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RESEARCH ARTICLE Indian Journal of Agricultural Research

Exploring the Biocontrol Potential of Rhizospheric Bacteria against *Bipolaris oryzae* L. Infecting Rice

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10.18805/IJARe.A-6443

ABSTRACT

Background: Rice brown spot, caused by *Bipolaris oryzae*, is one of the most significant diseases affecting rice cultivation and was a major contributor to the Bengal famine. The disease continues to threaten rice production, especially in Tamil Nadu. Excessive reliance on chemical fungicides for its control has led to environmental and health issues, necessitating eco-friendly alternatives such as biological control.

Methods: Infected rice leaf samples were collected from five major rice-growing districts of Tamil Nadu. The pathogen was isolated and morphologically identified as *Bipolaris oryzae*, characterized by fusiform, slightly curved conidia with septations. Bacillus isolates were obtained and screened for antifungal activity using dual culture assays. The isolates showing significant inhibition of *B. oryzae* were further analyzed. Molecular confirmation of the pathogen and bacterial isolates was performed using PCR (Polymerase Chain Reaction) analysis.

Result: Several bacterial antagonists exhibited notable effects against *B. oryzae*, with isolates BC34 (*Alcaligenes faecalis*-PV992712), BC 5 (*Alcaligenes faecalis*-PV991184) and MS8 (*Serratia marcescens*-PV992713) showing the highest levels of mycelial inhibition. PCR analysis confirmed the identity of both the pathogen as *B. oryzae* and the effective bacterial strains as *Bacillus spp.* The results demonstrate that these bacterial antagonists act through multiple mechanisms, including direct antagonism, enzymatic activity and the production of volatile organic compounds (VOCs), highlighting their potential as sustainable biocontrol agents in rice cultivation.

Key words: Antifungal activity, Bacterial antagonists, Biocontrol, *Bipolaris oryzae*, Volatile organic compounds.

INTRODUCTION

Rice (*Oryza sativa* L.) is a crucial crop belonging to the *Poaceae* family and serves as a staple food for approximately 60% of the global population. It is the primary source of nourishment for over a third of the planet's inhabitants. Globally, more than 3.5 billion people rely on rice for more than 20% of their daily caloric intake (Maclean *et al.,* 2013; Surendhar *et al.,* 2022). The majority of rice cultivation occurs in tropical areas such as South and Southeast Asia, West Africa and Central and South America. Rice is grown in over 100 countries, covering an area of around 162.06 million hectares, with a total annual production of 755.47 million tonnes and an average productivity of 4.66 tonnes per hectare (Anonymous, 2020a). Among the various rice diseases, brown spot caused by *Cochliobolus miyabeanus*) has been identified as a major concern, resulting in ongoing losses in both quality and quantity (Hossain *et al.,* 2011). The disease can escalate in severity up to 90%, adversely affecting plant growth, causing grain discoloration and diminishing market quality (Valarmathi and Ladhalakshmi, 2018). Bacterial antagonists that can produce the antimicrobial compounds, particularly those with antifungal properties, to control plant pathogens such as *Bacillus, Pseudomonas* and *Pantoea* spices, which have the potential to generate antifungal compounds targeting fungal pathogens like *Pythium* spp, *Fusarium oxysporum, Rhizoctonia solani* (Junaid *et al.,* 2013) and *Pyricularia oryzae* (Sha *et al.,* 2016). In terms of managing brown spot in rice, there has been limited exploration of strategies since resistant cultivars have not been extensively developed. The use of bacteria to reduce crop disease, especially brown spot disease, appears to be the most effective method for replacing synthetic

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How to cite this article: Mohana Priya, P., Rageshwari, S., Mohanasundaram, S. and Karpagavalli, S. (2025). Exploring the Biocontrol Potential of Rhizospheric Bacteria against *Bipolaris oryzae* L. Infecting Rice. *Indian Journal of Agricultural Research*. **1-8.** doi: 10.18805/IJARe.A-6443.

Submitted: 24-07-2025 **Accepted:** 04-08-2025 **Online:** 26-08-2025

fungicides. Therefore, employing the use of bacterial antagonists as biocontrol agents is expected to be an effective strategy for controlling foliar diseases such as brown spot. Nevertheless, the chemical control of *B. oryzae* has been demonstrated to be a quick, effective and economical method of management (Kumar *et al.,* 2017). Excessive use of chemical fungicides to manage this disease has raised environmental and health concerns, emphasizing the need for sustainable alternatives. In this context, the present study investigates the antifungal activity of bacterial antagonists against *B. oryzae*. bacterial antagonists are involved in the control of plant diseases through multifaceted action, *viz.*, competition, induction of systemic resistance and antibiotic production. Among them, antibiosis is one of the most important mechanisms (Thomashow and Weller, 1996). In the last few years in the Chengalpattu district, around 70% of disease incidence has mainly been observed in rice brown spot. It was severely infested in all areas of the Chengalpattu district. The study is focused on controlling the brown spot of rice by bacterial antagonists without any hazards to environmental conditions.

MATERIALS AND METHODS

Disease incidence and symptomatology

The survey has been conducted to record brown spot incidence in different rice growing districts of Tamil Nadu, *viz.,* Chengalpattu, Villupuram, Coimbatore, Thanjavur and Tiruvannamalai districts, from different varieties including Ponni, IR 20, CO 19, CR 1009 and ADT 37. The infected samples were also collected for pathogen isolation and pure culture maintenance. The severity and symptoms were recorded following a 0-9 scale (IRRI, 2002) and PDI (Per cent disease index) was calculated as proposed by Wheeler (1969).

Isolation and characterization of *Bipolaris oryzae*

Isolation and morphological characterization

The pathogen isolation was done following the protocol given by Valarmathi and Ladhalakshmi (2018). After incubation, the colonies' traits were recorded and tabulated (Tuite, 1969). Pure culture of the pathogen was done by hyphal tip method.

Molecular characterization

The mycelial mat of a 15-day-old virulent culture was utilized for DNA extraction. Fresh mycelial mat was pat dried onto sterile tissue paper to remove excess broth and used for DNA extraction. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Wu *et al.* (2001).

The DNA was subjected to PCR amplification of 18S-28S rRNA using Universal primer pair ITS-1 (5′-TCCGTA GGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCT TATTGATATGC-3′). The reaction mixture was prepared for a final volume of 30 µl and PCR was done following the protocol given by Sambrook *et al.* (1999). A 100 bp ladder was used to confirm the size of the targeted fragment (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplicons were visualized in a gel documentation unit (Vilber, E-Box model, France) (Monisha *et al.,* 2019) and documented.

Isolation and characterization of bacterial antagonists isolated from rhizosphere soil

Isolation and morphological characterization

Bacterial antagonists were isolated from the rhizosphere soil samples of different crops, *viz.*, thenai, rice, tamarind, neem, thorn tree and palmyra palm. The antagonists were isolated by serial dilution technique using the Nutrient Agar (NA) medium. A one-gram sample of rhizosphere soil was placed into a sterile test tube with 9 ml of sterile distilled water and then serially diluted up to 10^{-6} (Bhandhari *et al.,* 2024).

One ml from both the 10^{-5} and 10^{-6} dilutions of bacterial antagonists was drawn up and transferred into sterile petri dishes. Later, added with NA media with a gentle swirl and incubated at room temperature (28±2°C) for 24-48 h (Sethi and Mukherjee, 2018). The colony characters were recorded. The strains of bacterial antagonists were subjected to a dual plate assay against *B. oryzae*. Three bacterial antagonists that exhibited the best control were subjected to molecular characterization.

Molecular characterization of bacterial antagonists

The genomic DNA from three strains of bacterial antagonists were isolated using the TE buffer method as proposed by Odeyemi *et al.,* 2018. The final pellet was retained, washed with 70% ethanol and air dried. The pellets were resuspended in 50 µl TE buffer (pH 6.0).

The molecular characterization of the three isolates was done by analyzing the 16S rRNA gene sequence using universal primers, *viz.*, 27F: 5′ AGAGTTTGATCCTGGCTCAG 3′, 1492 R: 5′ TACGGCTACCTTGTTACGACTT 3′ (Frank *et al.,* 2008). The PCR reactions were carried out in a 30 µl reaction volume and the gel was visualized and documented in a gel documentation system (Vilber, E-Box model, France) (Rajashekhar *et al.,* 2017; Bhagyashree *et al.,* 2023).

Antagonistic activity of bacterial antagonists against *Bipolaris oryzae in vitro* (Dual method)

Bacterial isolates with antagonistic activity were screened *in vitro* against *Bipolaris oryzae* using dual culture technique. A total of 85 isolates were identified to have antagonistic potential and the best 15 isolates were subjected to screening. A mycelial disc of 5 mm was placed onto one end and the corresponding opposite end with a bacterial streak. These plates were incubated at 28±1°C till the mycelia covered the full plate. The results were validated using a completely randomized design (CRD) with three replications and the percentage suppression of pathogen mycelial growth was determined (Kishore *et al.,*

2022). Per cent inhibition was calculated using the standard formula (Dennis and Webster, 1971; Karan *et al.,* 2022).

RESULTS AND DISCUSSION

Disease incidence and symptomatology

The survey for the occurrence of brown spot in rice conducted in different district of Tamil Nadu including Chengalpattu, Villupuram, Coimbatore, Thanjavur and Tiruvannamalai revealed that the maximum incidence of 53.75 PDI was observed in CO 51 variety cultivated in Coimbatore district followed by 49.13 PDI in CR 1009 variety cultivated in Thanjavur district (Table 1) and the least incidence was observed in the Tiruvannamalai district of 27.91 PDI in variety of ADT 37. Irrespective of the varieties and their location, these findings are consistent with earlier reports by Valarmathi and Ladhalakshmi (2018), who have also reported higher disease prevalence in delta regions. The symptoms initially appeared as tiny brown dots, which later developed into cylindrical or oval-shaped dark brown spots similar to sesame seeds (Fig 1). The symptoms were also observed on panicles, glumes and infected grains. The symptoms were visible from the seedling stage till harvest and the initial symptoms were observed 35 to 45 days after sowing (DAS). The spots typically measured about 1/8 inch in diameter and varied in shape from circular to oval, depending on environmental factors and varieties.

Similarly, Rajasekar *et al.* (2017) have also indicated that the symptoms initially appear as small brown spots, which then develop into cylindrical or oval forms resembling sesame seeds. Tiny brown spots appear to be large in shape and sesame-like spots appear on the leaves of all stages of crops, from seedling to tillering stage.

Isolation and characterization of *Bipolaris oryzae*

Isolation and morphological characterization

The collected samples were isolated and a pure culture was maintained by the hyphal tip method. The pathogen was isolated from five districts, namely BOC1 (Chengalpattu), BOC2 (Villupuram), BOC3 (Coimbatore), BOC4 (Thanjavur) and BOC5 (Tiruvannamalai) and their mycelial character was found to be varied for different isolates.

The diameter of the mycelial growth was measured at the 7th day after incubation for all the isolates. The pathogen initially appeared white and later, turned to greyish black colour with fluffy mycelial growth on PDA medium (Fig 2). Among the five isolates, the isolate BOC3 collected from

Table 1: Survey on major rice growing areas of Tamil Nadu.

District	Village	GPS location	Variety	Isolate	PDI %
Chengalpattu	Munnakulam	Lat 12.355997° Long 79.723678°	Ponni	BOC 1	45.24
Villupuram	Gandhi nagar	Lat 12.203082° Long 79.68249°	IR 20	BOC 2	33.12
Coimbatore	TNAU	Lat 11.0122° Long 76.9354°	CO 19	BOC 3	53.75
Thanjavur	Paruthiapparkovil	Lat 10.663577° Long 79.263369°	CR 1009	BOC 4	49.13
Tiruvannamalai	Mallavadi	Lat 12.338398° Long 79.082688°	ADT 37	BOC 5	27.91

Table 2: Phenotypical characterization on different strains of *B. oryzae*.

Isolate code	Days of mycelial full growth	Radial mycelial growth (in mm)	Mycelial character	Sporulation (Days)
BOC 1	10	82	Mixture of grey and white with fluffy mycelium, raised margin	17
BOC 2	12	75	Greyish brown, fluffy mycelium with white dots and raised margin	23
BOC 3	7	90	Greyish brown, fluffy mycelium and raised margin	12
BOC 4	9	85	Greyish black mycelium with raised margin	15
BOC 5	15	70	Greyish white mycelium with fluffy growth, raised margin	28

Fig 1: Symptoms of rice brown spot.

Coimbatore district exhibited maximum mycelial growth of 90 mm compared to other isolates of *Bipolaris oryzae.* The colony characters and sporulation of each isolate were described in Table 2.

The microscopic observation revealed that the mycelia of all the isolates were light brown with septations. The hyphae was dark brown with branched. The conidia were olivaceous brown, fusiform, slightly curved with 6 to 14 transverse septations. The size of conidia and the number of septations also varied among different isolates of *B. oryzae* (Fig 3). The conidial characters of each isolate were described in Table 3.

According to Monisha *et al.,* (2019) the conidia were fusiform, multi-septate, brown and slightly curved. The fungal colonies were diverse in their characters and appeared white with a cottony texture, black with a fluffy

Fig 2: Cultural variations observed in different strains of *Bipolaris oryzae.*

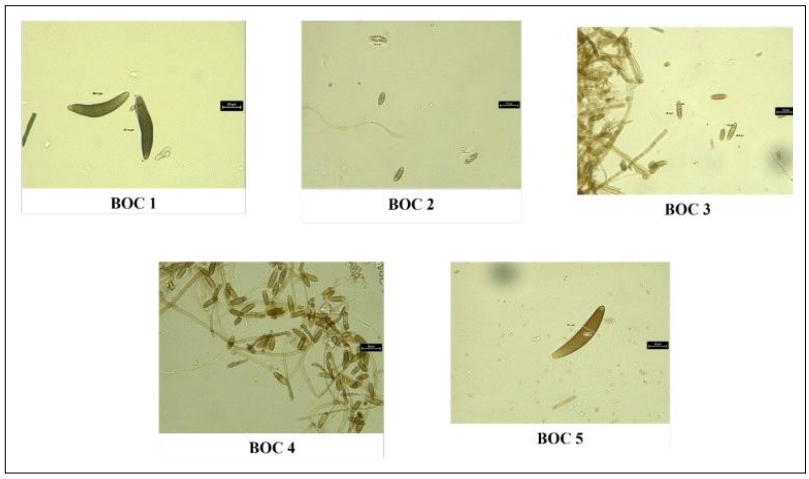

Fig 3: Microscopic character of conidial observation on different strains of *Bipolaris oryzae.*

Table 3: Conidial characters of *B. oryzae.*

Isolate code	Conidial characters	Mean conidia length (µm)	Mean conidia breadth (µm)	No. of septations
BOC 1	Dark brown with Multi septations, curved conidia and tapering ends	87.3	23.2	9
BOC 2	Light brown with septations, slightly curved and tapering ends	25.9	7.1	3
BOC 3	Light to dark brown with multi-septations and a tapering ends	26.6	6.8	6
BOC 4	Dark brown with multi-septations and tapering ends	54.2	8.2	5
BOC 5	Dark brown with no septations, curved conidia and tapering ends	96.4	21.1	0

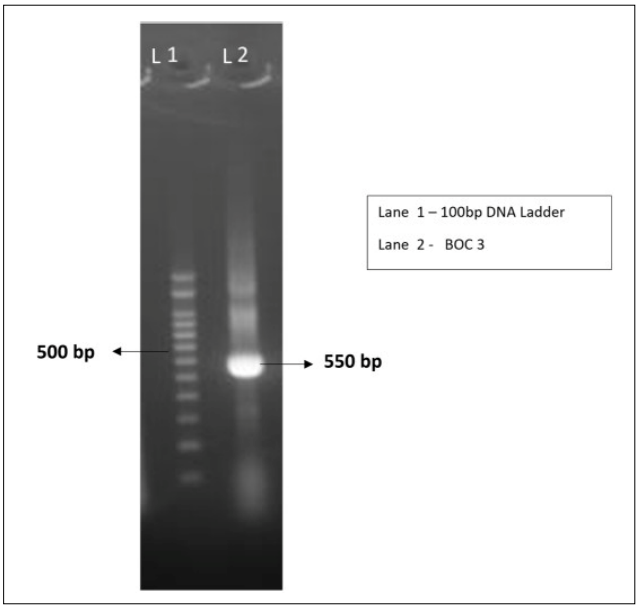

Fig 4a: Molecular characterization of *Bipolaris oryzae.*

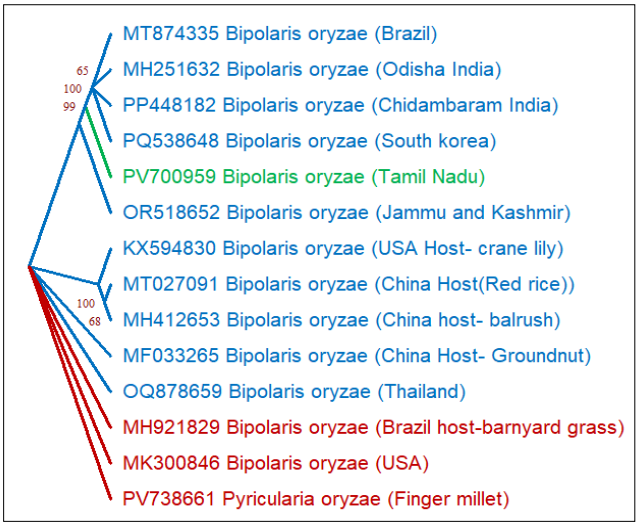

Fig 4b: Phylogenetic analysis of *Bipolaris oryzae* generated from 18S-28S rRNA gene sequences using mega 11.

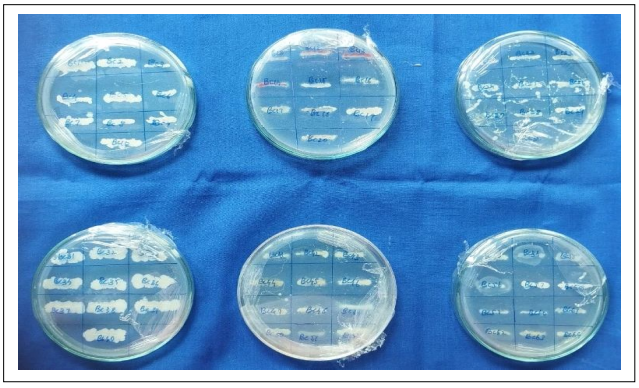

Fig 5: Isolation of bacterial isolates from rhizosphere soil.

growth pattern, black with stunted growth and black with a cottony texture (Kumar *et al.,* 2017).

Manamgoda *et al.* (2014) stated that *B. oryzae* conidiophores appeared either singly or in clusters. These conidiophores are multi-septate, branched or unbranched and ranged in color from brown to black. Conidia can be navicular, fusiform, obclavate, or nearly cylindrical and they are usually curved, though they can also be straight at times.

Molecular characterization

PCR analysis using the universal primers ITS1 and ITS4 resulted in an amplicon size of approximately 550 bp (Fig 4a). The sample was sequenced and submitted to the NCBI (National Center for Biotechnology Information, Gene Bank, New York, USA) under the accession number PV700959. Phylogeny analysis resulted in high similarity between genetic relationships among *Bipolaris oryzae* isolates from different geographical regions and hosts. Isolates are color-coded, with the Tamil Nadu isolate (green) clustering closely with other Asian strains. Bootstrap values on the branches indicate the confidence of branching patterns. Notably, *Pyricularia oryzae* and certain *Bipolaris oryzae* isolates (in red) form distinct clades, indicating evolutionary divergence (Fig 4b).

Genomic DNA isolated from the culture of all five effective isolates through the CTAB method and molecularly characterized by ITS primers ITS 1 and ITS 4 (Fungal universal primers). A single band of intact high molecular weight DNA was visualised through agarose gel electrophoresis. The size of the PCR fragments was approximately amplified at 550 bp (Frank *et al.,* 2008).

Isolation and characterization of bacterial antagonists isolated from rhizosphere soil

Isolation and morphological characterization

A total of 85 antagonistic bacterial strains were isolated from the rhizosphere of asymptomatic plants like rice, thorn tree, neem, tamarind, thenai, Palmyra palm tree, *etc.* (Fig 5). The morphology characters of bacteria observed were slimy, white to pink in colour, with smooth/ round/ filamentous/ lobate/ undulate margins (Table 4). Among them, 15 isolates exhibited antifungal activity.

Molecular characterization

PCR analysis using the universal primers 27F and 1492R resulted in an amplicon size of approximately 1600 bp, thus confirming the bacterial DNA (Fig 6). The isolates have been characterized molecularly and their identity is confirmed to be BC34 (*Alcaligenes faecalis*), BC5 (*Alcaligenes faecalis*) and MS8 (*Serratia marcescens*).

The sequences have been submitted in GenBank under the Accession numbers PV992712, PV991184 and PV992713, respectively. The BLAST search has revealed that the sequences have been found to have 100% identity with the sequences available in the database.

In vitro* screening of bacterial antagonists against *B. oryzae

Out of which, the isolate BC34 (*Alcaligenes faecalis)* recorded 26.6 per cent inhibition of mycelial growth over the control followed by MS8 (*Serratia marcescens*) and BC5 (*Alcaligenes faecalis*), which recorded 21.6 and 15.3 per cent inhibition, respectively (Fig 7). Minimum inhibition was exerted by the isolate BC 7, followed by BC 47, which recorded 6.67 and 7.33 per cent inhibition, respectively (Table 5).

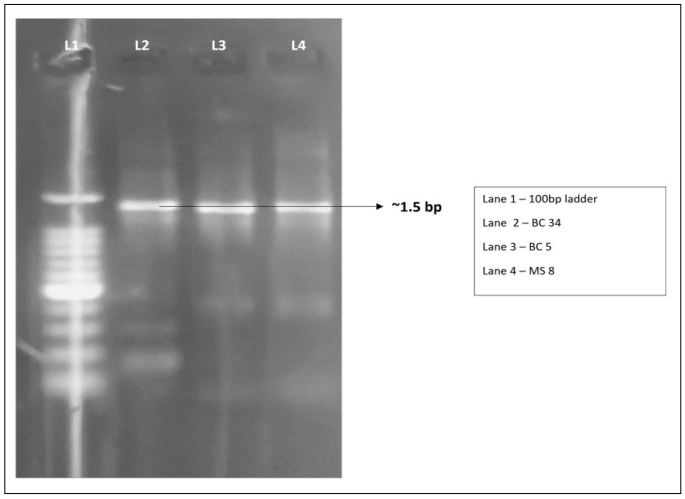

Fig 6: Gel electrophoresis for molecular conformation of 16S rRNA.

Fig 7: Antagonistic activity of *in vitro* with the best three isolates.

Table 4: List of bacterial isolates collected from various crop sources.

Crops	Isolates
Chickpea RRS pulses	BC 1, BC 2, BC 3, BC 4
Wheat RRS Bikaner	BC 5, BC 6, BC 7
Jaisdmer-Rajasthan	BC 8, BC 9, BC 10
Thenai (10^{-3}) R1	BC 11, BC 12, BC 13, BC 14, BC 15, BC 16. BC 17, BC 18, BC 19
Thenai (10^{-3}) R2	BC 20, BC 21, BC 22, BC 23, BC 24, BC 25, BC 26, BC 27, BC 28
Thenai (10^{6})	BC 29, BC 30, BC 31, BC 32, BC 33, BC 34
Tamarind (10^{-3}) R1	BC 35, BC 36, BC 37, BC 38, BC 39, BC 40, BC 41, BC 42, BC 43
Tamarind (10^{-3}) R2	BC 44, BC 45, BC 46, BC 47,
Rice (10^{-3})	BC 48, BC 49, BC 50, BC 51, BC 52, BC 53, BC 54
Acacia (10^{6})	BC 55, BC 56, BC 57, BC 58, BC 59, BC 60, BC 61, BC 62, BC 63, BC 64
Neem	MS 1, MS 2, MS 3, MS 4, MS 5
Palmyra palm tree	MS 6, MS 7, MS 8
Sesbania	MS 9, MS 10, MS 11
Tulsi (black)	MS 12, MS 13, MS 14, MS 15, MS 16
Tulsi (green)	MS 17, MS 18, MS 19, MS 20, MS 21

Table 5: *In vitro* screening of bacterial antagonists against *Bipolaris oryzae.*

Isolate code	Inhibition zone (mm)			Mean
	R1	R2	R3	
BC 5	20	10	16	15.3
BC 7	9	4	7	6.67
BC 34	25	30	25	26.6
BC 36	10	12	16	12.6
BC 42	13	10	14	12.33
BC 45	12	15	13	13.33
BC 47	10	5	7	7.33
BC 54	15	10	10	11.6
BC 55	10	8	6	8.0
MS 5	16	18	11	15.0
MS 6	11	10	12	11.0
MS 8	10	25	30	21.6
MS 17	15	12	10	12.33
MS 18	15	12	9	12.0
MS 20	19	14	11	14.67
SD			3.188	
CV%			29.186	

*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

These results were supported by earlier studies by Utkhede and Sholberg (1986) and Thomashow and Weller (1996), which highlighted the suppressive effects of *Bacillus* species through mechanisms like antibiosis and competition.

Leifert *et al.* (1995) found that *B. subtilis* CL27 generated antibiotics that were effective against *Alternaria brassicicola* and *Botrytis cinerea* in laboratory conditions.

CONCLUSION

The current study demonstrated the promising potential of rhizospheric *Bacillus* spp. as effective biocontrol agents against *Bipolaris oryzae*, the pathogen responsible for brown spot disease in rice. Through systematic isolation, morphological and molecular characterization and dual culture assays, it was evident that certain isolates particularly BC34 (*Alcaligenes faecalis)* and BC5 (*Alcaligenes faecalis)* exhibited significant antifungal activity. These strains not only inhibited fungal growth but also possessed key biochemical traits such as catalase activity, gelatin liquefaction, starch hydrolysis and IAA production, which contribute to both plant health and pathogen suppression. Compatibility analysis further revealed that BC34 (*Alcaligenes faecalis)* and BC5 (*Alcaligenes faecalis)* can be co-utilized, enhancing their applicability in developing microbial consortia. These findings highlight the potential of integrating beneficial rhizobacteria into sustainable disease management strategies for rice cultivation. By adopting such eco-friendly biocontrol agents, farmers can reduce dependency on chemical fungicides, lower input costs and improve overall soil health and yield sustainability. Further *in vivo* and field evaluations are recommended to validate their efficacy under natural conditions and to develop commercial formulations. Importantly for farmers, these beneficial bacteria can be developed into cost-effective, eco-friendly biocontrol agents that can be applied directly in the field or, foliar sprays. Since farmers can use the bacterial consortia for the application at the field level.

ACKNOWLEDGEMENT

The present study was supported by SRM College of Agricultural Sciences, Baburayanpettai.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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