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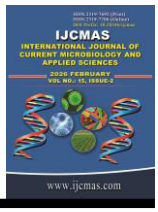

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Screening of Microorganisms from Soils of North Gujarat with Promising Antagonistic Activity against Phytopathogens Causing Damping Off Disease

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A B S T R A C T

Fungal phytopathogens cause serious losses of crop production worldwide. One of the most common diseases caused by fungal phytopathogens is damping off disease. Damping-off inhibits the initial stages of plant of growth and effectiveness, resulting in a serious hazard to horticulture and agriculture. Chemical control of these phytopathogens are hazardous to human health and the environment and lead to environmental pollution. Bacteria play a key role as biocontrol agents for plant disease control. For this reason, the present study was conducted to determine the antagonistic potential of rhizospheric soil bacteria against selected phytopathogenic fungi such as *Fusarium oxysporum, Rhizoctonia solani, Botrytis cinerea* and *Alternaria solani* by dual culture assay. The growth inhibition by each microbial strain of the fungal phytopathogens was measured and compared against a control. Among the tested pathogens, *Botrytis cinerea* was most inhibited (68.8%), followed by *Alternaria solani* (59.0%) and *Fusarium oxysporum* (57.7%). In contrast, *Rhizoctonia solani* exhibited the least inhibition (21.3%). Among the isolates, A5 showed the strongest broad-spectrum antagonism (74.8% inhibition), whereas B24 exhibited the weakest activity (27.4%). These findings highlight the potential of specific bacterial strains, particularly A5 (74.8%), C5 (64.2%), and C6 (60.2%), as promising biocontrol agents.

Introduction

Fungal phytopathogens are the most important factors that cause serious losses to agricultural products every year namely decreased seed germination, poor plant vigour, reduced crop yield, and post-harvest spoilage causing substantial financial losses to farmers. These pathogens account for an estimated 20–40% yield loss in major crops worldwide therefore, they have to be controlled to ensure the plant products are quantitative (Oerke, 2006). Qualitatively, they reduce nutritional value, cause seed discoloration, produce mycotoxins, and shorten the storage life of agricultural produce, ultimately decreasing its market acceptability (Dean *et al.*, 2012). Hence, effective and eco-friendly management strategies are urgently needed to safeguard agricultural productivity

and quality. Fungicides are commonly used to control the diseases in plants. Fungicides can be chemical or biological. However, frequent uses of chemical fungicides such as chlorothalonil, metalaxyl, and mancozeb are hazardous to human health and the environment and lead to environmental pollution. In contrast, biological methods employ beneficial microorganisms to tackle plant pathogens. Biological methods, as an alternative disease control approach, reduce the hazards caused by chemical agents and are eco-friendly as well as environmentally conscious.

One of the most common and severe diseases caused by fungal plant phytopathogens is damping-off disease. Damping-off inhibits the initial stages of plant of growth and effectiveness, resulting in a serious hazard to horticulture and agriculture (Figure 1). It is of two types: post-emergence damping-off, in which immature seedlings collapse at the soil line after emerging, and pre-emergence damping-off, in which seeds rot before emerging from the soil. This disease is caused primarily by soil-borne fungal and oomycete pathogens such as *Rhizoctonia solani*, and *Fusarium spp, Pythium spp.* These pathogens infect plants under unfavorable conditions, like higher soil moisture, poor drainage conditions, and at moderate to warm temperatures, and result in decreased crop stand and seedling death. The economic implications of damping-off are substantial, especially in vegetable and horticultural crops, where seedling loss can reach up to 60–80% under unmanaged conditions. Damping-off is widely observed in the seedlings of solanaceous crops (tomato, chili and brinjal), crucifers (cabbage and cauliflower), cucurbits and legumes such as chickpea and pea (Sharma & Gupta, 2004).

In recent years, the biological control of these phytopathogens has gained significant attention due to the limitations and ecological concerns associated with chemical fungicides. Among the various biological agents, plant growth-promoting rhizobacteria (PGPR) have emerged as promising candidates due to their safety and efficacy. They exhibit antagonistic activity against multiple phytopathogenic fungi. PGPR present in the rhizosphere soil suppress fungal growth by producing hydrogen cyanide (HCN) and siderophores (Raaijmakers *et al.,* 2002; Glick, 2012). The screening of such rhizosphere bacterial isolates for their antagonistic potential is a crucial step in identifying efficient biocontrol agents. In this study, rhizosphere soil was explored as a source of microorganisms capable of inhibiting common damping-off pathogens, including *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria solani*. Dual culture assay, one of the widely used methods for in vitro screening of antagonistic potential against plant pathogens, was used in the study. This approach aimed to contribute towards sustainable disease management strategies in agriculture by utilizing natural microbial resources.

Materials and Methods

Isolation of Rhizospheric bacteria

Rhizosphere soil was collected from 6 different places in North Gujarat, India. The soil samples were brought to the laboratory in sterile polyethylene bags and stored at 4ºC for the isolation of microorganism (Verma & Pal, 2020). Rhizosphere soil bacteria were isolated by the serial dilution method from soil samples. In brief, 1 g of soil was suspended in sterile distilled water and kept for shaking at 120 rpm for 5 to 3 min in a rotary shaker. Then it was diluted with distilled water (1:9) up to 10^{-5}. Aliquots of 200 µl from 10^{-3} to 10^{-5} dilutions were spread on to the nutrient agar (NA) media and by a sterile glass spreader. The plates were incubated at 35°C for 3 days. The isolates obtained on NA plates after 3 days were subcultured on the same medium multiple times to obtain morphologically distinct isolates. The pure isolates were stored as glycerol stock at -30ºC and used for further study.

Assay for the detection of HCN

For the qualitative HCN detection, filter-sterilized glycine (4.4 g/L final concentration) was added to nutrient agar after sterilization (Verma & Pal, 2020). The isolates (70) were inoculated in NA plates with glycine, while an uninoculated plate was used as a negative control. Whatman paper strips, measuring 5 by 1 cm were immersed in a solution of 0.5% picric acid and 2% Na_2CO_3 (1:1 v/v). The strips were attached to the inner lid of each Petri plate, the dishes were sealed with Parafilm, and were incubated for 96 hours (4 days) at 28 ± 1°C (Manasa K *et al.,* 2017).

Morphological characterization of isolated bacteria

The morphological characterization was done on the selected 10 isolates. For this, the cell shape (like cocci,

bacilli, or spirilla), cell arrangement (such as chains or clusters), and colony appearance (including size, color, texture, and margin) were observed on culture media. This analysis used microscopy and Gram staining for initial identification and classification (Alam *et al.,* 2021).

Phytopathogen fungal

Pathogenic fungi (*Fusarium oxysporum* (ITCC No. 8115), *Rhizoctonia solani* (ITCC No. 7855), *Botrytis cinerea* (ITCC No. 8651), and *Alternaria solani* (ITCC No. 5350)) were obtained from accredited repositories, the Indian Type Culture Collection and the Identification Division of Plant Pathology, Indian Agriculture Research Institute. Fungal cultures were maintained on potato dextrose agar (PDA) slant at -80°C for stock storage.

Invitro Dual Culture Assay for Screening Potential Antagonists

Dual Culture Assay

The dual culture technique was used to evaluate the antagonistic activity of chosen 10 potent bacterial strains from the HCN production assay against phytopathogens fungi. Each bacterial isolate was spread onto Potato Dextrose Agar (PDA) plates 3 centimeters away from the fungal mycelial plug that was positioned in the center (Alam *et al.,* 2021).

Bacterial strains were added around the edges of the plates, while the fungal pathogens were inoculated in the middle.

Depending on the growth requirements of the individual organisms, the plates were incubated for 5–7 days at 28°C (Al-Daghari *et al.,* 2023; Legrifi *et al.,* 2022a).

Measurement of Growth Inhibition

The inhibition of fungal growth was measured in centimeters (cm), and the percentage of inhibition was calculated using the formula as described in (Legrifi *et al.,* 2022b)

Growth zone data and inhibition percentages were recorded for all bacterial strains and fungi after 7 days of incubation (Alam *et al.,* 2021; Mis *et al.,* 2024; Ramona, 2021a).

Results and Discussion

Screening of Microorganisms from Rhizosphere Soil Sample

The six rhizosphere soil samples of North Gujarat were collected for the screening of potent bacterial strains. A total of 70 bacterial isolates were obtained using the serial dilution plate technique. A wide range of colonies were developed on the NA plates, which were purified through repeated streaking to obtain pure cultures. Following this rigorous process, a total of 70 bacterial isolates were successfully recovered. The isolates displayed marked variations in colony size, pigmentation, margin, and elevation, reflecting the presence of a diverse microbial community within the rhizosphere. For long-term preservation, all purified isolates were transferred into 20% glycerol stock solutions and stored at -30ºC for subsequent analysis and characterization.

Hydrogen Cyanide (HCN) Production Assay

All 70 bacterial isolates were first screened for the production of hydrogen cyanide (HCN). The assay was carried out on nutrient agar supplemented with glycine, which acts as a precursor of cyanogenesis. For detection, sterile Whatman No. 1 filter papers impregnated with an alkaline picrate solution (0.5% picric acid and 2% Na_2CO_3, 1:1 v/v) were fixed on the inner side of the Petri dish lids. Ten potent isolates (coded A2, A5, B24, C1, C5, C6, C14, C15, C17, and D4) from the examined strains produced HCN with significant results. This was evident by distinct colour transition of the indicator paper from yellow to orange-brown/reddish-brown (Figure 2). This reaction occurs when volatile HCN released by the isolates interacts with picric acid under alkaline conditions, reducing it to isopurpuric acid derivatives that impart the brownish hue. The observed variation in colour intensity indicated differential cyanogenic capacity among the positive isolates (Ahmad *et al.,* 2008; Lorck, 1948).

Two isolates displayed strong and consistent color development, indicating higher HCN production compared to the remaining isolates. These high-producing isolates showed activity comparable to reported strains of *Pseudomonas fluorescens*, *P. aeruginosa,* and *Chromobacterium violaceum* which are recognized as efficient HCN producers involved in the biocontrol of soil-borne pathogens (Alemu, 2016; Budge

& Whipps, 2001). Such findings indicate that the rhizospheric bacteria isolated in the present study may also serve as effective cyanogenic biocontrol agents. In addition to this, the antagonistic capability of each HCN-positive bacterial strain was evaluated against the phytopathogenic fungal cultures.

Morphological characterization of isolated bacteria

The bacterial isolates obtained from rhizospheric soil exhibited diverse colony morphologies on nutrient agar with differences in colony shape, pigmentation, and surface characteristics (Table 1). Microscopic examination post Gram staining revealed a predominance of Gram-negative bacteria (8 isolates), while only 2 isolates were Gram-positive. Several studies have reported a higher abundance of Gram-negative bacteria near the soil surface compared to Gram-positive bacteria. This is also evident in the present study, where the rhizospheric soil sample collected from 10–20 cm depth showed a similar pattern (Budge & Whipps, 2001; Naylor *et al.,* 2022; Zhao *et al.,* 2022).

Screening of antagonistic activities from isolated bacteria through an in vitro assay

Screening of antagonistic activity of each isolated bacterium was accomplished through an in vitro assay, as shown in Figure 3. Among them, six (6) isolates showed different degrees of antagonism (25-100%) against all pathogenic fungi.

The inhibitory effects of various rhizosphere soil bacterial strains against *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Alternaria solani* were evaluated using the dual culture assay via direct antagonism. The results displayed varied degrees of antagonism, which is tabulated in Table 2. The table summarizes the inhibitory effects of bacterial strains on pathogenic fungal growth, measured as radial percentage inhibition via direct antagonism (spread method).

Inhibition of Phytopathogenic fungi

The *Fusarium oxysporum* control showed a fungal growth zone of 7 cm (Figure 3(a)). Among the tested strains, A5, C5, and C14 completely inhibited fungal growth, achieving 100% inhibition. The rate of inhibition of strain C6 was 71.43%, whereas that of strains C17 and B24 was 57.14% and 42.86%, respectively (Figure 3(b)). The bacterial strains *Pseudomonas fluorescens*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Streptomyces spp*, exhibit antifungal properties which suppress *Fusarium oxysporum* by producing antifungal metabolites like 2,4-diacetylphloroglucinol (DAPG) and lipopeptides, reducing vascular wilt and root rot (Table 4) (Raaijmakers *et al.,* 2002; Tan, 2014; Verma & Pal, 2020).

The *Rhizoctonia solani* control showed a fungal growth zone of 8 cm (Figure 3(b)). The highest inhibition was observed in strains A5, C6, and D4, each achieving 37.5% inhibition. Overall, *R. solani* appeared more resilient as the tested strains remained below 40%. However, previous studies have reported higher antagonistic activity, with some bacterial isolates achieving >50% inhibition of *R. solani* (Farhaoui *et al.,* 2023). Antifungal mechanisms have been reported in bacterial strains *Bacillus subtilis, B. velezensis and Pseudomonas fluorescens*, which are well-documented producers of cell wall-degrading enzymes effective against *Rhizoctonia* spp. These bacteria produce hydrolytic enzymes, particularly chitinases and β-1,3-glucanases, which enzymatically degrade fungal cell walls (Tan, 2014).

Botrytis cinerea control growth was recorded at 5.13 cm (Figure 3(c)). Significant inhibition was seen in strains A2, A5, C1, C15, and C17, each achieving 80.5% inhibition. Strain C14 showed moderate inhibition at 70.76 %. *B. cinerea* is highly susceptible to microbial inhibition. One important necrotrophic fungal pathogen that causes grey mold is *Botrytis cinerea* (Haider *et al.,* 2016).

More than 1400 plant species are infected by the *Botrytis* genus, which has a wide range of hosts. *B. cinerea* modes of action (MoAs) include: i) synthesis of anti-fungal metabolites, such as antibiotics, cell wall-degrading enzymes and volatile organic compounds; ii) induction of host resistance. *Pseudomonas aeruginosa* has been extensively studied for its strong antagonistic activity against *B. cinerea*. Similarly, species of *Paenibacillus* and *Bacillus* are well known for producing a wide range of secondary metabolites, including lipopeptide antibiotics, antifungal proteins, volatile compounds, and lytic enzyme (Choquer *et al.,* 2007; Roca-Couso *et al.,* 2021).

Table.1 Morphological characteristics, Gram reaction, and antagonistic activity of bacterial isolates against four phytopathogenic fungi. Antagonistic activity is indicated as presence (+) or absence (–) of fungal inhibition.

Sr. No.	Isolated	*Fusarium oxysporum*	*Rhizoctonia solani*	*Botrytis cinerea*	*Alternaria solani*	Morphological characters	Gram reaction
1.	**A2**	+	+	+	+	Cream white, Punctiform, Circular, Smooth	- ve, Short rod
2.	**A5**	+	+	+	+	Whitish, Small, Circular, Smooth	- ve, Short rod
3.	**B24**	+	+	+	-	Creamy White, Small, Circular, Mucoid	- ve, Short rod
4.	**C1**	+	-	+	+	Creamy White, Small, Circular, Smooth	- ve, Short rod
5.	**C5**	+	+	+	+	Creamy White, Small, Circular, Smooth	- ve, Short rod
6.	**C6**	+	+	+	+	Creamy White, Small, Circular, Smooth	- ve, Short rod
7.	**C14**	+	-	+	+	Whitish, Large, Irregular, Circular, Mucoid	+ ve, Big rod
8.	**C15**	+	+	+	+	Creamy white, Punctiform, Circular, Smooth	- ve, Short rod
9.	**C17**	+	+	+	+	Yellow, Small, Circular, Mucoid	+ ve, Big rod
10.	**D4**	+	+	+	+	Whitish, Small, Circular, Mucoid	- ve, Short rod

(indicated as Gram-positive (+ve) and Gram-negative (–ve))

Table.2 Comparative antifungal activity of bacterial strains against soil-borne fungal pathogens using spread diffusion assays

Bacterial PGPR Strains	HCN	Spread Method (% Inhibition)			
		Fusarium oxysporum	*Rhizoctonia solani*	*Botrytis cinerea*	*Alternaria solani*
A2	+++	21.43	25	80.5	61.24
A5	+++	100	37.5	80.5	80.62
B24	+++	42.86	25	41.52	0
C1	++++	14.29	0	80.5	61.24
C5	++++	100	25	51.26	80.62
C6	+++	71.43	37.5	61.01	70.93
C14	+++	100	0	70.76	61.24
C15	+++	35.71	12.5	80.5	41.86
C17	+++	57.14	12.5	80.5	61.24
D4	+++	34.29	37.5	61.01	70.93

Table.3 Categorization of bacterial strains based on their inhibitory potential against phytopathogenic fungi

Fungal Pathogen	Control Growth (cm)	High Inhibition Strains (75 – 100%)	Moderate Inhibition Strains	Low/No Inhibition Strains
Fusarium oxysporum	7	A5 (100%), C5 (100%), C14 (100%)	C6 (71.43%), C17 (57.14%), B24 (42.86%)	—
Rhizoctonia solani	8	A5 (37.5%), C6 (37.5%), D4 (37.5%)	—	C1 (0%), C14 (0%)
Botrytis cinerea	5.13	A2, A5, C1, C15, C17 (all 80.5%)	C14 (70.76%)	—
Alternaria solani	5.16	A5, C5 (80.62%), D4 (70.93%)	C6 (70.93%), C17 (61.24%)	—

Table.4 Comparative antifungal activity and reported mechanisms of bacterial isolates against major phytopathogenic fungi

Sr. No	Pathogen	Observed Inhibition (This study)	Reported Mechanisms of Suppression	Representative Biocontrol Bacteria (from literature)	References
1	*Fusarium oxysporum*	Up to 100% inhibition (A5, C5, C14); moderate inhibition by other isolates	Production of antifungal metabolites (2,4-diacetylphloroglucinol, lipopeptides), HCN, hydrolytic enzymes; ISR (Induced Systemic Resistance)	*Pseudomonas fluorescens*, *Bacillus subtilis*	(Raaijmakers *et al.,* 2002; Weller *et al.,* 2002)
2	*Rhizoctonia solani*	Maximum inhibition 37.5% (A5, C6, D4)	Secretion of chitinases and β-1,3-glucanases degrading fungal cell walls	*Bacillus subtilis*, *Pseudomonas fluorescens*	(Farhaoui *et al.,* 2023; Tan, 2014)
3	*Botrytis cinerea*	Strains A2, A5, C1, C15, C17 showed 80.5% inhibition (high); C14 showed 70.76% (moderate)	Lipopeptides (surfactin, iturin, fengycin), ISR induction in host plants	*Bacillus amyloliquefaciens*, *Paenibacillus* and *Streptomyces* spp.	(Ongena & Jacques, 2008; Roca-Couso *et al.,* 2021; Weller *et al.,* 2002)
4	*Alternaria solani*	A5 and C5 showed 80.62% inhibition (high); D4 showed 70.93% (moderate)	Antifungal metabolites (phenazines, pyrrolnitrin, DAPG, lipopeptides), hydrolytic enzymes	*Pseudomonas fluorescens*, *Bacillus subtilis*	(Farag & Khalifa, 2025)

Figure.1 Damping-off disease cycle: showing pre-emergence and post-emergence stages.

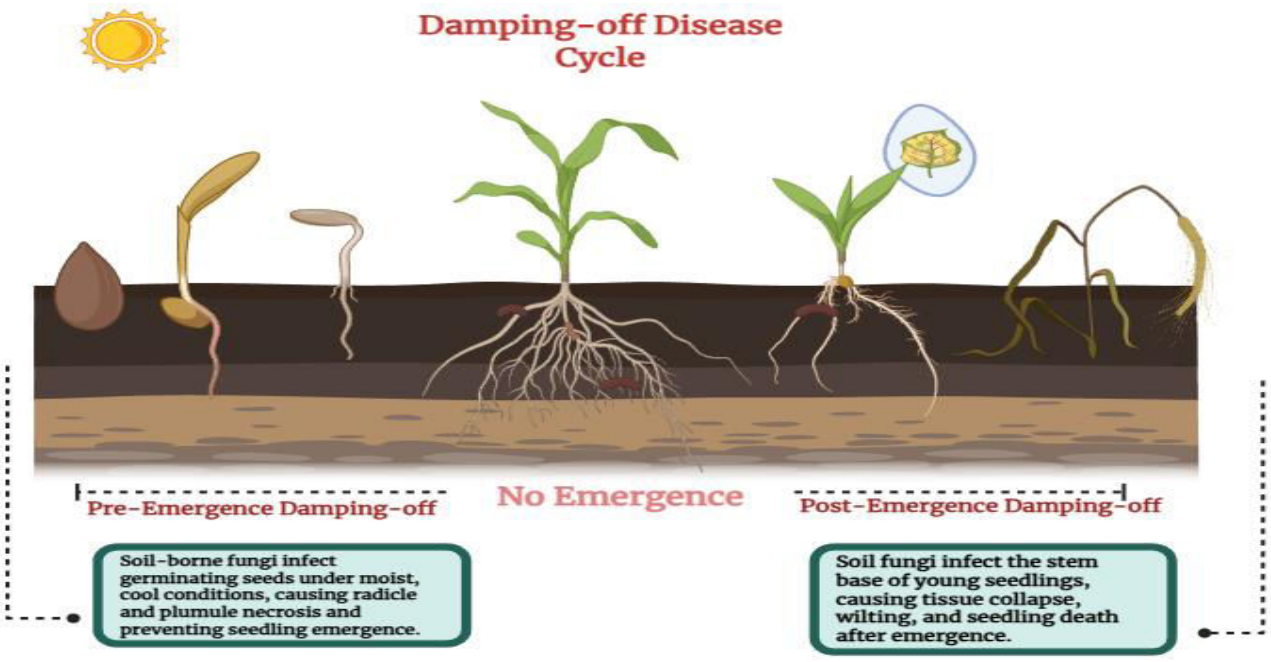
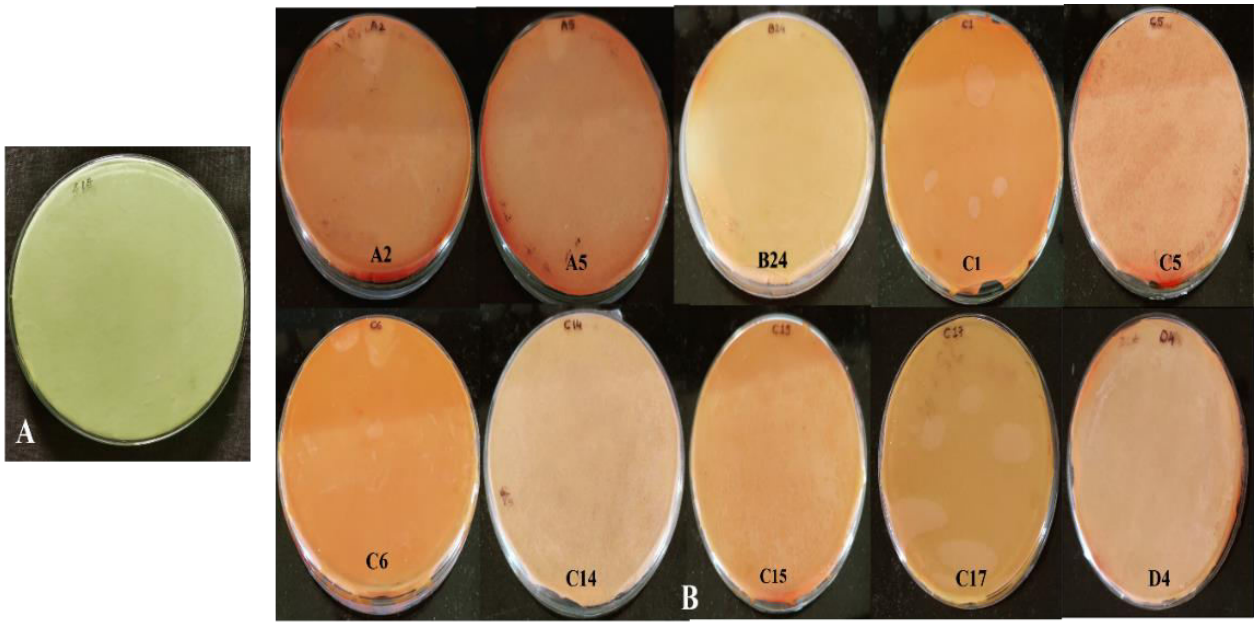


Figure.2 Hydrogen cyanide (HCN) production Assay. Negative control showed no colour change on nutrient agar (A) while positive HCN production was indicated by a colour change from yellow to orange-



brown/reddish-brown (B).

Figure.3 In vitro dual culture assay showing antagonistic activity of bacterial isolates against phytopathogenic fungi on PDA medium after 7 days of incubation at 25 °C against *(a) Fusarium oxysporum, (b)Rhizoctonia solani, (c)Botrytis cinerea and (d) Alternaria solani*

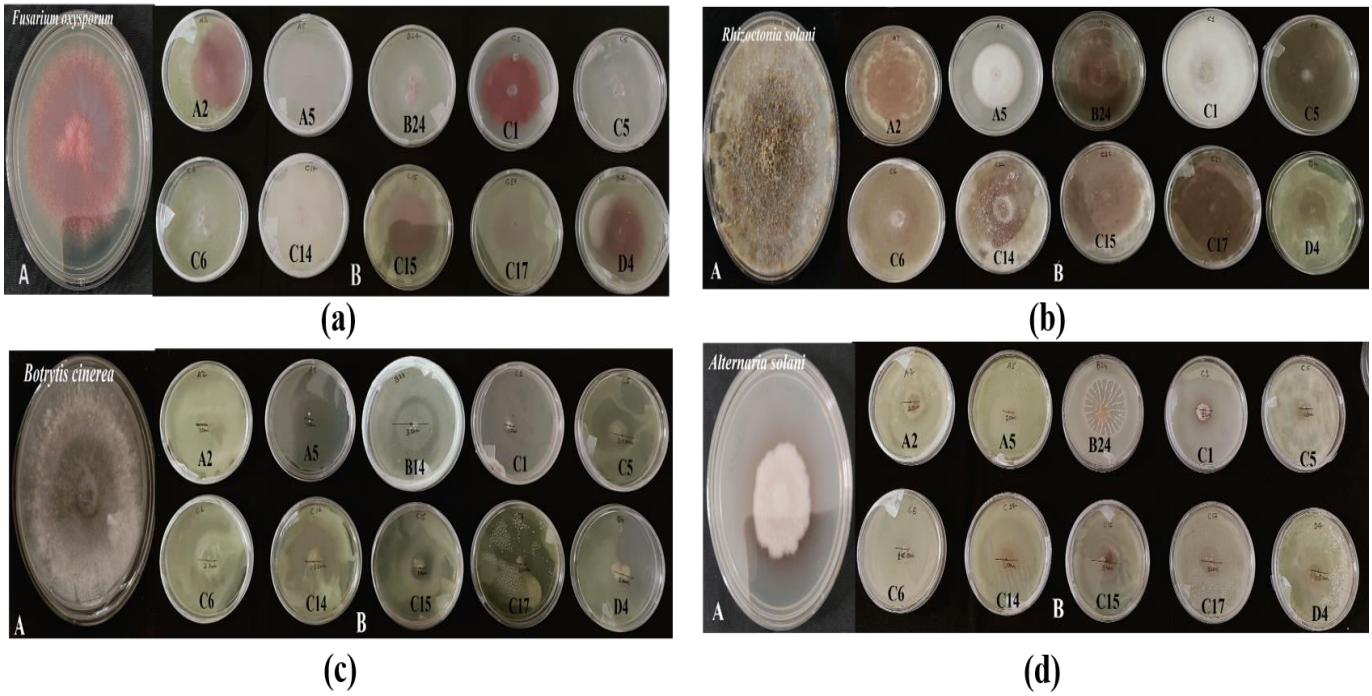
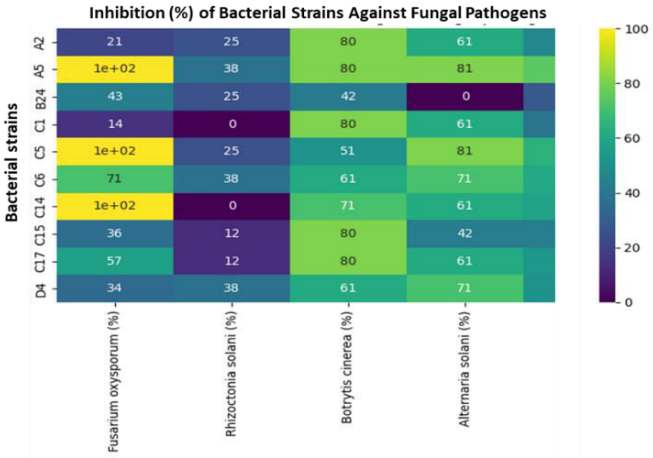


Figure.4 Heatmap showing the percentage inhibition of *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Alternaria solani* by different bacterial isolates.



Alternaria solani control growth reached 5.16 cm (Figure 3(d)). The most effective strains were A5 and C5, both demonstrating 80.62% inhibition, and D4 at 70.93%. Moderate inhibition was also recorded for C6 (70.93%) and C17 (61.24%). Antifungal mechanisms have been reported in bacterial strains *Bacillus spp (Bacillus velezensis, Bacillus subtilis)* and *Pseudomonas fluorescens* which produce various metabolites, including antifungal lipopeptides (like fengycins from *B. subtilis*), that can directly suppress the growth and pathogenicity of *A. solani* (Table 4) (Camlica & Tozlu, 2019; Farag & Khalifa, 2025).

Heatmap Analysis of Antifungal Activity

The results of antagonistic activity can also be summarized using a heatmap to provide a visual comparison of bacterial efficacy against four phytopathogenic fungi (*Fusarium oxysporum, Rhizoctonia solani, Alternaria solani,* and *Botrytis cinerea*). The color gradient represents the percentage of fungal growth inhibition, with darker shades indicating higher inhibition.The heatmap clearly illustrates variation in antagonistic potential among isolates and highlights the most promising candidates for biocontrol applications (Figure 4).

In conclusion, the antagonistic activity of bacterial isolates against phytopathogenic fungi varied significantly. Among the tested pathogens, *Botrytis cinerea* was most inhibited (68.8%), followed by *Alternaria solani* (59.0%) and *Fusarium oxysporum* (57.7%). In contrast, *Rhizoctonia solani* exhibited the least inhibition (21.3%). Among the isolates, A5 observed the strongest broad-spectrum antagonism (74.8% inhibition), whereas B24 exhibited the weakest activity (27.4%). These findings highlight the potential of specific bacterial strains, particularly A5 (74.8%), C5 (64.2%), and C6 (60.2%), as promising biocontrol agents. The study supports the hypothesis that select microbial isolates possess antifungal properties and can serve as biocontrol agents. Further field validation and formulation development are necessary to realize their potential in agricultural applications.

Author Contributions

Zinkal Rathod: Investigation, formal analysis, writing—original draft. Mayursinh Chavda: Validation, methodology, writing—reviewing. Chetan Patel:—Formal analysis, writing—review and editing. Mahesh Parmar: Investigation, writing—reviewing. Anil Kumar Gupta: Resources, investigation writing—reviewing. Swasti Dhagat: Validation, formal analysis, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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