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In vitro Evaluation of Phylloplane and Spermosphere Bacterial Antagonists from Rice Land Races against *Sarocladium oryzae* causing Sheath Rot Disease of Rice

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ABSTRACT: Rice, an inevitable staple food crop, is affected by various fungal diseases, including sheath rot caused by *Sarocladium oryzae*. Sheath rot disease is considered as an important disease since it causes quantitative and qualitative yield loss. This devastating disease causes yield loss ranging from 10 to 85 per cent. Most of the chemicals used to manage sheath rot disease causes remnant of the fungicides in rice seeds. As an environmentally safer method, biocontrol using spermosphere and phylloplane bacterial antagonists were tested against *Sarocladium oryzae* under *in vitro*. Among the isolated antagonistic bacteria, *Bacillus megaterium* (SB 4-spermosphere bacteria) and *Bacillus aryabhattai* (PB 4-phylloplane bacteria) were found to be effective in inhibiting the growth of *S. oryzae in vitro*. *Bacillus subtilis* (TNAU liquid formulation) is recommended as seed treatment, seedling dipping, soil application and foliar spraying to manage sheath rot disease of rice.

Keywords: Rice, sheath rot, *Sarocladium oryzae*, spermosphere, phylloplane.

INTRODUCTION

Rice, the world's most important cultivated food crop serving as staple food for 90 per cent of world population (IRRI, 2019) and it is affected by many fungal, bacterial and viral diseases. Among the fungal diseases, rice sheath rot disease caused by *Sarocladium oryzae* is an important disease. Sheath rot has gained importance in recent years because of severe disease incidence in high yielding varieties (Nithin Kumar and Rai 2021). This severe endemic disease affects all growth stages, but is most destructive in the booting stage before panicle emergence. Manasa *et al.* (2022) reported that sheath rot disease causes yield loss up to 85 per cent. Pushpam *et al.* (2019) reported that sheath rot disease damages the boot leaf sheath that protects young panicles and delays panicle emergence. The disease also reduces grain production and quality due to discoloured and sterile seeds produced in infected panicles (Pramunadipta *et al.,* 2020). *S. oryzae* is primarily seed-borne and disperse through wind borne conidia. The pathogen produces brown lesions on the flag leaf sheath, discoloration of grains, glumes and seeds, reduced seed germination and poor grain filling due to the blocking of nutrient movement from foliage to panicle (Bigirimana *et al.,* 2015). Most pathogens associated with sheath rot have a latent stage in their life cycles until the plant undergoes stress before infecting it. Helvolic acid and cerulean in are the two phytotoxins produced by the pathogen which are responsible for the production of brown lesion in the flag leaf sheath (Hittalmani *et al.,* 2016).

Chemical control measures cannot effectively prevent sheath rot disease and moreover continuous use of chemical fungicides leads to resistance in pathogen, residual toxicity and environmental pollution. Instead, bio control agents which are more cost-effective and environmentally safer than chemical control method could be exploited for the management of sheath rot pathogen. The seed microbiome reveals the complex interactions with microorganisms throughout the plant life cycle and reveals new possibilities for studying plant-microbe interactions (Nelson, 2018). Phylloplane

and spermosphere microbes could be used to manage the sheath rot disease (Sobanbabu, 2016; Ragul, 2021). Sahu *et al.* (2021) observed that phyllopshere micro-organisms has biocontrol potential by examining culturable bacterial communities of dark brown necrotic lesions on rice leaf. Nayak *et al.* (2022) tested various bioagents against sheath rot pathogen *Sarocladium oryzae* both *in vitro* and *in vivo*.

MATERIALS AND METHODS

A. Isolation and identification of pathogen

The pathogen *Sarocladium oryzae* was isolated by direct plating method (Shomari, 1999) from the sheath rot infected rice plants collected from Peikulam village of Thoothukudi district, Tamil Nadu. The infected sample was cut into small bits of 0.5 cm and surface sterilized for 30 seconds with 0.1% mercuric chloride solution. Then washed three times with sterile distilled water to get rid of mercuric chloride residues. Potato Dextrose Agar (PDA) medium was prepared and sterilized at 121°C for 20 minutes. To avoid bacterial contamination, PDA medium was incorporated with streptomycin sulphate at 100 ppm. The sterilized media was placed onto a Petri dish and allowed to cool and solidify before use. The sterilized leaf bits were transferred to the Petri plate containing Potato Dextrose Agar medium, aseptically. These Petri plates were incubated at 28±2°C for 7-9 days in incubator then observed for the growth of fungi. The fungal colony was sub-cultured from each plate by single hyphal tip method (Sobanbabu *et al.,* 2018). On PDA slants, the pure culture of the pathogen was maintained and kept at 4°C for future study. The isolate was identified based on cultural, morphological and molecular characteristics.

B. Isolation and purification of phylloplane micro-organisms

The healthy rice leaves from rice genotypes *viz.*, IR 64, Chithiraikar, Seeraga samba, Karupukavuni and Norungan were collected from the fields maintained by the Department of Genetics and Plant Breeding, Agricultural College Research Institute, Killikulam, Vallanad, Thoothukudi district. The leaf samples were collected in sterile polythene bags, stored in an ice box and then brought to the Plant Pathology laboratory. The phylloplane microbes were isolated from the leaves by leaf impression method. The melted and cooled NA medium was poured onto the Petri plate, after solidification of the media the collected leaves were placed in the medium and gently pressed by glass rod to get the leaf impression of adaxial surface. After five minutes, by using sterile forceps the leaf was turned to face the abaxial surface over the medium. The Petri plates were maintained at 28°C for 24 hrs. For the growth of bacteria single bacterial was purified by streak plate method as suggested by Verona (1958) (Table 1).

Table 1: Phylloplane microbes isolated from rice genotypes.

Sr. No.	Rice genotypes	Phylloplane bacterial isolates (PB)
1.	IR 64	PB 1
		PB 2
2.	Chithiraikar	PB 3
		PB 4
3.	Seeraga samba	PB 5
		PB 6
4.	Karupukavuni	PB 7
		PB 8
5.	Norungan	PB 9

C. Isolation and purification of spermosphere micro-organisms

Garden soil was collected and sterilized by autoclaving twice at 121°C at 20 lbs on two consecutive days to remove the sporulating micro-organisms. After sterilization of soil, rice seeds of rice genotypes *viz.*, IR 64, Chithiraikar, Seeraga samba and Karupukavuni were sown in the sterile containers filled with sterilized garden soil and stored in a controlled atmosphere for two to three days. After germination, seedlings were washed and the washings of all the samples were collected separately and serially diluted up to 10^{-5} and 10^{-6}. Separate plating on nutrient agar media was carried out by transferring one ml of aliquot from 10^{-5} and 10^{-6} dilutions and the incubated at 28±2°C for 24 hours. After 24 hours of growth, the bacterial colonies were isolated and purified by streak plate method as suggested by Verona (1958) (Table 2).

Table 2: Spermosphere microbes isolated from rice genotypes.

Sr. No.	Rice genotypes	Spermosphere bacterial isolate (SB)
1.	IR 64	SB 1
		SB 2
2.	Chithiraikar	SB 3
		SB 4
3.	Seeraga samba	SB 5
		SB 6
4.	Karupukavuni	SB 7
		SB 8

D. In-vitro evaluation of phylloplane and spermosphere bacterial antagonists against Sarocladium oryzae.

(i) Dual culture assay (Dennis and Webster 1971). Antagonistic ability of phylloplane and spermosphere bacteria against the *S. oryzae* was employed by dual culture method (Dennis and Webster 1971) under *in vitro* condition. Sterilized PDA medium was melted and after cooling at 45ºC was poured aseptically into sterilized Petri dishes at the rate of 20 ml per plate and allowed to solidify. Mycelial disc of 9 mm diameter from actively growing 10 days old culture of *S. oryzae* was punched out with the help of a sterilized cork borer and placed at the periphery of the Petri plate around one cm from the edge and incubated for 36 hr at 28 ± 2 ºC. Similarly, on the opposite side of the Petri plates, respective bacterial isolates of phylloplane were streaked. Three replications were maintained for each treatment. The Petri plates containing PDA medium inoculated with the *S. oryzae* alone served as control. All the Petri plates were incubated at 28 ± 2 ºC temperature and observation for inhibition of the *S. oryzae* was recorded from 3rd day onwards up to 15th day from inoculation.

The radial growth of *S. oryzae* in each treatment was measured and compared with radial growth of control. The per cent inhibition of the mycelial growth of *S. oryzae* was calculated for each treatment by adopting the following formula (Vincent, 1947).

Per cent Inhibition over control (%) = C-T/C × 100

Where,

C = Diameter of fungus colony (cm) in control plate

T = Diameter of fungus colony (cm) in treated plate

By following the same procedure, the spermosphere bacterial isolates were tested against *S. oryzae.* The best identified phylloplane and spermosphere bacterial isolates were characterized by DNA analysis by using 27F and 1115R primer pairs as per the standard procedure (Knapp *et al.,* 1996).

RESULTS AND DISCUSSION

A. Isolation and identification of pathogen

The pathogen isolated from the infected samples collected from Peikulam village of Thoothukudi district was identified as *Sarocladium oryzae* based on the cultural characteristics *viz.,* white cottony aerial mycelial growth and hyaline cylindrical conidia. In addition, it was confirmed as *Sarocladium oryzae* through molecular characterization (Accession number-OR298274). Amin *et al.* (1974); Gams & Hawksworth (1975) isolated *Sarocladium oryzae* on PDA medium and sub-cultured by single spore method. Sobanbabu *et al.* (2018) obtained three isolates of the pathogen on PDA medium from infected rice leaf sheath collected from Thoothukudi, Tirunelveli and Coimbatore districts.

B. Isolation of phylloplane bacterial antagonists

Nine isolates of phylloplanebacterial antagonists were isolated from leaf surface of rice genotypes *viz.,* IR 64, Chithiraikar, Seeraga samba, Karupukavuni and Norungan. The isolated phylloplane bacterial antagonistic efficacies were tested against rice sheath rot pathogen *Sarocladium oryzae.* Aswathy *et al.* (2017); Sobanbabu *et al.* (2018); Jeyashri *et al.* (2019) isolated phyllosphere bacteria by leaf imprinting method from rice leaves. Arunkumar *et al.* (2019) obtained eight bacterial isolates from phyllosphere region of rice by leaf impression method. Kumar *et al.* (2019) isolated phyllosphere micro-organisms from leaf samples of five different varieties by leaf imprinting method (Fig. 1).

C. Isolation of spermosphere bacterial antagonists

Eight isolates of spermosphere bacterial antagonists were isolated from spermosphere region of rice genotypes *viz.,* IR 64, Chithiraikar, Seeraga samba and Karupukavuni. Bio-control agents were isolated from rice ecosystem to control rice seed borne pathogens (Srinivas, 2002). Similarly, the spermosphere bacteria *Acinetobacter schindleri* was isolated from wild paddy Navara (Ragul, 2021). The isolated spermosphere bacterial antagonist efficacies were tested against sheath rot pathogen *Sarocladium oryzae* (Fig. 2).

D. In vitro evaluation of phylloplane bacterial antagonists isolates against Sarocladium oryzae

Nine phylloplane bacterial isolates were tested against the rice sheath rot pathogen *Sarocladium oryzae* by dual plate technique. Among the nine isolates, PB 4 isolated from rice genotype Chithiraikar was found to be effective in inhibiting the mycelial growth (39.68%) followed by PB 8 (37.68 %) and PB 1 (36.23 %) and the least mycelial growth inhibition was observed in PB 2 (24.63 %) which was isolated from IR 64. Wiraswati *et al.* (2019) observed that rice phyllosphere bacteria can be effectively used as biocontrol agents tocontrol rice diseases (Table 3 & Fig. 3). The molecular characterization of effective phylloplane bacteria PB 4revealed that the organism was*Bacillus aryabhattai* (Accession number-OR287195).

E. In vitro evaluation of spermosphere bacterial antagonists isolates against Sarocladium oryzae

Among the eight spermosphere bacterial isolates tested against *S. oryzae*, SB 4 isolated from Chithiraikar rice spermosphere was found to be effective in inhibiting the mycelial growth (35.61 %) followed by 32.88 and31.50 per cent inhibition by SB 1 and SB 3, respectively (Table 4 & Fig. 4).

Ragul (2021) reported that the spermosphere antagonistic bacteria isolates from wild paddy Navaraand wild bhendi. Twenty endophytic bacterial antagonists were isolated from rice plants for the management of false smut disease of rice (Navarasu, 2022). The molecular characterization of effective spermosphere bacteria SB 4 revealed that the organism was *Bacillus megaterium* (Accession number-OR287430).

Table 3: *In vitro* testing of phylloplane bacterial antagonists against *Sarocladium oryzae.*

Sr. No.	Treatment Number	Antagonistic bacterial isolate	Mycelial growth (cm)	Per cent inhibition over control (%)
1.	T1	PB 1	4.40^{cd}	36.23 (37.57)^{b}
2.	T2	PB 2	5.21^{a}	24.63 (30.42)^{g}
3.	T3	PB 3	4.82^{b}	30.43 (33.07)^{d}
4.	T4	PB 4	3.82^{e}	39.68 (38.73)^{a}
5.	T5	PB 5	5.11^{a}	26.09 (30.33)^{g}
6.	T6	PB 6	4.90^{b}	28.99 (32.27)^{e}
7.	T7	PB 7	5.00^{a}	27.54 (31.32)^{f}
8.	T8	PB 8	4.31^{d}	37.68 (37.61)^{b}
9.	T9	PB 9	4.50^{c}	34.78 (35.86)^{c}
10.	T10	Control	6.90	0.00
		CD P=(0.05)	**0.20**	**0.53**

*Mean of three replications; The treatment means are compared using Duncan Multiple Range Test (DMRT).

Table 4: *In vitro* testing of spermosphere bacterial antagonists against *Sarocladium oryzae.*

Sr. No.	Treatment Number	Antagonistic bacterial isolate	*Mycelial growth (cm)	*Per cent inhibition over control (%)
1.	T1	SB 1	4.90^{d}	32.88 (34.03)^{b}
2.	T2	SB 2	5.21^{c}	28.77 (31.98)^{d}
3.	T3	SB 3	5.00^{d}	31.50 (33.77)^{bc}
4.	T4	SB 4	4.71^{e}	35.61 (36.08)^{a}
5.	T5	SB 5	5.70^{a}	21.92 (27.48)^{f}
6.	T6	SB 6	5.12^{cd}	30.14 (32.99)^{c}
7.	T7	SB 7	5.40^{b}	26.03 (30.34)^{e}
8.	T8	SB 8	5.50^{b}	24.66 (29.52)^{e}
9.	T9	Control	7.3	0.00
		CD P=(0.05)	**0.19**	**0.86**

*Mean of three replications; The treatment means are compared using Duncan Multiple Range Test (DMRT).

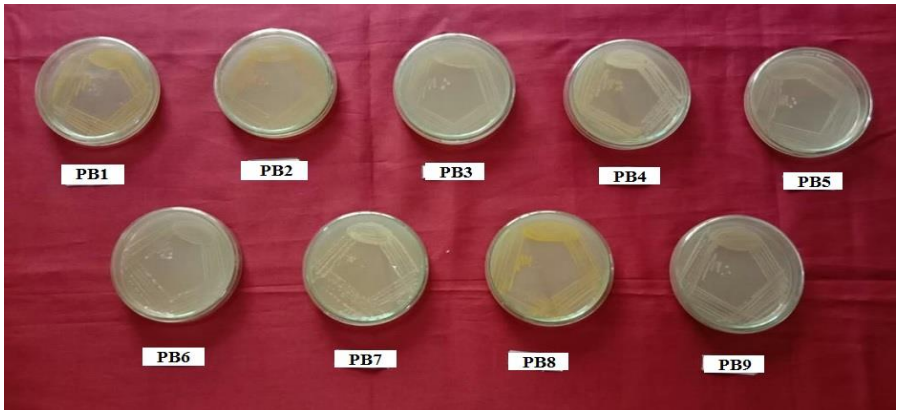

Fig. 1. Isolates of phylloplane bacterial antagonists, PB- Phylloplane bacteria.

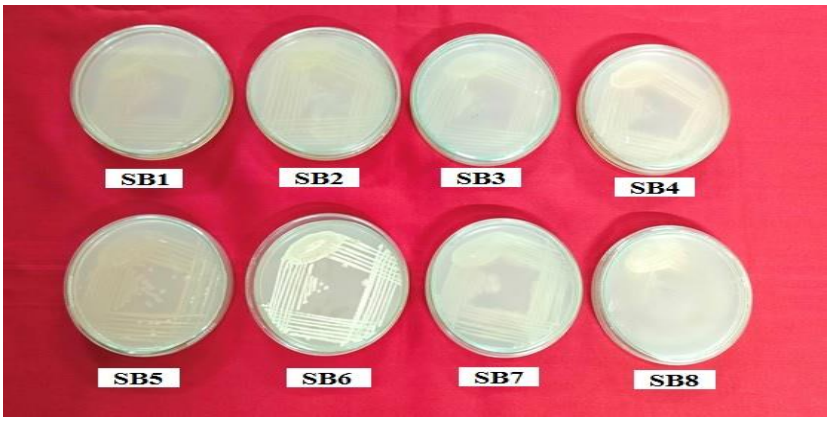

Fig. 2. Isolates of spermosphere bacterial antagonists, SB- Spermosphere bacteria.

Fig. 3. *In vitro* testing of phylloplane bacterial antagonists against *Sarocladium oryzae.*

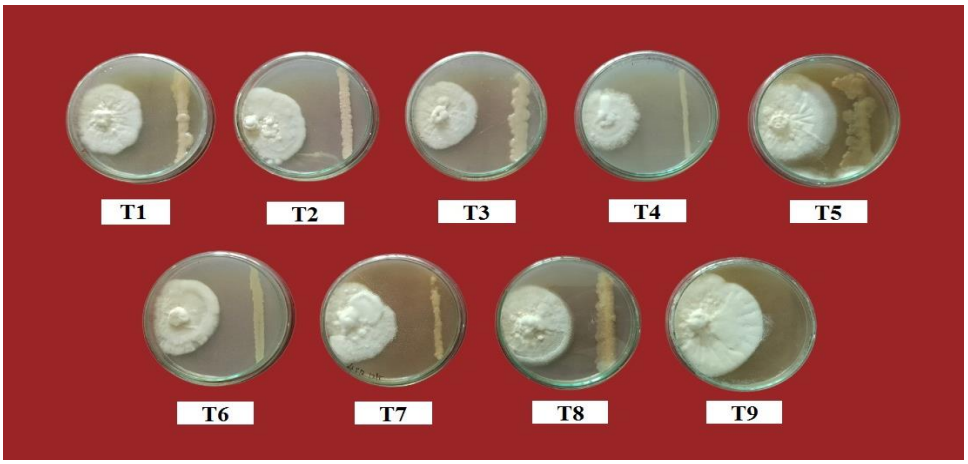

Fig. 4. *In vitro* testing of spermosphere bacterial antagonists against *Sarocladium oryzae.*

CONCLUSIONS

The use of biocontrol agents which are more cost-effective and environmentally safer than chemical control method is studied against sheath rot pathogen. The phylloplane and spermosphere bacteria *viz.*, *Bacillus aryabhattai* (PB 4) and *Bacillus megaterium* (SB 4), respectively were identified as effective bacterial antagonists in inhibiting the mycelial growth of rice sheath rot pathogen *Sarocladium oryzae.*

FUTURE SCOPE

The mechanism of phylloplane and spermosphere bio-agents will be focused for the management of sheath rot disease of rice. Commercial formulation of the best combination could be identified.

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Conflict of Interest. None.

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