VOLUME 11 ISSUE 1 2025

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 11, No. 1, 851-859 (2025)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Integrated Assessment of Plant Growth-Promoting Rhizobacteria from Millet Rhizosphere: Antagonistic Efficacy, Enzyme Production, and Interaction with Fungicides

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Received: 6th May, 2025; **Accepted:** 12th June, 2025; **Published online:** 30th June, 2025

https://doi.org/10.33745/ijzi.2025.v11i01.089

Abstract: Plant Growth-Promoting Rhizobacteria (PGPR) especially from the climate resilient millets represent a sustainable alternative to chemical pesticides and fertilizers, offering eco-friendly solutions for managing phytopathogens and preserving soil health. In this study, five rhizobacterial strains isolated from the millet rhizosphere were identified through 16S rRNA sequencing as *Pseudomonas* sp. (SA6 and ATE5), *Bacillus* sp. (KV18), *Pseudomonas azotoformans* (ATE9), and *Agrobacterium* sp. (ASA6). These isolates were evaluated for their antagonistic activity against key fungal and bacterial pathogens, including *Fusarium oxysporum*, *Macrophomina phaseolina*, *Colletotrichum* sp., *Xanthomonas oryzae*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. Strains ASA6, SA6, and ATE5 exhibited significant antifungal activity, with inhibition rates of 41.18%, 51.11%, and 32.2%, against *Fusarium oxysporum*, *Macrophomina phaseolina* and *Colletotrichum* sp., respectively. ATE5 also demonstrated antibacterial activity against *Xanthomonas oryzae* with a 1.5 cm inhibition zone. The strains were also tested for hydrolytic enzyme activity and interaction with fungicides. All strains showed protease activity, with SA6 displaying the highest enzymatic index (4.6), while amylase and glucanase activities were strain-specific. Notably, all isolates were resistant to Bavistin (Carbendazim 50% WP), while KV18 showed sensitivity to higher concentrations of Beam (Tricyclazole 75% WP) and Sixer (Mancozeb 63% WP + Carbendazim 12% WP). These findings highlight the potential of these rhizobacterial strains as biocontrol agents in integrated disease management strategies for sustainable agriculture.

Keywords: Plant Growth-Promoting Rhizobacteria, Antagonistic activity, Protease, Amylase, Glucanase, Fungicides

Citation: Punitha S. and Kavitha S.: Integrated assessment of plant growth-promoting rhizobacteria from millet rhizosphere: Antagonistic efficacy, enzyme production, and interaction with fungicides. Intern. J. Zool. Invest. 11(1): 851-859, 2025.
https://doi.org/10.33745/ijzi.2025.v11i01.089

Introduction

Plant pathogens are the major cause for the reduction in yield and productivity of crop plants. Many chemical fertilizers and pesticides are being used to overcome this yield loss, which in a long

run affects the fertility of the soil (Jiao *et al*., 2021).The adverse impact of these synthetic chemicals on human health has driven the pursuit of biological control methods as a safer alternative strategy for protecting the crops (Barratt *et al*., 2010; Gay, 2012). One such strategy is the use of Plant Growth Promoting Rhizobacteria (PGPR) as a sustainable solution to protect the crop plants without harming the environment. PGPR act as potential biocontrol agents by utilizing diverse strategies, that include the synthesis of antibiotics, antimicrobial peptides, bacteriocins, toxins, and enzymes that inhibit or eliminate pathogenic organisms (Raaijmakers *et al*., 2002; Compant *et al*., 2005). Plant growth-promoting rhizobacteria play a pivotal role in sustainable agriculture not only by enhancing plant growth but also by contributing to biological control through the production of various hydrolytic enzymes. These enzymes, such as chitinases, cellulases, proteases, and glucanases, degrade the structural components of pathogenic fungi and other harmful microbes, thereby suppressing their proliferation in the rhizosphere. By disrupting the cell walls of phytopathogens, these enzymatic activities reduce disease incidence and enhance plant resilience against biotic stress. Furthermore, the production of such enzymes often works synergistically with other mechanisms like antibiotic production, siderophore release, and induced systemic resistance, collectively reinforcing the protective barrier around plant roots. Thus, enzyme-mediated pathogen suppression by PGPR represents a crucial, eco-friendly strategy in integrated pest management systems. These bacteria reveal greater competence by efficiently colonizing plant roots and suppressing the growth of competing or harmful bacterial organisms (Salomon *et al*., 2017). *Pseudomonas fluorescens*, *P. stutzeri*, *Bacillus subtilis* and *B. amyloliquefaciens* have been recognized as few of the effective bacterial biocontrol agents that are widely used to combat various plant diseases (Panpatte *et al*., 2016; Islam *et al*., 2016). In addition, testing the compatibility of bacterial biocontrol agents with fungicides is essential to ensure their effective co-application in integrated disease management strategies. Rhizobacteria isolated from millets are particularly well-suited as biocontrol agents due to their natural adaptation to challenging and resource-limited environments, which often mirror the stress-prone conditions in many agricultural soils (Swamy, 2023). Millets are traditionally grown in arid and semi-arid regions with low-input farming systems, where they rely heavily on their root-associated microbiota for nutrient acquisition and stress tolerance. As a result, the rhizobacteria associated with millet roots tend to possess enhanced traits such as drought resistance, efficient root colonization, and robust antagonistic activity against phyto-pathogens. The current study aims to evaluate the antagonistic potential along with their enzyme production capabilities of the rhizobacteria isolated from little, foxtail and barnyard millet rhizosphere. The compatibility of the rhizo-bacteria with commercial fungicides was also evaluated to assess their resistance to these chemical agents.

Materials and Methods

Rhizobacterial isolates

Five rhizobacterial isolates from foxtail, barnyard and little millet rhizosphere collected at the Centre for Excellence in Millets, Athiyandal, Thiruvannamalai, India were used for the current study and they were characterized for the antagonistic activity, enzyme production and fungicide compatibility.

Molecular identification of rhizobacterial isolates by 16S rRNA

The bacterial genomic DNA was isolated and amplified by following the protocol of Sharma and Singh (2005). The amplified product was sequenced using Sanger dideoxy sequencing and the sequences were deposited in the NCBI GenBank and the accession numbers were obtained for the five rhizobacterial isolates namely SA6, KV18, ATE5, ATE9 and ASA6.

Enzyme production of rhizobacteria

The enzyme production of rhizobacteria was tested for different enzymes namely Amylase, Cellulase, Protease, Pectinase and Glucanase by following the methods of Dogan and Taskin (2021) and Oo *et al*. (2020) with modifications. Based on the Enzymatic Index (EI) the rhizobacterial isolates were classified as follows: non-producer (EI = 0); low producer (0 > EI ≤ 2); moderate producer (2 > EI ≤ 4); and high producer (EI > 4) (Dornelas *et al*., 2017).

Antagonistic activity of rhizobacteria against fungal and bacterial pathogens

The antagonistic activity of the rhizobacteria was tested against the fungal pathogens using dual culture method (Mardanova *et al*., 2016) and bacterial pathogens using agar spot assay (Hossain, 2024).

In vitro interaction of rhizobacteria with commercial fungicides

The compatibility of the rhizobacteria with the commercial fungicides was tested by zone inhibition method using three different fungicides namely Bavistin (Carbendazim 50% WP), Beam (Tricyclazole 75% WP) and Sixer (Mancozeb 63% WP and Carbendazim 12% WP) in the concentrations of 0.05%, 0.1% and 0.2% and the control was maintained with distilled water (Martensson, 1992; Nandini *et al*., 2018). The presence and absence of zone of inhibition indicated that the strains were sensitive and resistant to the fungicides.

Statistical analysis

All the experiments were conducted in replicates and the data obtained from the antagonistic and enzyme activity assays were statistically evaluated using Analysis of Variance (ANOVA) in Microsoft Excel, 2021.

Results and Discussion

The five rhizobacterial strains were identified as *Pseudomonas* sp. (SA6 and ATE5), *Bacillus* sp. (KV18), *Pseudomonas azotoformans* (ATE9) and *Agrobacterium* sp. (ASA6) with the following accession numbers PP938950, PP938947, PP938949, PP938948 and PP956881.

Five rhizobacterial isolates were assessed for the production of hydrolytic enzymes namely Amylase, Cellulase, Protease, Pectinase and Glucanase. All the rhizobacterial isolates were found to exhibit protease activity with the highest activity shown by SA6 with the enzymatic index (EI) of 4.6 followed by ATE9, ASA6, ATE5 and KV18 with the enzymatic indices of 2.82, 1.57, 1.4 and 1.34, respectively. Similarly, *Pseudomonas congelans* produced protease with an EI of 4.22 (Dogan and Taskin, 2021). Amylase production was observed in SA6 and ATE5 with an EIs of 2.5 and 2.79 which is comparable with the results obtained by Yasmin *et al*. (2020) where *Pseudomonas putida* exhibited an EI of 2.63. Glucanase activity was recorded in SA6, KV18 and ATE5 with EIs of 2.85, 2.5 and 2.33, respectively. Similar results were observed in the study by Oo *et al*. (2020) in which the strains *Pseudomonas plecoglossicida* and *Pseudomonas aeruginosa* showed moderate glucanase production. In contrast, cellulase and pectinase activities were absent in all the rhizobacterial strains (Table 1, Fig. 1). These hydrolytic enzymes produced by PGPR play a vital role in the biocontrol of phytopathogens by breaking down their cell wall components, ultimately leading to structural disruption and pathogen suppression (Mishra *et al*., 2020). This enzymatic degradation compromises the structural integrity of the pathogen, leading to cell lysis and inhibition of infection. Studies have shown that higher levels of such enzyme production are often associated with greater biocontrol efficacy. In many such biocontrol agents, such enzyme activity is considered a key biomarker of a potential effectiveness.

Rhizobacterial isolates were assessed for their antagonistic effects against three fungal pathogens: *Fusarium oxysporum* (OFO), *Macrophomina phaseolina* (MP), and *Colletotrichum* sp. (CO). Among the tested rhizobacterial strains, ASA6 exhibited the strongest

Table 1: Hydrolytic enzyme production by rhizobacterial strains

S. No.	Rhizobacterial strains	Enzymatic Index (EI)		
		Protease	Amylase	Glucanase
1.	SA6	4.6±0 *	2.5±0 *	2.86±0.48*
2.	KV18	1.34±0.05 *	0	2.5±0*
3.	ATE5	1.4±0 *	2.79±0.54 *	2.33±0 *
4.	ATE9	2.82±0.19 *	0	0
5.	ASA6	1.57±0 *	0	0

Non-producer (EI = 0); low producer (0 > EI ≤ 2); moderate producer (2 > EI ≤ 4); high producer (EI > 4); Values are represented as Mean ± Standard Error (SE); *p<0.05indicates statistically significant

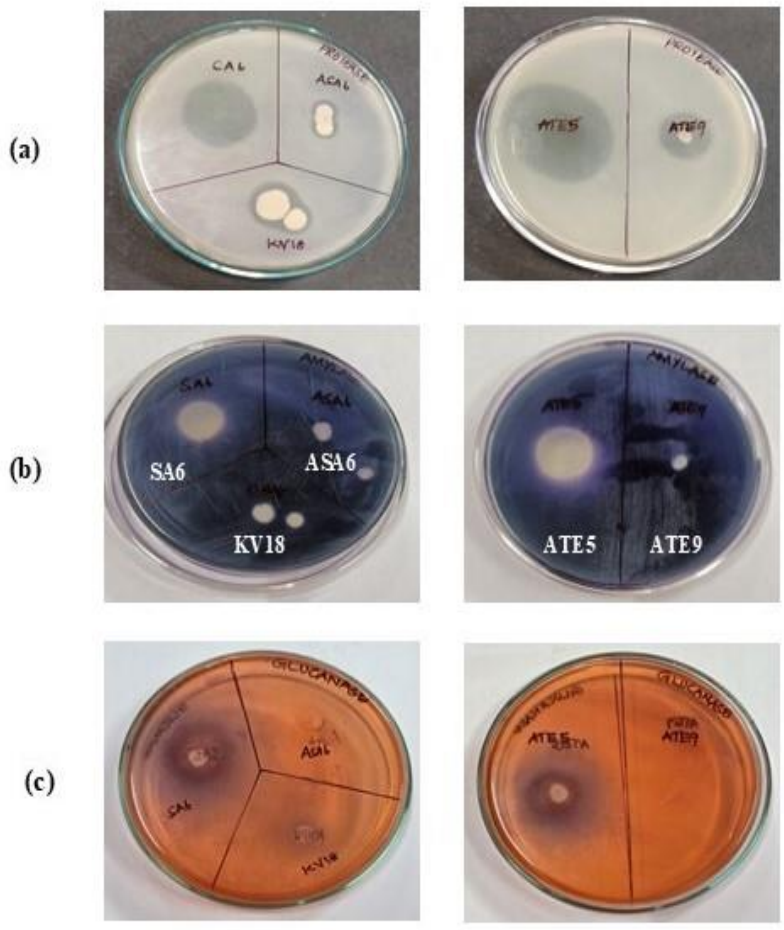

Fig 1: Hydrolytic enzyme production by rhizobacterial strains (a) Protease (b) Amylase (c) Glucanase.

Table 2: Inhibition of fungal pathogens by rhizobacterial strains after 7 days in dual culture method

S. No.	RHIZOBACTERIAL STRAINS	% INHIBITION		
		***Fusarium oxysporum* (OFO)**	***Macrophomina phaseolina* (MP)**	***Colletotrichum* sp. (CO)**
1.	SA6	28.83±0.59 *	51.11 *	26.03±4.11 *
2.	KV18	31.18±2.94 *	11.11±3.33 *	25.35±3.43 *
3.	ATE5	30.59±1.18 *	31.11±0.0 *	32.2±0.69 *
4.	ATE9	0	8.89±3.33 *	0
5.	ASA6	41.18±0.0 *	0	0

Values are represented as Mean ± SE; *p<0.05 indicates statistically significant

antagonistic effect against *Fusarium oxysporum* (OFO), with an inhibition rate of 41.18%, followed by KV18 (31.18%), ATE5 (30.59%), and SA6 (28.83%). In a study conducted by Khalifa *et al*. (2022) the *Pseudomonas* isolates E2PP6 and E1PP2 exhibited an inhibition percentage of 30.10% and 27.36% against *Fusarium oxysporum* (Foc-S1 and Foc-S2) which is in closest similarity with the results obtained in the present study. *Bacillus subtilis* GM2 and *Bacillus subtilis* GM5 were also found to inhibit the growth of *Fusarium oxysporum* (Mardanova *et al*., 2016).

SA6 demonstrated the strongest antagonistic activity against *Macrophomina phaseolina* (MP), showing 51.11% inhibition, whereas ATE5 and ATE9 exhibited moderate (31.11%) and minimal (8.89%) effects, respectively. A comparable result was observed in the study by Pothiraj *et al*. (2018), where *Pseudomonas fluorescens* isolate Pfkkm9 reduced mycelial growth by 50.91% over the control. An inhibition rate of 30.26% was observed in *Pseudomonas putida* CW1 (Yasmin *et al*., 2020), which is consistent with the results of the current study.

ATE5 showed the highest inhibition against *Colletotrichum* sp. (CO) at 32.2%, followed by SA6 (26.03%) and KV18 (25.35%). *Paenibacillus polymyxa* C1 and *Bacillus siamensis* C7B was reported to inhibit the mycelial growth of *Colletotrichum scovillei* (Suprapta *et al*., 2020) (Table 2, Fig. 2).

The antagonistic potential of the rhizobacterial strains against the bacterial pathogens was evident as SA6 and ATE5 produced inhibition zones of 14 mm and 15 mm, respectively, against *Xanthomonas oryzae* (XO). Notably, ATE5 also exhibited activity against *Bacillus subtilis* (BS), forming a 12 mm inhibition zone, whereas no inhibitory effect was detected against *Pseudomonas aeruginosa* (PA) (Fig. 3). The present results are in agreement with the observations of Sharma and Kaur (2010) who reported that *Pseudomonas* isolates demonstrated superior antagonistic activity against various plant pathogens in comparison to *Bacillus* isolates. Moreover, it is a proven fact that there is a well-established correlation between enzymes produced by rhizobacterial isolates and pathogen inhibition in plants (Khedher *et al*., 2021; Dobrzynski *et al*., 2023). Analysis of Variance (ANOVA) performed on the antagonistic activity and enzyme production assay results using Microsoft Excel, 2021 showed statistically significant differences ($p < 0.05$).

In vitro analysis of the interaction between rhizobacterial strains and commercial fungicides showed that all strains were tolerant to Bavistin

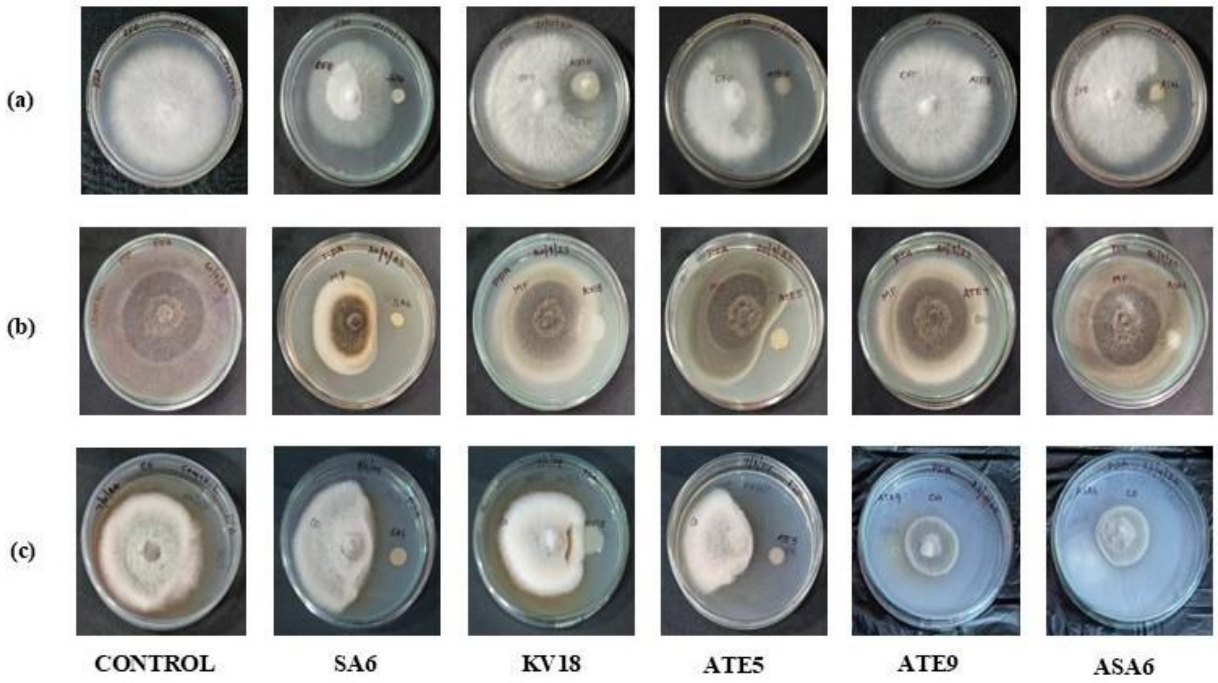

Fig. 2: Antifungal activity of rhizobacterial strains against (a) *Fusarium oxysporum* (OFO); (b) *Macrophomina phaseolina* (MP) and (c) *Colletotrichum* sp. (CO).

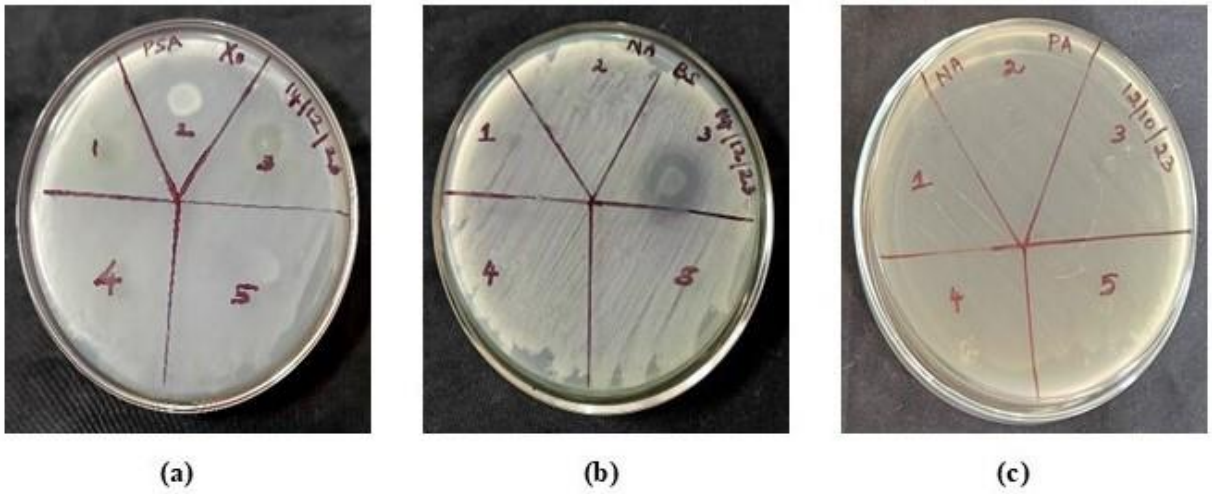

Fig. 3: Antibacterial activity of rhizobacterial strains against (a) *Xanthomonas oryzae* (XO); (b) *Bacillus subtilis* (BS) and (c) *Pseudomonas aeruginosa* (PA).

(Carbendazim 50% WP) across the three tested concentrations. However, strain KV18 exhibited sensitivity at the highest concentration (0.2%) of Beam (Tricyclazole 75% WP). Both KV18 and ATE9 were sensitive to all tested concentrations of Sixer (Mancozeb 63% WP and Carbendazim 12% WP) (Table 3, Fig. 4). According to Mubeen *et al*. (2006), *Pseudomonas* strains showed sensitivity to Mancozeb. In line with the current study's findings, Sarvani *et al*. (2021) reported that *Pseudomonas* and *Bacillus* isolates were resistant to carbendazim, even when applied at

Table 3: In vitro interaction between rhizobacterial strains and commercial fungicides

S. No.	Rhizobacterial strains	Zone of Inhibition								
		Bavistin (Carbendazim 50% WP)			Beam (Tricyclazole 75% WP)			Sixer (Mancozeb 63% WP and Carbendazim 12% WP)		
		0.05 %	0.1 %	0.2 %	0.05 %	0.1 %	0.2 %	0.05 %	0.1 %	0.2 %
1.	SA6	-	-	-	-	-	-	-	-	-
2.	KV18	-	-	-	-	-	+	+	+	+
3.	ATE5	-	-	-	-	-	-	-	-	-
4.	ATE9	-	-	-	-	-	-	+	+	+
5.	ASA6	-	-	-	-	-	-	-	-	-

(-) indicates absence of zone of inhibition (Resistant); (+) indicates presence of zone of inhibition (Sensitive)

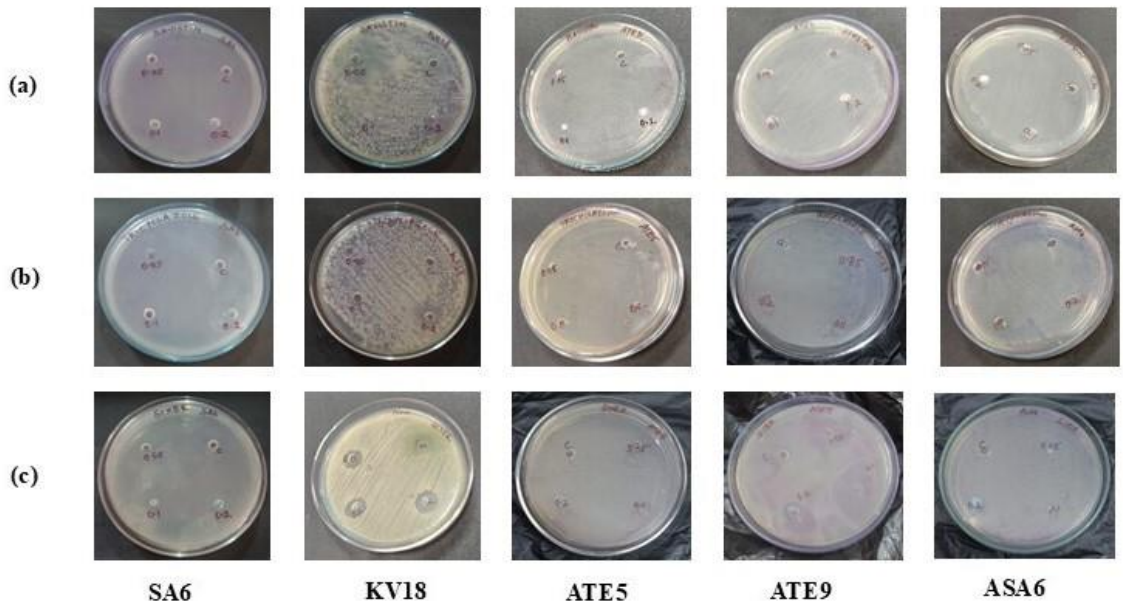

Fig 4: *In vitro* interaction between rhizobacterial strains and fungicides (a) Bavistin (Carbendazim 50% WP); (b) Beam (Tricyclazole 75% WP); and (c) Sixer (Mancozeb 63% WP and Carbendazim 12% WP).

recommended and half-recommended doses. The compatibility of these bacterial isolates with fungicides can be advantageous in disease management when they are used as biocontrol agents in a way without compromising on their viability, colonization ability, or metabolic activity. These compatibility assessments are crucial for developing synergistic or at least non-antagonistic combinations in integrated disease management strategies that maximize disease control while minimizing chemical inputs and preserving microbial biodiversity.

Conclusion

The rhizobacterial strains isolated from the barnyard, little and foxtail millet rhizosphere exhibited significant antagonistic activity against both fungal and bacterial pathogens, which may be attributed to their production of hydrolytic enzymes. Additionally, several of these strains exhibited compatibility with commercially available fungicides. These combined traits highlight the potential of these PGPR isolates as an environmentally sustainable approach for the biocontrol of phytopathogens and their practical application in agricultural fields.

Ethical Statement

All experimental procedures involving microbial cultures were conducted in accordance with institutional biosafety guidelines and relevant national guidelines.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors would like to thank the Principal, Head and Faculty members of the Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women for their invaluable support and the facilities provided by the institution. We would also like to extend our gratitude to Madurai Kamaraj University, Centre for Advanced Studies in Botany (University of Madras), Department of Plant Biology and Biotechnology (Loyola College) and the Department of Microbiology (Ethiraj College for Women) for providing us with the pathogens.

Conflict of Interest

The authors declare no conflicts of interest.

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