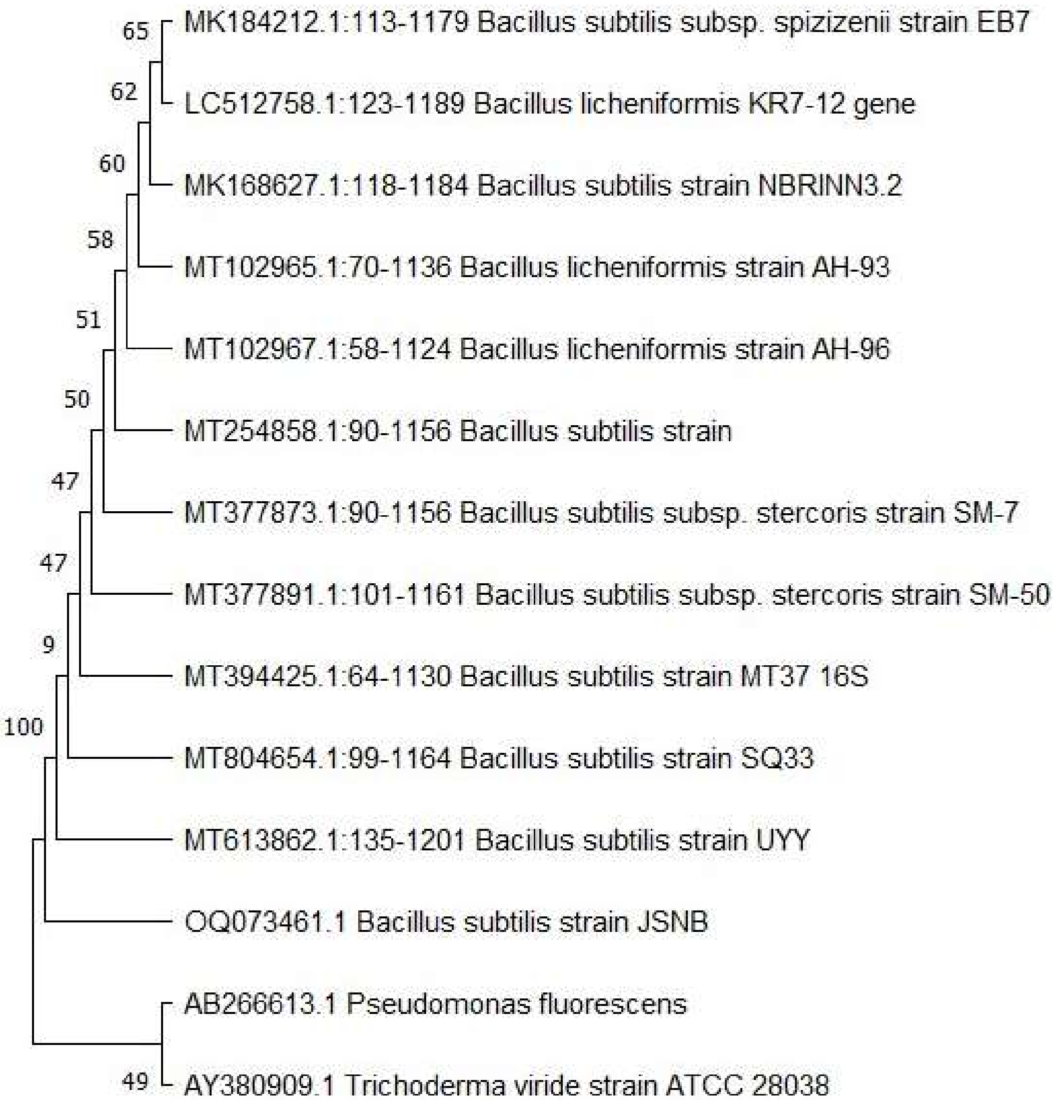


Fig.3. Neibhour joining phylogenetic tree of SPKKM 5 constructed using Mega 11.0 software.

Antagonistic potential of spermosphere bacteria against brown spot disease (Pot culture): Among the different treatments evaluated in dual plate assay, the three most effective bacterial isolates were subjected to challenge against the test pathogen under pot culture study. It was found that foliar spraying with the standard chemical metominostrobin at a concentration of 0.1% exhibited the most significant effectiveness in reducing brown spot disease. This treatment recorded a Plant Disease Index (PDI) of 12.54 and achieved an impressive 80.80% disease reduction compared to the control group. Following closely in efficacy was the treatment involving *Bacillus subtilis* (SPKKM 5) seed treatment (ST) at a rate of 10 ml kg^{-1} combined with foliar spraying at a concentration of 0.5% at flowering and 15 days after the first spray. This treatment recorded a PDI of 22.50 and a disease reduction of 65.86%, which was on par with the *Bacillus subtilis* BS 1 TNAU liquid formulation seed treatment (ST) at 10 ml kg-1 in combination with foliar spraying at 0.5% at flowering and 15 days after the first spray. This later treatment achieved a PDI of 23.43 and a disease reduction of 62.60% (Table 5). Seed priming with *Bacillus amyloliquefaciens* was potent in the reduction of rice blast disease (Patil *et al*., 2022) A reduction in the occurrence of rice sheath blight disease was demonstrated in seed bacterization with a mixture of PGPR (Nandakumar *et al*., 2001). Additionally, Ramamoorthy *et al*. (2001) gave an account of the positive impact of PGPR on various crop plants, enhancing their resistance to plant diseases through induced systemic resistance. The application of *Bacillus subtilis* through seedling dipping and foliar application proved effective in managing blast, sheath blight and bacterial leaf blight diseases in rice.

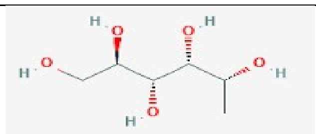
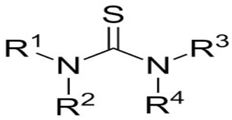
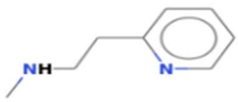
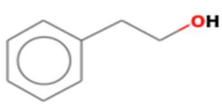
Table 5. Management of rice brown spot disease by spermosphere microbes (Pot culture)

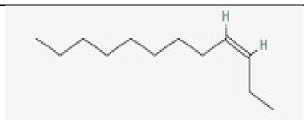
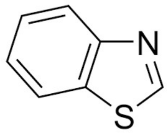
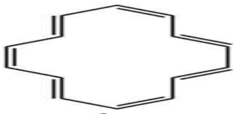
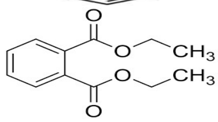
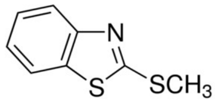
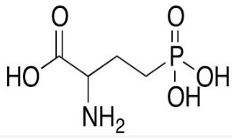
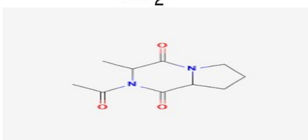
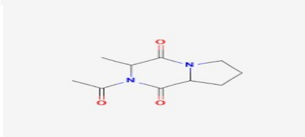
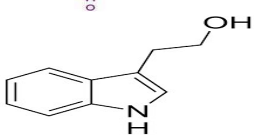
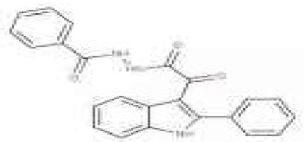
S.no.	Treatment details	Percent disease index (PDI)*	Percent disease reduction over control*
T_1	*Acinetobacter schindleri* (SPKKM 4) ST + Foliar spray 0.5% at flowering and 15 days after the first spray	32.00^{c}	51.07^{c}
T_2	*Acidovorax* sp. (SPKKM 2) ST (10 ml kg-1) + Foliar spray 0.5% at flowering and 15 days after the first spray	38.50^{b}	41.06^{d}
T_3	*Bacillus subtilis* (SPKKM 5) ST (10 ml kg-1) + Foliar spray 0.5% at flowering and 15 days after the first spray	22.30^{d}	65.86^{b}
T_4	*Bacillus subtilis* BS 1 TNAU liquid formulation (ST) (10 ml kg-1) + Foliar spray 0.5% at flowering and 15 days after the first spray	23.43^{d}	62.60^{b}
T_5	Metominostrobin 0.1% foliar spray at flowering and 15 days after the first spray	12.54^{e}	80.80^{a}
T_6	Inoculated Control	65.33^{a}	0.00^{e}
T_7	Uninoculated control	0.00^{f}	0.00^{e}
	CD ($P_{0.05}$)	**3.08**	**4.69**

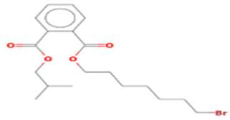
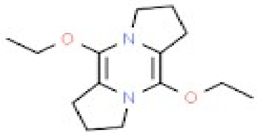
*Mean of three replications
The treatment mean was compared by using Duncan's Multiple Range Test (DMRT)

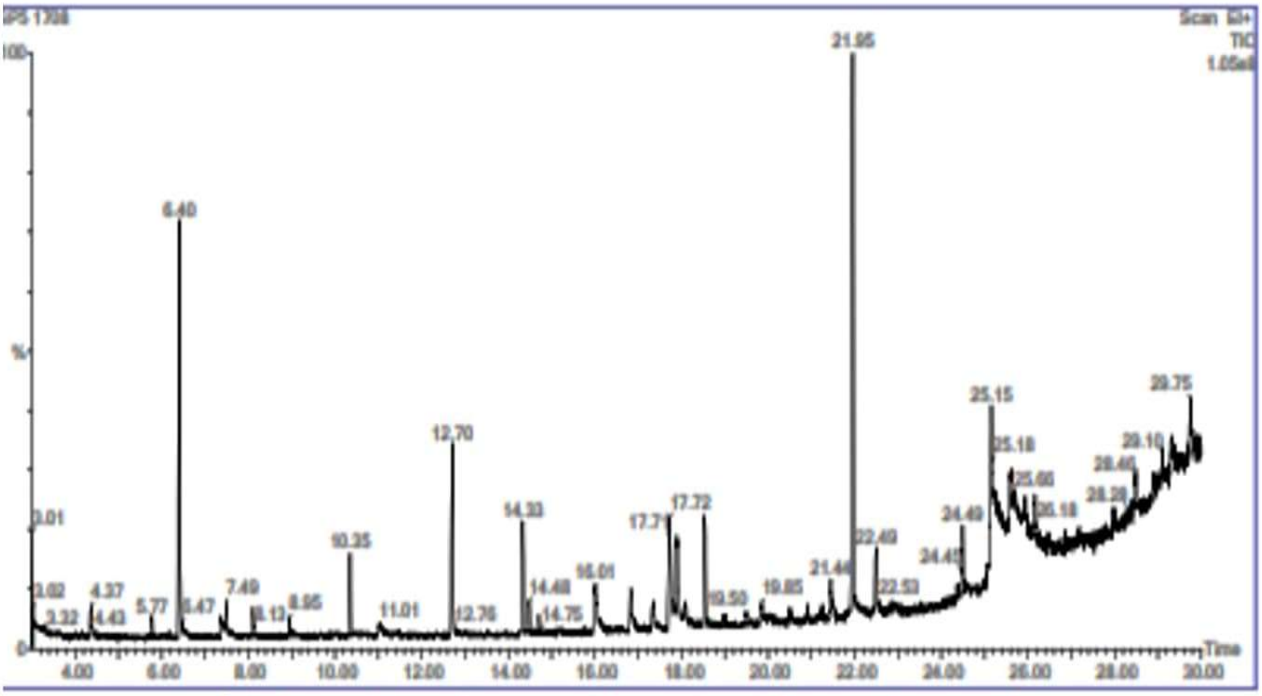
GCMS study: Gas chromatography-mass spectrometry (GCMS) study of SPKKM 5 extract indicated the occurrence of several compounds with potential antimicrobic properties produced by *B. subtilis*, which exhibited antifungal activities against *Bipolaris oryzae*, contributing to disease reduction (Fiddaman and Rossall, 1993). On corroborating with the reference literatures (Zhang *et al*., 2020; Awan *et al*., 2023), Thiourea, 2,4-Di-tert-butylphenol, Diethyl Phthalate, 2-(Methylmercapto) benzothiazole, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-, Benzohydrazide, N2-acethyl-N2-phenyl- and 1-Nonadecene were found to possess strong antifungal properties. Surya *et al.* (2020) carried out GC-MS investigation of *Bacillus* spp. crude metabolites confirmed the production of antimycotic compounds, including Pyrrolo(1,2a) pyrazine-1,4-dione, 9-Octadecenol, hexahydro-, 1-Propanol, Butanoic acid,2,2-dimethyl-acetate, Benzeneacetic acid and Phenol (Table 6 and Fig 4).

Table 6. Detection of anti-microbial compounds from SPKKM 5 by GCMS analysis

Peak	Retention time	Area %	Volatile compound	Biological activity	Chemical structure	Chemical formula
1	3.103	0.331	1-Deoxy-d-mannitol	Antibacterial		$C_6H_{14}O_5$
2	3.043	1.232	Thiourea	Antibacterial and Antifungal		CH_4N_2S
3	4.369	0.580	á-Histine	Antibacterial		$C_8H_{12}N_2$
4	6.405	4.343	Phenylethyl Alcohol	Antimicrobial		$C_8H_{10}O$

5	7.485	0.541	3-Dodecene, (Z)-	Antimicrobial		$C_{12}H_{24}$
6	8.105	0.347	Benzothiazole	Antibacterial, Antimicrobial and Antioxidant		C_7H_5NS
7	10.346	0.891	1-Tetradecene	Antibacterial and Antioxidant		$C_{14}H_{28}$
8	12.702	2.397	2,4-Di-tert-butylphenol	Antibacterial and Antifungal		$C_{14}H_{22}O$
9	14.333	1.508	Cyclotetradecane	Antimicrobial		$C_{14}H_{28}$
10	14.478	0.453	Diethyl Phthalate	Antifungal		$C_{12}H_{14}O_4$
11	14.698	0.322	2-(Methylmercapto) benzothiazole	Antifungal		$C_8H_7NS_2$
12	16.009	1.283	4-Phosphonobutyric acid	Antibacterial		$C_4H_9O_5P$
13	16.829	0.857	3-Methyl-1,4-diazabicyclo [4.3.0]nonan-2,5-dione, N-acetyl-	Antimicrobial		$C_{10}H_{14}N_2O_3$
14	17.339	0.647	3-Methyl-1,4-diazabicyclo [4.3.0]nonan-2,5-dione, N-acetyl-	antimicrobial		$C_{10}H_{14}N_2O_3$
15	17.724	2.175	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro--	Antifungal		$C_{14}H_{16}N_2O_2$
16	17.889	1.533	Tryptophol	Antimicrobial		$C_{10}H_{11}NO$
17	18.804	0.374	Benzohydrazide, N2-acethyl-N2-phenyl-	Antibacterial and Antifungal		$C_{16}H_{13}N_3O_2S$
18	18.530	1.509	1-Nonadecene	Antifungal		$C_{19}H$

19	19.845	0.367	Phthalic acid, 7-bromoheptyl isobutyl ester	Antimicrobial		$C_{19}H_{27}BrO_4$
20	21.441	0.800	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'd]pyrazine	Antibacterial		$C_{14}H_{22}N_2O_2$



#	RT	Scan	Height	Area	Area %	Norm %
14	17.339	2867	5,435,298	329,476.0	0.647	5.72

Pk #	RT	Hit	Compound Name	Match	R.Match	Prob.	CAS	Library
14	17.339	1	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl-	619	704	23.4		mainlib
		2	Uric acid	618	666	22.5	69-93-2	replib
		3	Uric acid	598	651	22.5	69-93-2	mainlib
		4	Glycyl-L-glutamic acid	560	607	4.1	7412-78-4	mainlib
		5	3-Ethoxy-4-methoxyphenol	549	629	2.8	65383-57-5	replib
		6	1H-Pyrazole-1-carboxaldehyde, 4-ethyl-4,5-dihydro-5-propyl-	547	632	2.6	54411-09-5	mainlib
		7	Bicyclo[2.2.1]heptane-1-carboxylic acid, 7,7-dimethyl-	543	585	2.2	937-75-7	mainlib
		8	Ethyl dodecyl ether	541	581	2.0	7289-37-4	replib
		9	1-(2-Ethyl-1,2,4-triazol-3-yl)ethanamine	540	741	2.0	1015846-51-1	mainlib
		10	3-Pyrrolidin-2-yl-propionic acid	538	788	1.8		mainlib

Fig 4. Results of GC-MS analysis of the crude antibiotic extract obtained from SPKKM 5 isolate

Statistical Analysis: Completely Randomized Block Design was followed in conduction of all the laboratory experiments and pot culture experiments. Statistical analysis was done by using WASP - Web Agri Stat Package (WASP 2.0) analysis software.

Conclusions: A total of 32 spermosphere bacteria were isolated from rice landraces while eight isolates of *Bipolaris oryzae* isolated and tested for the virulence. Among the 32 spermosphere bacteria, six bacteria reduced the growth of the mycelium of *Bipolaris oryzae* (*in vitro*). These three bacteria were further characterized (biochemical and molecular) and identified as *Bacillus subtilis* (SPKKM 5), *Acinetobacter schindleri* (SPKKM 4) and *Acidovorax spp*. (SPKKM 2). Among the seven treatments tested, foliar spraying with standard chemical metominostrobin (0.1%) was found to be the best in controlling the brown spot disease which recorded 12.54 PDI and 80.80 per cent disease reduction over control. The next best treatment was *Bacillus subtilis* (SPKKM 5) ST (10 ml kg^{-1}) + Foliar spray 0.5% at flowering and 15 days after first spray which recorded 22.50 PDI and 65.86 per cent disease reduction over control which was on par with *Bacillus subtilis* BS 1 TNAU liquid formulation (ST) (10 ml kg^{-1}) + Foliar spray 0.5% at flowering and 15 days after first spray (23.43 PDI and 62.60 per cent disease reduction over control). Hence, the spermosphere bacterial antagonist *Bacillus subtilis* can be used to manage rice brown spot disease.

Authors' contribution: MA supplied the seeds of all landraces (Rice) from the Department of Genetics and Plant Breeding, VOC Agricultural College and Research Institute, Killikulam, Vallanad, Thoothukudi (District), Tamil Nadu, India. AA conceived and designed the review. SR and MPT executed the laboratory experiments. All authors systematically reviewed the manuscript for significant intellectual content, and authorized the final version.

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