

***In vitro* antagonism of native microbes against cotton root rot pathogen**

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ABSTRACT

This study was carried out to screen indigenous rhizospheric fungal and bacterial antagonists for their capacity to antagonize cotton root rot pathogen *Macrophomina phaseolina* (Tassi) Goid. Five fungal strains and seven bacterial strains were isolated and assessed for their ability to inhibit the growth of *M. phaseolina*, under *in vitro* condition. Among them, the isolate five of *Trichoderma* and isolate five of *Bacillus* have been identified as potent antagonistic agents. Best performing isolates were molecularly identified as *Trichoderma longibrachiatum* Accession No. PP800312 and *Bacillus cereus* Accession No. PP800283.

Key words: Cotton, root rot, indigenous antagonists

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INTRODUCTION

Cotton (*Gossypium* spp.) is an economically valuable crop species that is cultivated around the world. Cotton possesses the most miraculous fibre under the Sun for several years. No other fibre has quality like cotton fibre. The fibre of thousand faces and many uses, cotton is noted for its versatility, appearance, performance and above all, its natural comfort. Cotton is grown all around the globe and is traded internationally as well. Cotton production is influenced by repeated outbreaks of pests and diseases and these are the major factors responsible for lower yield of cotton in India (Vimala and Vijayaraghavan, 2024). Among these, vulnerability of cotton crop to several diseases especially root rot (*Macrophomina phaseolina*) is the most limiting factor responsible for low productivity of cotton (Kumar *et al.*, 2022). The incidence of this disease in individual fields ranged from 0-69 per cent in different parts of the country (Mongol and Raj, 1996). Due to its devastating nature, there is an urgent need to manage the disease for better returns to adverse effect of fungicides on the environment and increased interest in sustainable agriculture, biological control with antagonistic

microorganisms has been tried as an effective approach for the management of soil borne plant pathogens (Bapat and Shar, 2000; Ahmad and Khan 2024; Indra *et al.*, 2024). Although several studies have reported the antagonistic activity of *Trichoderma* spp., *Bacillus* spp., and other beneficial microbes against *Macrophomina phaseolina*, significant gaps still remain. However, *M. phaseolina* is highly variable, and its pathogenic behaviour differs across agro-climatic zones. Hence, region-specific antagonists adapted to local soil and climatic conditions have not been thoroughly explored. Moreover, many studies relied solely on morphological identification of antagonists without confirming their identity through molecular characterization, which limits reproducibility and confidence in strain-level performance. An experiment was undertaken to determine the antagonistic effect of indigenous *Trichoderma* and *Bacillus* isolates against *Macrophomina phaseolina* causing root rot disease of cotton.

MATERIALS AND METHODS

Isolation and purification of *Macrophomina phaseolina*

For isolation, the diseased roots of cotton (SVPR 2) were collected from fields of Cotton Research Station, Srivilliputtur and washed gently in several changes of tap water for five minutes to remove dirt and cut into small bits of 2-5 mm dimension. These bits were surface sterilized by 0.1% mercuric chloride solution for thirty seconds followed by three washings by sterile distilled water, then drying on blotting paper. After drying, infected plant parts placed aseptically into Petri plates poured with potato dextrose agar medium under aseptic condition and incubated at $28 \pm 2^\circ\text{C}$ for complete growth of the fungi (Hemalatha *et al.*, 2018). After seven days of incubation a bit of hyphal growth was transferred aseptically to fresh potato dextrose agar slants. The fungus was purified by employing single hyphal tip method (Singh and Srivastava, 1988). The cultures were maintained by periodical transfer to potato dextrose agar slants for further studies.

Isolation and identification of native bioagents

To isolate native antagonists from soil, rhizosphere soil samples were collected from five different locations viz., Natchiarpatti ($9.46^\circ\text{N}; 77.65^\circ\text{E}$), Saminathapuram ($11.15^\circ\text{N}; 77.72^\circ\text{E}$), Thombakulam ($9.38^\circ\text{N}; 77.65^\circ\text{E}$), Keelarajakularaman ($9.39^\circ\text{N}; 77.63^\circ\text{E}$) and Cotton Research Station, Srivilliputtur ($9.08^\circ\text{N}; 77.10^\circ\text{E}$). Soil samples were collected from the rhizosphere of healthy cotton plants at 5-15 cm depth in poly ethylene bags and brought to the laboratory. Serial dilution method (Johnson and Curl, 1972) was used for the isolation of fungal antagonists which are abundant in rhizospheric soil of healthy plants. The *Trichoderma* isolates were isolated by using *Trichoderma* selective medium at the dilution of 10^3 hundred microliter of prepared soil suspension from the test tube was poured into sterilized Petri plate contained the medium. Then, the plates were incubated at room temperature for the growth of colonies. The fully grown colonies were picked with the help of a needle and identified on the basis of mycological keys described by Barnett and Hunter (1986).

Antagonistic bacteria were isolated from the rhizosphere of healthy cotton plants by dilution

plating on to nutrient agar medium. These bacterial isolates were identified by standard bacteriological tests (Schaad, 1992) and 16s-23 s ribosomal RNA gene sequencing (Ramesh kumar *et al.*, 2002).

Evaluation of antagonists on the growth of *Macrophomina phaseolina*

Microorganisms isolated were tested for their antagonism to *Macrophomina phaseolina* on potato dextrose agar medium in Petri dishes by Dual culture method as described by (Sabalpara, 2009).

The antagonistic potential of fungal antagonist was studied. A 9mm diameter disc of fungal antagonist was placed individually at one end of the Petri dish (9cm) containing potato dextrose agar medium and just opposite to that a 9 mm diameter disc of the pathogen was placed. Three replications were maintained for each antagonist.

The purified bacterial isolates were evaluated for their antagonistic activity against *Macrophomina phaseolina* by streaking the bacterial isolates on one side of a Petri dish (1 cm in from the edge) containing the potato dextrose agar medium and a 9 mm mycelial disc from a 7day old culture was placed on the opposite side of the Petri dish and the plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 3-5 days. The zone of inhibition (mm) (Dennis and Webster, 1971) was recorded by measuring the distance between the edges of the growing mycelium and the antagonistic bacterium. Three replications were maintained for each isolate. The Petri dishes were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for seven days and observations on the growth of antagonists and pathogen were recorded. In control, the pathogen alone was inoculated.

Statistical Analysis

Data were analyzed using a Completely Randomized Design (CRD). Percent inhibition values were subjected to ANOVA, and treatment means were compared using CD at 5% significance level.

RESULTS

An experiment was conducted to identify the antagonistic activity of indigenous fungal and bacterial antagonists. Five *Trichoderma* and seven

Bacillus isolates were obtained. The biochemical characters of bacterial isolates are given in Table 1. The antagonistic action of five isolates of *Trichoderma* spp. and seven isolates of *Bacillus* spp. was evaluated against the test fungus *M. phaseolina* by dual culture technique.

Based on the observations of radial growth of native antagonists and test fungus per cent inhibition was calculated. The results of the dual culture assay revealed significant variation in the antagonistic efficacy of native rhizosphere isolates against *M. phaseolina* (Table 2). All the tested antagonists significantly reduced the mycelial growth of the pathogen when compared to the control, which recorded a maximum radial growth of 88.66 mm.

Among the native rhizosphere isolates, *Trichoderma*-5 was found to be the most effective antagonist, exhibiting the minimum mycelial growth of 21.33 mm and the maximum per cent reduction of 75.94% over control. This was closely followed by *Bacillus*-5, which recorded 22.66 mm mycelial growth with 74.44% inhibition. The standard check *Trichoderma asperellum* also showed strong antagonistic activity with 70.30% reduction, while *Bacillus subtilis* Bbv57 resulted in 69.92% inhibition of the pathogen (Figure 1).

Moderate antagonistic activity was observed in *Trichoderma*-4 (54.88%), *Trichoderma*-2 (50.37%), *Trichoderma*-1 (41.35%), *Bacillus*-4 (40.97%), *Bacillus*-6 (41.34%) and *Bacillus*-7 (40.98%), with mycelial growth ranging from 40.00 to 56.66 mm. Minimum levels of inhibition were recorded in *Bacillus*-1 (16.54%), *Bacillus*-2 (26.31%) and *Bacillus*-3 (30.45%).

The variations among treatments were statistically significant, confirming the effectiveness of selected rhizosphere antagonists, particularly *Trichoderma* spp. and *Bacillus* spp., in suppressing *M. phaseolina* under *in vitro* conditions.

The DNA of the effective bioagents (*Trichoderma*-5 and *Bacillus*-5) were extracted by cetyl trimethyl ammonium bromide method amplified by using the ITS 1; 4 primers for *Trichoderma* sp. (T5) and 27F;1492 R for *Bacillus* spp.(B5). Accordingly, the best performing isolates (isolate 5 of *Trichoderma* spp. and isolate 5 of *Bacillus* spp.) were

molecularly identified as *Trichoderma longibrachiatum* Accession No. PP800312 and *Bacillus cereus* Accession No. PP800283 (Figure 2).

Table 1. Biochemical characterization of bacterial isolates

Biochemical test	BACILLUS 1	BACILLUS 2	BACILLUS 3	BACILLUS 4	BACILLUS 5	BACILLUS 6	BACILLUS 7
Gram Staining	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Phosphate solubilisation	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-
HCN	+	+	+	+	+	+	+

* Positive (+) Negative (-)

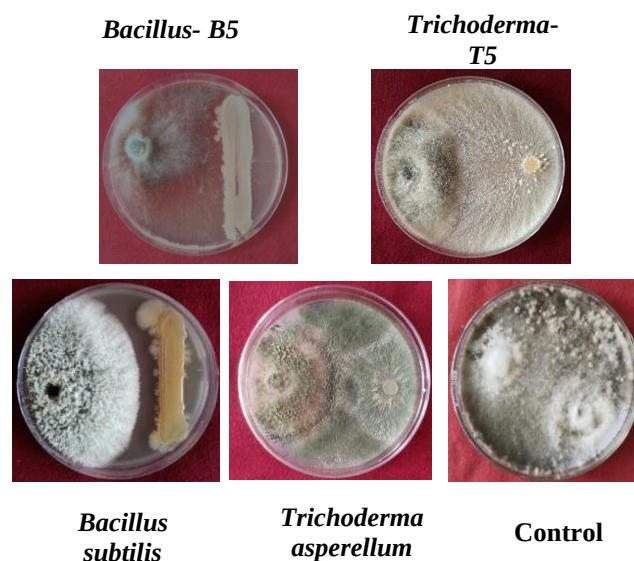


Figure 1. Screening of rhizosphere antagonistic isolates against the *in vitro* growth of *Macrophomina phaseolina* (Dual culture technique)

Table 2. *In vitro* evaluation of rhizosphere antagonistic isolates against *Macrophomina phaseolina* using Dual culture technique

Antagonistic isolates	Mycelial growth of the pathogen (mm)*	Per cent reduction over control
<i>Bacillus</i> -1	74.00	16.54
<i>Bacillus</i> - 2	65.33	26.31
<i>Bacillus</i> - 3	61.66	30.45
<i>Bacillus</i> - 4	52.33	40.97
<i>Bacillus</i> - 5	22.66	74.44
<i>Trichoderma</i> - 1	52.00	41.35
<i>Trichoderma</i> -2	44.00	50.37
<i>Trichoderma</i> - 3	56.66	36.09
<i>Trichoderma</i> - 4	40.00	54.88
<i>Trichoderma</i> -5	21.33	75.94
<i>Bacillus</i> - 6	52.11	41.34
<i>Bacillus</i> - 7	52.33	40.98
<i>Trichoderma asperellum</i>	25.33	70.30
<i>Bacillus subtilis</i> Bbv57	26.67	69.92
Control	88.66	-
SE	3.88	
CD (p=0.05)	3.18	

DISCUSSION

The hazardous effect of chemicals used in the plant disease management has diverted plant pathologists to find out the alternative methods having little or no adverse effect on environment. There is a possibility of developing biological control agent for management of plant diseases under field condition. Majumdar *et al.* (1996), Sindhan *et al.* (2002), Kaswate *et al.* (2003), Kaur *et al.* (2004), Mathur and Srivastava, (2005), Karthikeyan *et al.* (2006), Sreedevi and Devi (2012), Yadav *et al.* (2021), Nagrale *et al.* (2022), Degani *et al.* (2024), found *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp. and other microbes antagonistic to *M. phaseolina* in their studies.

In the current study also, among the fungal antagonists, *Trichoderma longibrachiatum* (*Trichoderma* -5) recorded the highest mycelial growth inhibition (75.94%), while *Bacillus cereus* (*Bacillus* -5) exhibited maximum inhibition (74.44%) among bacterial isolates. The superior performance of these two indigenous isolates confirms the potential of native microbial strains to act as effective biocontrol agents against cotton root rot pathogen.

The strong antagonistic activity of *Trichoderma* spp. observed in this study aligns with several recent reports demonstrating that species such as *T. harzianum*, *T. viride*, *T. asperellum*, and *T. longibrachiatum* suppress *M. phaseolina* through mechanisms like mycoparasitism, competition, and secretion of antifungal metabolites (Yadav *et al.*, 2021; Nagrale *et al.*, 2022). Degani *et al.* (2024) also highlighted that *Trichoderma* spp. produce cell-wall degrading enzymes (β -1,3-glucanases and chitinases) and volatile organic compounds that significantly reduce the pathogenicity of *M. phaseolina*. The inhibitory percentage recorded in our study is comparable to these findings, further reinforcing the antifungal capabilities of *Trichoderma* species. Several recent reports highlight the effectiveness of *Trichoderma* species against *M. phaseolina* and related soil-borne pathogens. For instance, *Trichoderma harzianum* significantly inhibited the mycelial growth of *M.*

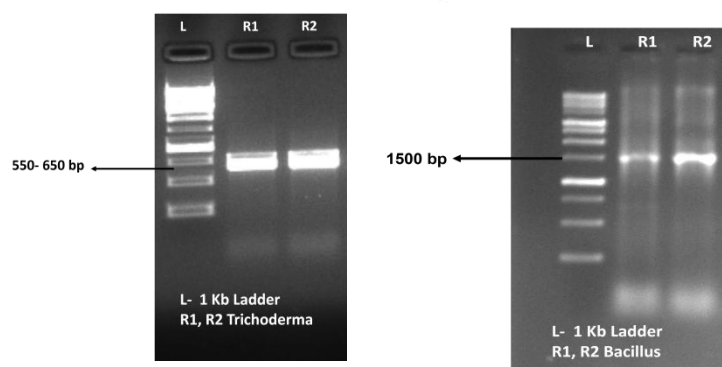


Figure 2. PCR amplification of the ITS 1 & 4 primers for *Trichoderma* - T5 and 27F & 1492 R primers for *Bacillus*-B5

phaseolina in legumes under controlled conditions, confirming the strong antagonistic potential of this genus *in vitro* and *in vivo* (Chaudhari *et al.*, 2025). Similarly, *Bacillus cereus* expressed significant inhibitory activity, which agrees with the recent findings of Ahmad and Khan (2024), who reported that *Bacillus* strains isolated from legume rhizospheres inhibited *M. phaseolina* by producing lipopeptide antibiotics such as fengycins and surfactins.

The molecular identification of the most effective isolates (*Trichoderma longibrachiatum* and *Bacillus cereus*) provides scientific validation and allows future use of these strains in standardized biocontrol formulations. Many recent studies stress the importance of integrating molecular characterization with classical assays to ensure strain specificity and reproducibility (Indra *et al.*, 2024).

Importantly, the present investigation also highlights the ecological relevance of using indigenous antagonists. Native rhizosphere microbes are naturally adapted to the cotton-growing soil environment of the region, which may provide them superior competitive ability, environmental tolerance, and persistence compared to exotic strains. This supports the idea that region-specific antagonist selection can greatly enhance the success of field-level biocontrol programs.

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