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# Screening of fungicides, biocontrol agents, and PGPR for *in vitro* Suppression of cotton wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum*

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## Abstract

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), poses a significant threat to cotton production. In this study, the efficacy of five biocontrol agents and ten fungicides was evaluated under *in vitro* conditions against FOV isolate FOV-5. Antagonistic potential of three fungal biocontrol agents *Trichoderma viride* (SKDTV-22), *T. harzianum* (SKDTH-8), *T. asperellum* (SKDTA-1) and two bacterial isolates *Pseudomonas fluorescens* (SKDPF-6) and *Bacillus subtilis* (SKDBS-19)—was assessed using the dual culture technique. Among these, *T. viride* showed the highest inhibition (54.76%) of FOV growth, followed by *T. harzianum* (51.46%) and *T. asperellum* (49.84%). *P. fluorescens* also displayed strong antagonism with 69% inhibition. Fungicidal efficacy was tested using the poisoned food technique at four concentrations (100, 250, 750 and 1000 ppm). Complete inhibition of mycelial growth at 1000 ppm was observed with Carbendazim, Zineb, Chlorothalonil, Copper oxychloride, and Azoxystrobin, indicating their high potency. Other fungicides such as Propiconazole, Thiophanate methyl, Captan, and Mancozeb showed significant dose-dependent suppression. The study highlights the potential of specific BCAs and fungicides as effective tools for managing Fusarium wilt of cotton, warranting further validation under greenhouse and field conditions for integrated disease management strategies.

**Keywords:** Fusarium wilt, *Fusarium oxysporum* f. sp. *vasinfectum*, cotton, biocontrol agents, PGPR, fungicides, *in vitro*, *Trichoderma*, *Pseudomonas fluorescens*, disease management

## Introduction

Cotton (*Gossypium* spp.) is one of the most important fiber crops cultivated worldwide, playing a vital role in the global agricultural economy (Cusser *et al.*, 2016; Voora *et al.*, 2020) [6, 16]. Its primary value lies in the production of natural fiber used in the textile industry. Additionally, cotton also contributes significantly as an oilseed crop and as a source of animal feed (Chen *et al.*, 2015) [5]. Despite its economic and industrial significance, the productivity and fiber quality of cotton are often severely constrained by a range of abiotic and biotic stresses, as well as competition from weeds. These stress factors together account for substantial reductions in crop yield (Halpern *et al.*, 2018; Hussain *et al.*, 2024) [11, 9]. Among the biotic stress factors, a wide spectrum of pathogens including fungi, bacteria, viruses, and nematodes are known to cause extensive damage to cotton crops globally (Kamburova *et al.*, 2018; Tarazi *et al.*, 2020) [12, 14]. It has been estimated that these pathogenic organisms can lead to up to 30% losses in fiber yield annually (Tarazi *et al.*, 2020) [14], underscoring their significance in cotton disease management. A variety of both soil-borne and foliar diseases affect cotton, the most prevalent of which include: black arm disease caused by *Xanthomonas campestris* pv. *malvacearum*, anthracnose (*Colletotrichum gossypii* or *C. capsici*), Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*), grey mildew (*Ramularia areola*), root rot (*Rhizoctonia bataticola*), cotton leaf curl disease (Cotton leaf curl virus), Verticillium wilt (*Verticillium dahliae*), and leaf blight (*Alternaria macrospora*). Among these diseases, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) is considered one of the most destructive soil and seed-borne diseases affecting cotton crops globally. The pathogen not only hampers crop establishment and vigor but also significantly limits fiber yield and quality (Hillocks, 1992; Sanogo and Zhang, 2016; Zhu *et al.*, 2022) [10, 13, 17]. Infected plants typically exhibit early symptoms such as chlorosis and yellowing of the leaves, followed by premature leaf complete plant death. A distinguishing

feature of *Fusarium* wilt is the reddish-brown vascular discoloration visible in cross-sections of the stem and roots, which serves as a diagnostic characteristic of the disease (Ayubov *et al.*, 2024) <sup>[1]</sup>. A major challenge in managing this pathogen is its ability to persist in the soil for several years as chlamydospores, even in the absence of a suitable host. This longevity makes FOV extremely difficult to eradicate from infested fields (Bani *et al.*, 2018) <sup>[2]</sup>. Historical yield loss data illustrate the seriousness of this disease. Between 1953 and 2012, *Fusarium* wilt was responsible for cotton fiber yield losses ranging from 0.19% to 1.36% annually in affected regions (Blasingame and Patel, 2013) <sup>[4]</sup>. In the United States alone, it was reported that the disease led to a reduction of approximately 109,000 bales (each weighing around 227 kg or 500 lbs) in 2004, according to estimates by the National Cotton Disease Council (Blasingame and Patel, 2005) <sup>[3]</sup>.

Given the economic importance of cotton and the persistent threat posed by *Fusarium* wilt, it is essential to explore effective disease management strategies. One promising approach is the use of biocontrol agents, including beneficial fungi and bacteria, as well as chemical fungicides, to suppress the pathogen's growth. Therefore, to assess the potential of such biological and chemical interventions, an *in vitro* study was conducted to evaluate the antagonistic or inhibitory effects of selected biocontrol agents and fungicides on the mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum*.

## Materials and Methods

### Isolation of *Fusarium oxysporum* f. sp. *vasinfectum*

To isolate the causal organism of wilt disease in cotton, ten symptomatic cotton plants exhibiting typical signs of wilting were carefully collected from infested cotton fields. The selection of plants was based on visual symptoms such as yellowing, wilting, leaf drop, and vascular browning indicative of *Fusarium* infection. Each plant sample was placed in separate sterile polyethylene (poly) bags to avoid cross-contamination and ensure aseptic conditions during transport. These bags were sealed and promptly transported to the laboratory for further analysis and fungal isolation.

Upon arrival at the laboratory, a standard isolation procedure was followed to recover *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) from the collected plant materials. Small sections of plant tissue, approximately 2 to 5 mm in size, were excised from the stem and root portions of each sample plant, particularly from areas showing discoloration or vascular browning, as these regions are typically colonized by the pathogen.

The tissue pieces were then subjected to surface sterilization in order to eliminate any epiphytic or contaminant microorganisms that might interfere with the isolation process. The sterilization was done using a 2.5% sodium hypochlorite (NaOCl) solution (volume/volume) for about 30 seconds. After sterilization, the samples were thoroughly rinsed two to three times with sterile distilled water to remove any residual bleach, which could otherwise inhibit fungal growth. The cleaned tissues were then blotted dry using sterile tissue paper to remove excess moisture.

Following sterilization, the tissue sections were aseptically placed onto Petri dishes containing solidified Potato Dextrose Agar (PDA), a nutrient-rich medium commonly used for the cultivation of fungi. These Petri plates were sealed and incubated at a controlled temperature of 25±2°C

in a BOD (Biological Oxygen Demand) incubator to allow the growth of fungi from the plant tissue.

Over a period of several days, fungal colonies began to emerge from the plant tissues. The growing colonies were carefully observed for morphological characteristics typical of *Fusarium oxysporum* f. sp. *vasinfectum*. To confirm the identity of the isolate, microscopic examination of the fungal structures such as hyphae, conidia (macro- and microconidia), and chlamydospores was conducted using a compound microscope. The identification was carried out by comparing the morphological traits with standard descriptions provided in taxonomic keys and manuals (Upadhyay and Rai, 1992; Gilman, 2001) <sup>[15, 8]</sup>.

Through this process, the pathogen responsible for wilt in the collected cotton samples was successfully isolated and identified as *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), confirming its presence in the surveyed field samples. This isolate was then used for further pathological and antagonistic studies under *in vitro* and *in vivo* conditions.

### Dual culture test of biocontrol agents and wilt fungus

To assess the antagonistic potential of various fungal and bacterial bio-control agents against *Fusarium oxysporum* f. sp. *vasinfectum* (FOV5), an *in vitro* experiment was conducted using the dual culture technique as described by Zivkovic. The study involved nine different isolates of microbial antagonists, comprising seven fungal species from the genus *Trichoderma* and two bacterial strains. The fungal bio-agents used included *Trichoderma viride* (SKDTV-22), *T. harzianum* (SKDTH-8), *T. asperellum* (SKDTA-1). The bacterial antagonists evaluated were *Pseudomonas fluorescens* (SKDPF-6) and *Bacillus subtilis* (SKDBS-19).

For fungal antagonists, dual culture assays were performed on sterile Petri plates containing solidified Potato Dextrose Agar (PDA). In each plate, two 5 mm diameter mycelial discs were aseptically cut from actively growing cultures of the test pathogen (FOV5) and the respective fungal bio-agent. These discs were then placed on the PDA medium at a distance of 3 cm from each other to allow simultaneous growth. The Petri plates inoculated with *F. oxysporum* f. sp. *vasinfectum* alone, without any bio-agent, were used as controls to compare the growth behavior of the pathogen in the absence of antagonistic interference.

Each treatment was replicated five times to ensure statistical reliability and to account for any experimental variation. After inoculation, the plates were incubated in a Biological Oxygen Demand (BOD) incubator at 25±2 °C for a period of 14 days. The growth interaction between the pathogen and each fungal antagonist was observed and recorded.

For bacterial bio-agents, a slightly modified method was followed. The bacterial cultures of *Pseudomonas fluorescens* and *Bacillus subtilis* were streaked on PDA plates at a distance of 3 cm from the mycelial disc of FOV-5. These plates were incubated at a slightly higher temperature of 35±2 °C for 7 days, which favors bacterial growth. The interaction zone between the bacterial antagonist and the fungal pathogen was observed for any signs of inhibition. In both fungal and bacterial assays, observations were made on the radial growth of the pathogen as well as the antagonists. The presence of any zone of inhibition—an area where the growth of FOV5 was clearly suppressed by the bio-agent—was noted and measured. The degree of inhibition was used as a primary indicator of the antagonistic potential of each isolate.

$$PI = \frac{C - T}{C} \times 100$$

Where,

C = the test pathogen's growth (mm) in control.

T = the test pathogen's growth (mm) in the amended medium.

This formula provided a quantitative assessment of the degree of fungal growth suppression achieved by each fungicide at varying concentrations. The results from this experiment were used to determine the most effective fungicide and concentration for managing FOV-5 under laboratory conditions. The fungicides showing high inhibition rates *in vitro* could be selected for further evaluation under greenhouse and field conditions to develop integrated disease management strategies for Fusarium wilt in cotton.

## Results and Discussion

The five biocontrol agents (fungi and bacteria) and ten fungicides were screened *in vitro* conditions to evaluate their effectiveness against *F. oxysporum* f. sp. *vasinfectum* (FOV7).

### Effect of biocontrol agents on the colonization of *Fusarium oxysporum* f. sp. *vasinfectum*

The antagonistic potential of five isolates of biocontrol agents (BCAs) was evaluated against *Fusarium oxysporum* f. sp. *vasinfectum* isolate FOV-5 under *in vitro* conditions using the dual culture technique. The BCA's tested included three fungal species belonging to the genus *Trichoderma* and two bacterial isolates. Specifically, the tested agents were: *T. viride* (SKDTV-22), *T. harzianum* (SKDTH-8), *T. asperellum* (SKDTA-1), along with *Pseudomonas fluorescens* (SKDPF-6) and *Bacillus subtilis* (SKDBS-19).

The results clearly demonstrated that all tested biocontrol agents were effective in suppressing the mycelial growth of the wilt-causing pathogen *F. oxysporum* f. sp. *vasinfectum* to varying degrees. The antagonistic activity was evident by the formation of inhibition zones and reduced radial growth of the pathogen on PDA plates. However, there was considerable variation in the level of inhibition among the different isolates, with percent inhibition ranging from 45% to 55% compared to the untreated control.

Among the nine BCAs, four isolates exhibited significantly higher antagonistic effects, reducing the mycelial growth of the pathogen by more than 69%. The most effective isolate was *Trichoderma viride* (SKDTV-22), which achieved maximum inhibition of 54.76%, followed closely by *T. harzianum* (SKDTH-8) with 51.46%, *T. asperellum* (SKDTA-1) with 49.84%, and *P. fluorescens* (SKDPF-6) with 69% inhibition. The differences in performance among these top-ranking BCAs were statistically significant ( $P \leq 0.05$ ), highlighting their potential as strong antagonists against FOV5.

The lowest yet still notable inhibition was observed with *B. subtilis* (SKDBS-19) at 44.55% and *P. fluorescens* (SKDPF-6), which showed the least inhibitory effect of 45.45% over control.

These findings (summarized in Table-1) suggest that certain

strains, particularly *T. viride*, *T. harzianum*, *T. asperillum* possess strong antagonistic properties against *F. oxysporum* f. sp. *vasinfectum* and could be effectively employed as part of an integrated management strategy for controlling Fusarium wilt in cotton. Further studies under greenhouse and field conditions are warranted to validate their performance and compatibility with other control measures.

### *In vitro* effectiveness of fungicides against *Fusarium oxysporum* f. sp. *vasinfectum*

A total of ten fungicides namely Thiram, Mancozeb, Zineb, Chlorothalonil, Copper oxychloride

Captan, Carbendazim, Thiophanate methyl, Propiconazole, Azoxystrobin- were evaluated *in vitro* for their efficacy against *Fusarium oxysporum* f. sp. *vasinfectum* (FOV-5). The assessment was conducted using the poisoned food technique to determine the minimum inhibitory concentration (MIC) of each fungicide. Fungicides were tested at four concentrations: 100 ppm, 250 ppm, 750 ppm, and 1000 ppm of active ingredient.

The poisoned food method revealed that, in general, the inhibitory effect of fungicides increased with increasing concentrations, as indicated by a progressive reduction in the radial growth of the fungal colonies. This dose-dependent suppression confirms the fungistatic or fungicidal properties of the tested compounds against FOV-5.

Remarkably, complete inhibition (100%) of mycelial growth of *Fusarium oxysporum* f. sp. *vasinfectum* was observed at the lowest tested concentration (1000 ppm) for the following fungicides: Carbendazim, Zineb, Chlorothalonil, Copper oxychlorite, Azoxystrobin.

These results suggest that these four fungicides are highly effective even at low concentrations, indicating a low minimum inhibitory concentration (MIC) and strong fungicidal activity against FOV7. Their early and complete inhibition makes them potential candidates for effective disease control in integrated disease management (IDM) strategies.

The other fungicides- Propiconazole, Thiophenate methyl, Captan, Mancozeb and Copper Oxychloride-also demonstrated significant suppression of the pathogen's growth, although complete inhibition was not achieved at 1000 ppm. Their efficacy increased progressively with higher concentrations, reflecting a positive dose-response relationship. While not achieving full inhibition at the lowest concentration, these fungicides still produced substantial reductions in colony diameter across all tested doses (as presented in Table 2), indicating their potential utility, especially in combination with other control measures. These findings underscore the variability in the effectiveness of different fungicidal compounds against *F. oxysporum* f. sp. *vasinfectum*, and they highlight the importance of selecting appropriate active ingredients and concentrations for optimal control. The most potent fungicides identified in this study (Carbendazim, Zineb, Chlorothalonil and Copper oxychlorite) may serve as key components in seed treatment or soil drench applications for effective wilt management in cotton. However, further testing under greenhouse and field conditions is essential to validate these laboratory results and assess the environmental safety and phytotoxicity of these chemicals.



**Table 1:** Effect of different fungicides on the radial growth of *Fusarium oxysporum* f. sp. *vasinfectum* at different concentrations

Treatment Name	Fungicides concentration (ppm)								Overall Mean Inhibition (%)
	100ppm		250ppm		500ppm		1000ppm		
	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)	
Thiram	51.54	38.46	44.78	45.22	40.33	49.67	36.67	53.33	46.67
Mancozeb	23.33	66.67	20.17	69.83	18.35	71.65	15.47	74.53	70.76
Zineb	32.17	57.83	16.47	73.53	0.00	100	0.00	100	82.84
Chlorothalonil	38.67	51.33	32.94	57.06	25.80	64.20	0.00	100	68.14
Copper oxychloride	11.67	78.33	0.00	100	0.00	100	0.00	100	94.58
Captan	17.67	72.33	15.87	74.13	15.58	74.42	14.15	75.47	74.08
Carbendazim	0.00	100	0.00	100	0.00	100	0.00	100	100
Thiophanatemethyl	39.92	50.08	36.68	53.14	14.67	75.33	12.87	77.13	63.92
Propiconazole	36.88	53.12	33.60	56.40	15.81	74.19	12.54	77.46	65.29
Azoxystrobin	34.95	55.05	13.34	76.66	0.00	100	0.00	100	82.92
Control	90.00	0.00	90.00	0.00	90.00	0.00	90.00	00	00
SE (m)	0.89	1.68	1.72	2.51	2.61	1.85	2.65	2.98	3.17
CD (p=0.05)	1.87	2.52	2.98	3.51	3.98	4.28	3.28	4.42	5.31
CV	3.71	5.21	5.89	6.28	4.27	5.17	4.54	6.82	7.38

**Table 2:** *In vitro* antagonistic effect of five BCA's on the radial growth (cm) of *Fusarium oxysporum* f. sp. *Vasinfectum* (Fov) at 3, 5, 7 and 9 days after inoculation

Sr. No.	Isolates name	Colony diameter (mm)					Inhibition over control (%)				
		3	5	7	9	Mean	3	5	7	9	Mean
1	<i>T. viridae</i> (SKDTA-1)	35.00	31.35	28.26	25.35	29.99	31.03	52.26	63.92	71.83	54.76
2	<i>T. harzianum</i> (SKDTH-8)	37.67	34.33	30.15	26.25	32.10	25.77	47.72	61.51	70.83	51.46
3	<i>T. asperillum</i> (SKDTA-1)	38.45	35.07	31.22	28.45	33.30	24.24	46.60	60.14	68.83	49.84
4	<i>Pseudomonas fluorescens</i> (SKDPF-6)	37.79	34.73	30.18	27.63	32.71	26.18	43.48	56.95	64.73	45.45
5.	<i>Bacillus subtilis</i> (SKDBS-19).	36.23	33.54	29.73	26.29	31.26	25.92	41.51	54.51	63.18	44.55
	Control (FOV-5)	50.75	65.67	78.33	90.00	71.19	0.00	0.00	0.00	0.00	0.00
	SEm±	1.17	0.67	2.33	3.03	1.01	1.42	1.16	1.23	1.57	2.36
	CD (p=0.05)	4.13	3.23	5.34	4.27	4.07	5.55	4.23	3.55	5.67	4.67
	CV	0.23	2.20	1.33	3.67	1.01	0.45	0.33	1.26	0.11	0.38

## Conclusion

The present *in vitro* study clearly demonstrated differential antagonistic efficacy among the nine tested biocontrol agents (BCAs) against *Fusarium oxysporum* f. sp. *vasinfectum* (FOV-5), the causal organism of Fusarium wilt in cotton. Among the isolates evaluated, three BCAs exhibited the highest level of antagonism by significantly inhibiting the mycelial growth of the pathogen. These included *T. viride* (SKDTV-22), *Trichoderma harzianum* (SKDTH-8) and *T. asperillum* (SKDTA-1) all of which achieved inhibition percentages ranging from 69% to 73%. These agents were statistically superior ( $P \leq 0.05$ ) in suppressing the radial growth of FOV-5, indicating strong antagonistic potential. On the other hand, moderate to lower antagonistic effects were observed with *T. asperillum* (SKDTA-1), *P. fluorescens* (SKDPF-6), and *B. subtilis* (SKDBS-19), which exhibited comparatively less inhibition of the wilt pathogen, ranging from 44.55% to 49.84%. Although these isolates still showed antagonism, their suppressive ability was relatively weaker when compared to the top-performing isolates.

In a parallel *in vitro* evaluation, ten fungicides were tested against FOV-5 using the poisoned food technique to determine their efficacy at four different concentrations (100, 250, 750 and 1000 ppm). The results indicated that four fungicides- Carbendazim, Zineb, Chlorothalonil, Copper oxychlorite were highly effective, completely inhibiting the mycelial growth of FOV-5 at even the lowest tested concentration of 1000 ppm. This finding suggests that these fungicides possess strong fungicidal properties and

could be effective components of an integrated disease management program for Fusarium wilt in cotton.

The remaining fungicides- Propiconazole, Thiophenate methyl, Captan, Mancozeb- were found to be less effective at lower concentrations, although they still exhibited significant inhibitory effects as the concentration increased. While these fungicides did not achieve complete suppression at 1000 ppm, their ability to restrict fungal growth at higher doses indicates potential for use, especially in combination with cultural or biological control methods. Overall, the experiment highlights the importance of selecting both potent biocontrol agents and effective fungicides for the integrated management of Fusarium wilt. The most promising candidates from this study-including *T. viride*, *T. harzianum*, and fungicides like Carbendazim and Zineb-warrant further evaluation under greenhouse and field conditions to validate their efficacy in real-world cropping systems.

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