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MORPHOLOGICAL IDENTIFICATION AND BIOCHEMICAL ANALYSIS OF RHIZOBACTERIAL DIVERSITY IN CHILLI AND THEIR ANTAGONISTIC ACTIVITY AGAINST *Colletotrichum jasminigenum* SPTD17

Simran PANIGATTI[1], Thangadurai DEVARAJAN[1], Sangeetha JEYABALAN[2], Poojashree Nagappa KUMMUR[1], Smita SHINDE[1]

[1]Department of Botany, Karnatak University Dharwad, Karnataka, Pavate Nagar, Dharwad, India
[2]Department of Environnemental Sciences, Central University of Kerala, Periye, Kasargod, Kerala, India

Corresponding author email: simranjp786@gmail.com

Abstract

Anthracnose, a drastic fungal disease caused by Colletotrichum spp. is a significant constraint to chilli production all over the world, resulting in substantial yield losses across all major chilli-producing areas. Management of plant diseases using biocontrol agents is one of the best methodologies that may reduce the use of synthetic chemical based formulations. A total of fifteen rhizospheric bacteria were isolated from chilli fields. Preliminary morphological identification was done followed by scanning electronic microscope and then subjected for biochemical characterization. Antagonistic activities of all isolates were evaluated in vitro against Colletotrichum jasminigenum SPTD17. Based on the morphological and biochemical characterization six isolates categorized to Bacillus, two Lysinibacillus, one Paenibacillus, five Pseudomonas and one Acinetobacter genera were identified. Seven isolates (PS1, PS4, PS6, PS8, PS9, PS10 and PS11) showed strongest antagonistic activity and more than 80% zone of inhibition against Colletotrichum jasminigenum SPTD17. The results of this study suggest that chilli rhizosphere bacterial diversity can be resource for biocontrol of chilli anthracnose pathogens; further research may encourage the molecular characterization and PGRP applications of rhizospheric bacterial biocontrols.

Key words*: biocontrol, morphological characterization, scanning electron microscopy, biochemical characterization, antagonistic bacteria.*

INTRODUCTION

The rhizosphere contains a diverse range of microorganisms, with bacterial community composition influenced by various factors, including plant species and variety, plant growth stage and phenology, environmental stress and disturbance, occurrence of disease, pests and soil management practices (Marschner et al., 2011). The role of plant growth promoting rhizobacteria (PGPR) has emerged as a viable and eco-friendly strategy for enhancing plant growth, mitigating stress, and fostering sustainable agriculture practices, thereby providing a promising alternative to synthetic agrochemicals (Gupta & Pandey, 2019). As an ecological, efficient, and ecologically benign method of managing crop plant diseases, biological management is quickly gaining popularity. Kumar & Thirumalaisamy (2016) stated that the broad analysis of the biocontrol literature reveals a focus on soil borne pathogens relatively than foliar infections, with later receiving a more favorable response.

Rhizobacterial communities regulate key processes in ecosystem functioning, such as nutrient cycling, decomposition, and carbon sequestration, eventually influencing ecosystem productivity and flexibility (Kones et al., 2020). Soil represents the most productive reservoir of microbial life, surrounding a vast array of taxa, including fungi, actinomycetes, nematodes and protozoa, with bacterial populations showing the greatest richness and diversity (Yao et al., 2016). PGPR employ beneficial effects on host plants through two preliminary mechanisms among which one is plant growth promotion, which enhances plant development, another one is productivity, and biological disease control, which reduces the impact of plant pathogens (Kumar et al., 2011).

Paul & Frey (2023) revealed that biochemical examines contributes a fundamental approach

in microbial identification based on their specific biochemical responses and metabolic processes. The bacterial identification is facilitated by the analysis of various biochemical and physiological characteristics, including proteolysis, lipolysis, glycolysis, enzymatic activity, and substrate utilization patterns (Franco-Duarte et al., 2019). Optimizing the utilization of PGPRs necessitates the development of efficient strategies for their isolation and characterization from rhizospheric soil, which will enable the formulation of crop-specific biofertilizers, tailored to meet the unique requirements of various crops (Muthukumar et al., 2021). The bacterial antagonistic mechanisms are involved in competition for space or nutrients, antibiosis for enrichment of plant, root growth, for introduction of plant inactivation and resistance to the enzymes of pathogens (Alam et al., 2021). The escalating environmental degradation resulting from climate change and modern agricultural practices underscores the need for innovative solutions.

The ultimate identification of these bacterial species demands the employment of molecular-based methodologies; with PCR – mediated gene amplification being a basic technique, providing exceptional specificity and sensitivity (Erlich, 1989). The application of contemporary techniques, including DNA sequencing and MALDI-TOF mass spectrometry, is indispensible for advancing microbiological research; however, these practices demand specialized technical expertise and substantial financial assets (Assis et al., 2011). A comprehensive understanding of PGPRs imposes the examination of conventional identification and characterization methods, including morphological, microscopic, and biochemical approaches, which will be systematically discussed in the present study. The present study was aimed to identify rhizobacterial strains exhibiting biocontrol potential against a *Colletotrichum jasminigenum* SPTD17 pathogen of chilli anthracnose. The forthcoming view is to develop a dual-purpose inoculum exhibiting biofertilizer and biocontrol potential for profitable use in nutrient and integrated disease management for defendable agronomy.

MATERIALS AND METHODS

Isolation of Fungal pathogen

Colletotrichum jasminigenum SPTD17 was used in this study, which has been isolated from anthracnose infected chilli fruit samples.

Isolation of fifteen rhizobacteria

A total of 17 rhizospheric soil samples were collected from different fields of chilli growing areas of Karnataka (Table 1). The soil samples were brought to the laboratory in sterile polyethylene bags and stored at 4 ℃ for the isolation of bacteria. Soil bacteria were isolated using serial dilution method from collected soil samples. Suspended 1 g of soil in 100 ml of sterile distilled water then kept on rotary shaker at 120 rpm for 10 min. Then it was diluted with distilled water (1:9 ratios) up to 10^7 fold. 100 µl aliquots from 10^{-3} to 10^{-7} dilutions were spread on King's B agar and nutrient medium and slightly spread by a sterile glass spreader (Somasegaran & Hoben, 1994). Then all the plates were incubated 3 days at 35 ℃, all morphologically different colonies were picked and purified on nutrient and King's B agar plates by repeated sub-culturing. For the further confirmation all the isolates were subjected to other morphological and biochemical tests.

Table 1. Collection and geographical locations of soil samples collected from major chilli growing areas of Northern Karnataka

District	Village	Verity
Haveri	Agadi	Sangro
	Aladakatti	Syngenta 5531
	Hombaradi	Mailari
	Maranbeed	Tejashwini
	Krishnapur	Byadagi dabbi
	Teggihalli	Vaso
	Hedigonda	Jwala
Gadag	Shiratti	Byadagi dabbi
	Kurtakoti	Byadagi kaddi
Dharwad	Nelagudda	Devanur dabbi
	Kusugal	Delax
	Shiraguppi	Byadagi dabbi
	Annigeri	Devanur dabbi
Vijayanagar	Harapanhalli	Guntur
	Somalapur	Byadagi kaddi
Bellari	Kudligi	Byadagi dabbi
Raichur	Kochigudda	Guntur

Morphological characterization and Scanning Electronic Microscopic analysis of all fifteen rhizobacteria

The bacterial colonies obtained from the chilli rhizosphere underwent supplementary analysis, employing microscopic, were further analyzed by Scanning Electronic Microscopy (SEM), to elucidate their characteristics. Microscopic slide preparation involved a conventional Gram's staining technique, incorporating crystal violet as the primary stain and Safranin as the counterstain (Becerra et al., 2015). Prior to scanning electron microscopic analysis, the sample preparation was done by following a standard methodology, where 1 ml of overnight grown pure cultures of bacteria was washed with IX PBS (pH-7.4) incubated for 12 hrs after adding 2% of 1 ml glutaraldehyde. Additionally, samples were centrifuged for 10 min at 7000 rpm, then pellet was washed in sequence using 10-100% of ethanol and centrifuged, lastly 50 ml of 100% ethanol was added to pellet and drop of sample was smeared on a coverslip then stored in desiccators for overnight then examined in a ZEISS scanning electronic microscope at 15 kV.

Characterization of rhizospheric bacteria based on biochemical features

Biochemical assays including Gram's staining test (Fawole & Oso, 2004), mobility test (Oyeleke & Manga, 2008), Casein hydrolysis test (Muthukumar et al., 2021), indole test (MacFaddin, 1980), Hydrogen sulphide test (Cowan & Steel, 1970), Citrate test (Simmons 1976), Denitrification test (Seldin et al., 1984), methyl red test (McDevitt, 2009) Voges-Phoskaeuer (McDevitt, 2009), Oxidase test (Cheesbrough, 2006), Urea test (James & Sherman, 1992) and fermentation tests were performed using the procedure given by Fawole & Oso (2004). The bacterial colonies resulting from the sequential assays were systematically observed, recorded and archived for subsequent analysis and data interpretation (Talaiekhozani, 2013; Mahmud et al., 2023).

Screening of rhizospheric bacterial isolates for *in vitro* antagonistic activity against *C. jasminigenum* SPTD16

Bacterial isolates were tested for their antagonistic activity against the *Colletotrichum jasminigenum* SPTD17 using dual-culture method (Sakthivel & Gnanamanickam 1987). A fungal disc (≈5 mm^2) was placed at one side of the plate containing potato dextrose agar (PDA). Rhizobacterial isolates were streaked 3 cm away from plugged fungal disc. Plates inoculated with the fungal discs and control was maintained without the bacterial sample. Zone of inhibitions around the isolates were measured after 5 days of incubation at 28±2 °C and zone of inhibition was recorded by measuring the clear distance between margin of the test fungus and antagonistic bacteria. The colony diameter of the pathogen in control plate was also recorded. All strains were tested in triplicates and tests were carried out twice for each isolates with one control for maintaining only pathogen. The inhibition percent was calculated by using the following equation given by Ji et al. (2014).

$$Inhibition\ (\%) = \left[1 - \left(\frac{Fungalgrowth}{Control\ growth}\right)\right] \times 100$$

Data Analysis

Statistical parameters such as mean, standard deviation and analysis of variance were performed at P=0.05 using three replicate values in IBM SPSS Statistics version 20.00

RESULTS AND DISCUSSIONS

Identification of rhizospheric bacteria based on morphological, SEM and biochemical features

All the rhizospheric bacteria were successfully isolated and characterized on the bases of morphological, microscopic and biochemical features. The total of fifteen rhizobacteria were isolated and subjected to macro and microscopic studies by examining distinct colony colour, shape, texture, margin, elevation, opacity, and cell shape and pigmentation. The colonies of the isolates showed diverse range of variations in their shapes and sizes, either from circular to undulate or irregular to punctured, and size may be large or small. PS1, PS3, PS5 and PS12 showed rough colony texture whereas, rest all isolates possessed smooth. Colony margins were varied, some isolates showed entire to wavy and some were undulate to muciod.

Elevation was varied from umbonate, convex, raised, to flat. Opacity of the cells varies from opaque to translucent. Further Gram's staining test was carried out using standard protocol by Fawole and Oso (2004). Among all the 15 isolates, 9 isolates were Gram +ve, whereas the rest were Gram –ve. The cell shapes of bacterial isolated varied from rod, long rod to cylindrical rod. PS6, PS9, PS10 and PS14 showed yellowish green, PS8 showed brownish yellow and remaining 10 isolates showed white pigmentation. Table 2 explains the details of colony and cell morphology of rhizobacteria from different chilli fields of Karnataka.

For the morphological characterization, fifteen rhizospheric bacterial isolates were inoculated on nutrient agar and King's B agar plates, incubated for 24 h at 37±2 °C in incubator. The features such as shape, size, margin, elevation, texture, and opacity were observed and recorded. The microscopic characterization by Gram's staining and motility test were also performed. Fifteen rhizobacterial isolates were subjected to biochemical analysis, where bacterial isolates showed a diverse range of features which helps in identifying the bacteria up to genus level. PS7 was non-motile whereas, remaining all the isolates were motile, Casein hydrolysis was present in all the isolates except PS7. Indole test was negative in all the isolates. 7 isolates such as PS1, PS3, PS5, PS9, PS12, PS14 and PS15 showed positive hydrogen sulphide test. In present study all the isolates had citrate activity, which means all the 15 isolates can utilize the citrate as carbon source (Harold, 2002). Citrate utilization test was the indicated by the growth of bacteria, followed by an alkaline pH (Cappuccino & Sherman, 2002). The isolates tested negative for Methyl Red test except 4 isolates PS1, PS3, PS12 and PS15. For Voges-Proskaeur (VP) tests, deep red coloration indicated a positive result, in present analysis 5 isolates were found VP positive (PS3, PS4, PS11, PS12 and PS15). 11 isolates such as PS1, PS3, PS4, PS5, PS6, PS7, PS8, PS10, PS11, PS13 and PS15 showed positive result to denitrification test.

The population size of denitrifying bacteria were commonly observed in soils exceed the expected carrying capacity based on denitrifying respiration alone, suggesting that these microorganisms must employ additional metabolic strategies to sustain their growth (Murray et al., 1989). 2 isolates PS5 and PS7 were reported to be oxidase negative. 7 isolates such as PS1, PS2, PS3, PS7, PS12, PS13 and PS15 showed positive urease test. In acid fermentation activity 2 isolates such as PS4 and PS12 showed negative glucose fermentation, 2 isolates PS5 and PS11 showed negative fructose fermentation, 2 isolates such as PS5 and PS11, showed positive Mannitol test and 7 isolates PS1, PS2, PS3, PS5, PS7, PS13 and PS15 showed positive sucrose fermentation test (Table 3). In present study, identified isolates by microscopic and biochemical studies were further exposed for SEM analysis where distinct morphological features were comprehensively observed. The length and width of the bacterial cells recorded and ranges between 1 µm to 2 µm, comparable results were detected by Hagen et al. (1968) and Ibrahim et al. (2021). Figure 1 shows SEM images of representative members from the each genus of rhizospheric bacteria.

Table 2. Morphological and microscopic characteristics of rhizospheric bacterial isolates of chilli field soil

Codes of isolates	Colony morphology					Microscopic observation			Pigmentation	Identification based on morphology
	Colony size	Colony shape	Colony texture	Margin	Elevation	Gram's test	Opacity	Cell shape		
PS1	Large	Circular	Rough	Entire	Umbonate	Positive	Opaque	Rod	White	*Bacillus* sp.
PS2	Punctured	Circular	Smooth	Entire	Convex	Positive	Opaque	Rod	White	*Lysinibacillus* sp.
PS3	Irregular large	Circular	Rough	Wavy	Raised	Positive	Opaque	Long rods	White	*Bacillus* sp.
PS4	Irregular large	Circular	Smooth	Undulate	Convex	Positive	Translucent	Rod	White	*Paenibacillus* sp.
PS5	Large	Undulate	Rough	Wavy	Flat	Positive	Opaque	Rod	White	*Bacillus* sp.
PS6	Irregular large	Circular	Smooth	Undulate	Convex	Negative	Translucent	Cylindrical rod	Yellowish green	*Pseudomonas* sp.
PS7	Small	Circular	Smooth	Muciod	Slightly raised	Negative	Opaque	Rod	White	*Acinetobacter* sp.
PS8	Irregular large	Circular	Smooth	Undulate	Convex	Negative	Translucent	Cylindrical rod	Brownish yellow	*Pseudomonas* sp.
PS9	Irregular large	Circular	Smooth	Undulate	Convex	Negative	Translucent	Cylindrical rod	Yellowish green	*Pseudomonas* sp.
PS10	Irregular large	Circular	Smooth	Undulate	Convex	Negative	Translucent	Cylindrical rod	Yellowish green	*Pseudomonas* sp.
PS11	Irregular large	Circular	Smooth	Entire	Flat	Positive	Opaque	Rod	White	*Bacillus* sp.
PS12	Irregular large	Circular	Rough	Wavy	Raised	Positive	Opaque	Long rods	White	*Bacillus* sp.
PS13	Punctured	Circular	Smooth	Entire	Convex	Positive	Opaque	Rod	White	*Lysinibacillus* sp.
PS14	Irregular large	Circular	Smooth	Undulate	Convex	Negative	Translucent	Cylindrical rod	Yellowish green	*Pseudomonas* sp.
PS15	Small	Circular	Smooth	Entire	Raised	Positive	Opaque	Rod	White	*Bacillus* sp.

Table 3. Biochemical characterization of rhizospheric bacterial isolates of chilli field soil

Name of the test	**Rhizospheric bacterial isolates**														
	PS1	PS2	**PS3**	**PS4**	**PS5**	**PS6**	**PS7**	**PS8**	**PS9**	**PS10**	**PS11**	**PS12**	**PS13**	**PS14**	**PS15**
Motility	M	M	M	M	M	M	NM	M	M	M	M	M	M	M	M
Casein hydrolysis	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrogen sulphide	+	-	+	-	+	-	-	-	+	-	-	+	-	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red (MR)	+	-	+	-	-	-	-	-	-	-	-	+	-	-	+
Voges-proskaeur (VP)	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+
Denitrification	+	-	+	+	+	+	+	+	-	+	+	+	-	-	+
Oxidase	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
Urease	+	+	+	-	-	-	+	-	-	-	-	+	+	-	+
Acid fermentation															
Glucose	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+
Fructose	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
Mannitol	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
Sucrose	+	+	+	-	+	-	-	-	-	-	-	-	+	-	+
Identification based on biochemical test	*Bacillus* sp.	*Lysinibacillus* sp.	*Bacillus* sp.	*Paenibacillus* sp.	*Bacillus* sp.	*Pseudomonas* sp.	*Acinetobacter* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Bacillus* sp.	*Bacillus* sp.	*Lysinibacillus* sp.	*Pseudomonas* sp.	*Bacillus* sp.

*+ indicates present, - indicates absent result

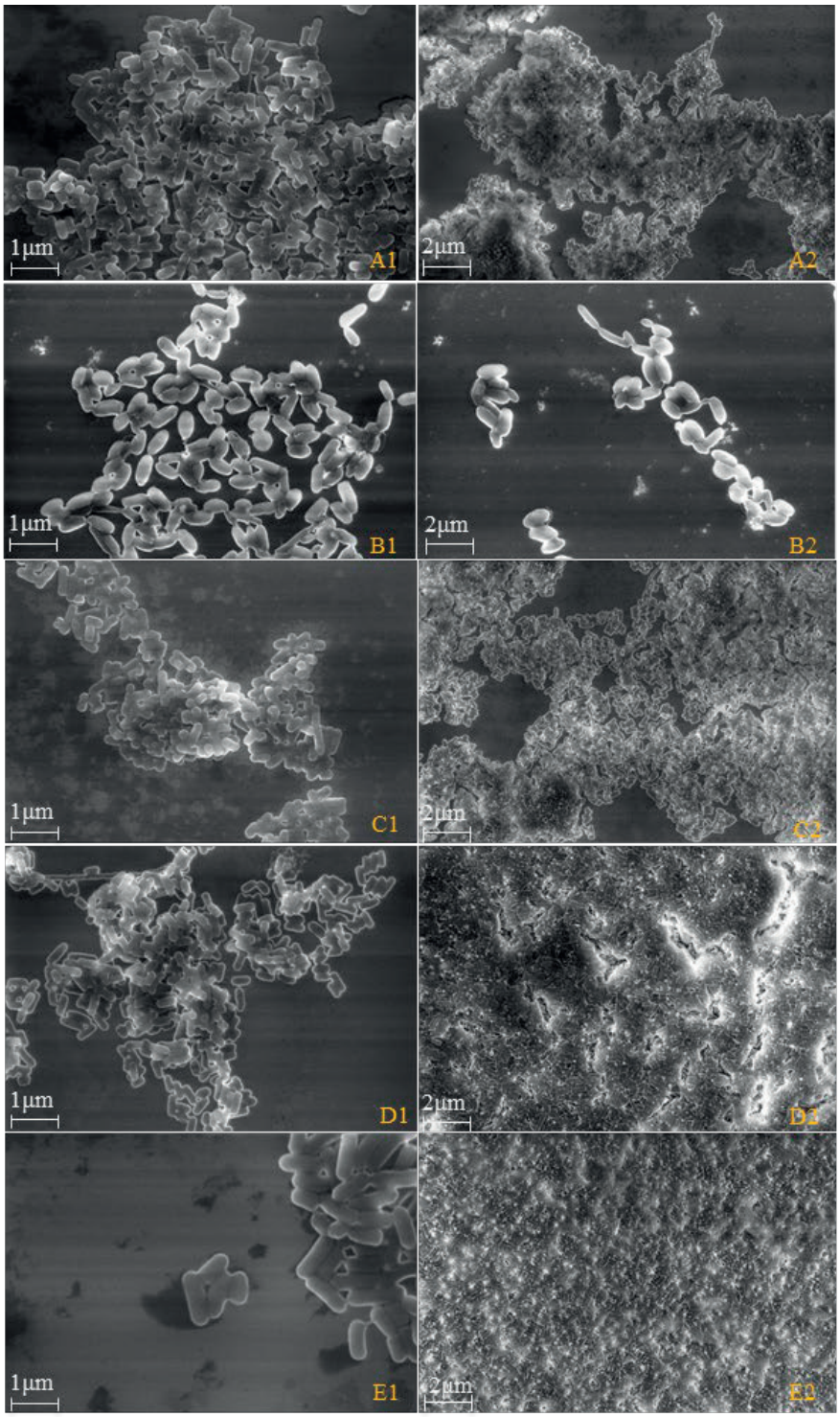

Figure 1. SEM images of representative bacterial genus, PS1 (A1-1 µm, A2-2 µm) - *Bacillus* sp.; PS2 (B1-1 µm, B2-2 µm - *Lysinibacillus* sp.; PS4(C1-1 µm, C2-2 µm) - *Paenibacillus* sp.; PS7 (D1-1 µm, D2-2 µm) - *Acinetobacter* sp.; PS8 (E1-1 µm, E2-2 µm) - *Pseudomonas* sp.

***In-vitro* antagonistic potential of rhizospheric bacteria against *Colletotrichum jasminigenum* SPTD17**

A substantial number of *Bacillus* species from rhizosphere soil was detected by Panigatti et al. (2025).

According to Rosch et al. (2002) bacteria belonging to these groups are widely distributed in the soil. Results revealed that the majority of organisms obtained from screening included *Bacillus*, *Lysinibacillus*, *Paenibacillus*, *Acinetobacter* and *Pseudomonas* sp.

Particular rhizobacterial strains possess inherent biocontrol properties, facilitating the elicitation of plant resistance and/or protection against a diverse array of plant pathogens (Compant et al., 2010).

There are a limited number of studies that have identified biocontrol agents possessing dual functionality, namely antagonistic activity against fungal pathogens and the ability to promote plant growth.

Present study was, therefore, designed to explore the potential of rhizosphere bacteria to control broad-host range fungal pathogen *Colletotrichum jasminigenum* SPTD17, with parallel stimulatory effects on the disease management.

There were 15 isolated and purified rhizospheric bacterial isolates from various regions of chilli growing areas of Karnataka.

In our investigation, we found that seven isolates designated as PS1 (93.33%), PS4 (86%), PS6 (90.66%), PS8 (92.66%), PS9 (87.33%), PS10 (91.66%) and PS11 (88.33%) were strongly inhibited the growth of *Colletotrichum jasminigenum* SPTD17 and they have been showed above 80% zone of inhibition those were grouped into a, whereas PS2 (71.6%), PS5 (77.33%), PS13 (70.33%) and PS14 (77.66%) were showed moderate zone of inhibition i.e., 70-80% under the group b, and PS3 (42.33%), PS7 (25.33%), PS12 (38.33%) and PS15 (40.66%) have been showed week activity with zone of inhibition 25-41% belong to c (Figure 2), and the bar graph showed different degrees of inhibition against *Colletotrichum jasminigenum* SPTD17 in dual culture assay was represented in Figure 3.

Bacillus strain has been demonstrated that decreased mycelial growth of pathogen *in vitro* and enhanced chilli seedling growth by Kumar et al. (2021).

Che et al. (2017) have been demonstrated that the antifungal VOCs produced by *Lysinibacillus* sp. FJAT-4748 were potentially useful as agents for controlling anthracnose caused by *Colletotrichum acutatum*. Darmadi et al. (2020) demonstrated that *Paenibacillus polymyxa* C1 and *Bacillus siamensis* C7B, were proven to possess strong antifungal activities against *Colletotrichum scovillei* in chilli.

Among the various rhizospheric bacteria, the bacteria belonging to *Pseudomonas*, which colonize roots of a wide range of crop plants, were reported to be antagonistic to soil borne plant pathogens.

Work done by Jisha et al. (2018) demonstrated that *Pseudomonas aeruginosa*, Ps 2 showed maximum inhibition of 93.41% whereas the other isolate Ps 3 showed 72.5% of inhibition against *Colletotrichum capsici* of chilli anthracnose after 5 days of incubation.

Several bacterial strains, including *Bacillus velezensis* and actinobacteria, have demonstrated significant antagonistic effects against *Colletotrichum capsici*, with some achieving up to 100% control efficacy (Yanti et al., 2023).

Work done by Sarmah et al. (2023) illustrated that in dual culture analysis against *Colletotrichum gloeosporioides* of chilli anthracnose, the endophytic bacteria, *B. velezensis* exhibited the highest inhibition (68.67%) against pathogen followed by *B. altitudinis* (52.89%), *B. cereus* (45.33%) and *B. mycoides* (65.33%).

Phenotypic characterization, based on biochemical and morphological attributes, may be insufficient for accurately assessing rhizosphere microbial diversity, due to potential homology among distinct species.

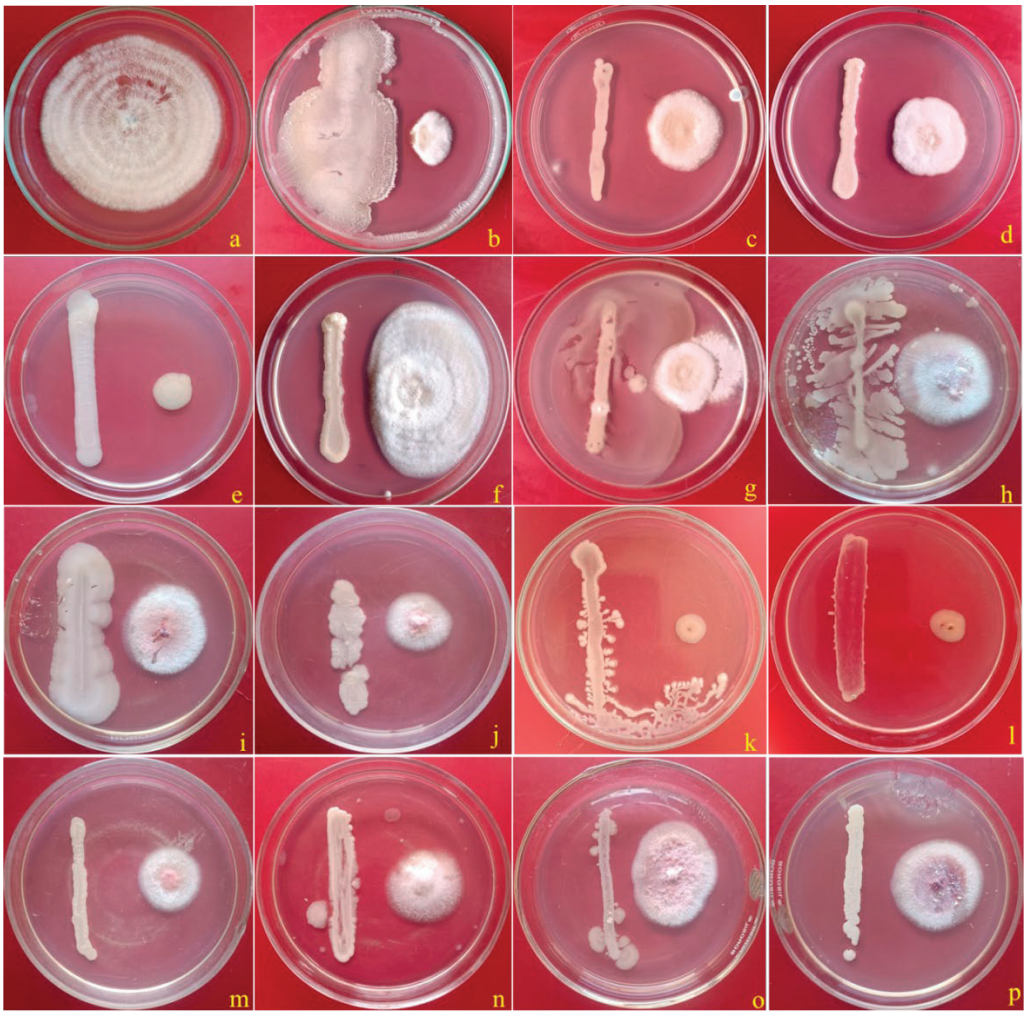

Figure 2. Antagonistic activity of 15 bacterial isolates against *Colletotrichum jasminigenum* SPTD17: control plate inoculated with only the pathogen (a) and fungal growth was inhibited towards the direction of inoculated bacteria (b-p); b-PS1, c-PS2, d-PS3, e-PS4, f-PS5, g-PS6, h-PS7, i-PS8, j-PS9, k-PS10, l-PS11, m-PS12, n-PS13, o-PS14, p-PS15

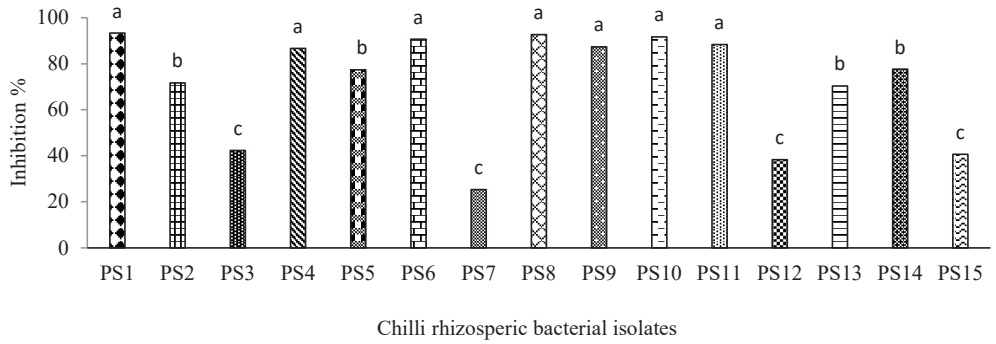

Figure 3. Inhibition percent of fifteen rhizospheric bacterial isolates against *Colletotrichum jasminigenum* SPTD17

Morphological identification of biocontrol agents is hindered by the paucity and homoplasy of distinguishing characteristics, which can be exacerbated by the occurrence of cryptic species, necessitating the adoption of molecular-based identification approaches (Kullnig et al., 2001). The application of molecular techniques, particularly those leveraging DNA analyses, is indispensable for elucidating the complex composition and functional dynamics of rhizosphere bacterial communities (Lagos et al., 2015). A polyphasic approach, combining morphological, molecular, and other characteristics, is essential for accurate species identification and assessing microbial diversity.

CONCLUSIONS

Rhizobacteria exhibit multifunctional plant-beneficial properties, including growth promotion and disease suppression, mediated by the elicitation of secondary metabolites with antimicrobial and plant-protective activities. The first and foremost step in biological control is the selection of effective antagonistic microbe. On the basis of this study, it is concluded that biochemical and morphological characterization of these isolates revealed the most abundant bacteria in the rhizosphere of chilli such as *Bacillus*, *Lysinibacillus*, *Paenibacillus*, *Acinetobacter* and *Pseudomonas* sp. The result of this study discloses that, in dual culture technique, bacterial antagonists strongly inhibited the spore germination and mycelial growth of the chilli anthracnose pathogen. The integration of biochemical, morphological and DNA-based approaches is necessary for a comprehensive understanding of rhizospheric microbial diversity, as DNA-evolution. Molecular characterization and screening of the isolated bacteria will be based methods provide critical insights into phylo-genetic relationships and microbial performed to elucidate their biocontrol importance and facilitate the development of innovative biocontrol manufacture technologies.

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