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Greenhouse Evaluation of Biocontrol Agents for Managing Fusarium Wilt Disease in Tomato Pusa Ruby (Solanum lycopersicum L.): A Comparative Study

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Abstract: The present greenhouse study was conducted to determine the biocontrol efficacy in preventive (prophylactic) and curative applications against Fusarium wilt disease on the tomato susceptible Pusa Ruby variety. An *in-vitro* dual assay revealed the biocontrol antagonistic property of the *Bacillus amyloliquefaciens* (JUB2024-002), *Bacillus subtilis* (JUB2024-001), and *Trichoderma harzianum* (JUF2024-006) against phytopathogen *Fusarium oxysporum* (NFCCI-3426) as compared to *Pseudomonas fluorescens* (JUB2024-003) with moderate control on Fusarium. The nature of the pathogenicity of *F. oxysporum* (NFCCI-3426) on the selected Pusa Ruby variety showed that the disease severity of wilt in tomato plants was greater in soil application than foliar. Using the methodology of prophylactic and curative treatment under greenhouse conditions over diseased plants revealed the results of the applied dosage of biocontrol, *i.e.*, 2L/ac. It turned out as a result that *B. amyloliquefaciens* (JUB2024-002) outperformed other applied cultures of *B. subtilis* (JUB2024-001) and *T. harzianum* (JUF2024-006), and *P. fluorescens* (JUB2024-003). The suggested 2L/ac dosage of biocontrol *B. amyloliquefaciens* (JUB2024-002) can be checked under field conditions to measure the efficiency, along with the combination with other biocontrol agents.

Keywords: Fusarium Wilt, Bacillus Amyloliquefaciens Biocontrol Agents, Tomato Disease Management, Greenhouse Study

1. Introduction

Numerous pests and diseases cause significant yearly losses in crop output in agriculture globally. Plant diseases caused by bacteria and fungi pose a danger to agricultural productivity and food security. Approximately 80% of agricultural illnesses that cause significant crop damage and financial loss are caused by fungi and bacteria, which are the most prevalent and extensively distributed plant pathogens [1]. Plant diseases in crops are soilborne infections that lead to a yearly loss in yield of 10-20% [2]. The most common soil-borne disease is wilt, which leads to a yield loss of up to 70% in the agricultural fields, ultimately leading to major economic loss to the farmers in different geographical locations [3]. Wilting is essentially defined as the plant's non-woody portions becoming less stiff. The turgor pressure drops to zero, which causes the water content in that region to decrease [4] Wilt is very common in solanaceous crops like tomatoes, potatoes, lentils, eggplant, and other crops like cucumbers, sugarcane, soyabean, chili, bananas, cabbage, cotton, onions, muskmelon, watermelon, tulips, gladioli, flax, etc. Wilt is mostly caused by fungal and bacterial pathogens [5].

The pathogens responsible for fungal wilting include fungi such as *Ceratocystis* spp., *Fusarium* spp., *Verticillium spp., Botrytis Cineria, Sclerotinia Sclerotiorum, Thielaviopsis, Alternaria alternata,* and

others [5]. Furthermore, *Xanthomonas campestris* pv. *Musacearum, Xanthomonas translucens* pv. *Graminis, Erwinia tracheiphila, Pantoea stewartii, Erwinia chrysanthemi, Enterobacter mori,* and *Curtobacterium flaccumfaciens* subsp. *Flaccumfaciens* are among the pathogens that induce bacterial wilting [6].

Plants that are damaged by fungal wilt exhibit distinguishable symptoms like drooping of plants towards the ground, leaves in the initial stages show discoloration and yellowing, referred to as "plant wilting and rotting," in response to heat, dryness, or disease [7]. When a plant starts to exhibit wilting signs, bacterial wilt is referred to as "green wilt" illness since the infected plant's leaves do not turn yellow [8]. Because of its excellent adaptation to soil conditions, the bacteria may thrive in the rhizosphere. It invades vascular tissues through manufactured or natural holes in the roots and carries a wide range of virulence factors, resulting in both internal and external symptoms that ultimately kill the plant [9].

Nowadays, to protect crops from wilt disease, several conventional methods are practices are being employed, such as the use of chemical fungicides as a prophylactic (preventive) treatment. Using chemical fungicides and bactericides is a crucial technique for managing bacterial and fungal illnesses [10-12]. Biological management of Fusarium wilt in chickpea was also investigated by Kala *et al.* (2016) [13]; that crop-specific study provides background on integrated wilt management.

Plants can protect from wilt disease to some extent with the help of existing endophytes, which can be found associated with the roots (rhizosphere), leaves (phyllosphere), or within the plant (endosphere). Another innate mechanism to reduce disease load is enhancing plant defenses and physiological immunity, and raising soil mineral bioavailability can all be achieved by using microorganisms that support plant development [11, 12] Apart from the existing solutions, the use of biocontrol to prevent plants from is becoming prevalent these days. The idea of biocontrol of plant diseases [14, 15]. Rhizobacteria that stimulate plant growth have been shown to biocontrol parasitic diseases. Antibiotic microorganisms may be used to biologically manage bacterial wilt illness, according to recent research [16].

Additionally, a relationship between soil bacteria and wilting has been discovered. It implies that an impact would occur [4]. Understanding these dynamics is critical for developing effective disease control measures and maintaining crop health and output [7]. Therefore, the present study aims to screen the effectiveness of biocontrol agents (existing) for control of *Fusarium* wilt *in vitro*, assess the pathogenicity of the *Fusarium* wilt in a tomato (*Solanum lycopersicum*) susceptible variety, and evaluate the effectiveness of bacterial and fungal antagonists in suppressing *Fusarium* wilt disease and mitigating disease severity in a greenhouse study.

2. Materials and Methods

2.1 Culture Procurement and Maintenance

The plant pathogenic pure fungal cultures with the ability to cause wilt disease were obtained from the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India. The obtained fungal cultures were *Fusarium oxysporum* (NFCCI-3426) and *Alternaria alternata* (NFCCI-3474). The other microbial cultures, *Bacillus subtilis* (JUB2024 001), *Bacillus amyloliquefaciens* (JUB 2024 002), *Pseudomonas fluorescens* (JUB 2024 003), and *Trichoderma harzianum* (JUF 2024 006) for the purpose of biocontrol testing were obtained from JU Agrisciences Pvt. Ltd., UPSIDC, Sikandrabad, India.

The obtained fungal cultures, *i.e.*, *Fusarium oxysporum* (NFCCI-3426), *Alternaria alternata* (NFCCI-3474), and *Trichoderma harzianum* (JUF2024-006), were sub-cultured on the prepared plates of Potato Dextrose Agar (PDA) and incubated at 25 ℃ for 7 days. Bacterial cultures, *i.e.*, *Bacillus subtilis* (JUB2024-001), *Bacillus amyloliquefaciens* (JUB2024-002), and *Pseudomonas fluorescens* (JUB2024-003), were sub-cultured on the prepared plates of Nutrient Agar (NA) media and incubated at 37 ℃ for 48 h.

2.2 Cultural and Morphological Identification

After the growth was obtained, the bacterial and fungal cultures were observed for their colony size, color, elevation, margins, surface, texture, spore formation, and pigmentation. In regards with microscopic observation of the culture, slides were prepared with standard microbiological methods. The fungal cultures were wet mount on the slides and stained with Lacto phenol cotton blue (LCB) stain, which was further visually observed under a 60X objective lens of a compound microscope [17]. Similarly, the bacterial cultures were treated with differential stain, *i.e.*, Gram's stain, and were visually observed under a compound microscope at 100X oil immersion objective lens [18].

2.3 In-Vitro Qualitative Evaluation of Antagonistic Activity

According to Nysanth *et al.* (2022) [19], the effectiveness of the antagonistic ability of biocontrol qualitatively for wilt disease in *In-vitro* conditions, an antagonistic assay was performed where phytopathogens *Fusarium oxysporum* (NFCCI-3426) was checked against *Bacillus subtilis* (JUB2024-001), *Bacillus amyloliquefaciens* (JUB2024-002), *Pseudomonas fluorescens* (JUB2024-003), and

Trichoderma harzianum (JUF2024-006). The pure culture of *Fusarium oxysporum* (NFCCI-3426) was cut into discs using a cork-borer and transferred to the prepared plates containing Potato Dextrose Agar (PDA) and Nutrient Agar (NA) plates. After that, the bacterial cultures *Bacillus subtilis* (JUB2024-001), *Bacillus amyloliquefaciens* (JUB2024-002), and *Pseudomonas fluorescens* (JUB2024-003) were streaked against the placed disc of *Fusarium oxysporum* (NFCCI-3426), *Trichoderma harzianum* (JUF2024-006) was also cut and placed against the disc of *Fusarium oxysporum* (NFCCI-3426). The plates were then incubated at 25 ℃, and the growth was monitored for one week.

2.4 *In-Situ* Evaluation of Pathogenicity of Phytopathogens

2.4.1 Preparation of Host Seedlings

The potting mixture was prepared by mixing sand, soil, cocopeat, and FYM in the ratio of 1:1:1:1, respectively accordingly and moist-sterilized twice. After the sterilization, 4 kg each of potting mixture was then transferred into the (12 inches diameter) pots, which were pre-sterilized twice with 70% Ethanol [20]. After the preparation of the potting mixture, the seeds of tomato (*Solanum lycopersicum*), Pusa Ruby susceptible variety, were sown, and the seedlings were prepared. After 15 days of sowing, the juvenile seedlings of tomato plants were then distributed according to the treatments and replicates of the experimental layout (Table 1).

2.4.2 Inoculum Preparation of Phytopathogens

The inoculum of *Fusarium oxysporum* (NFCCI-3426) was prepared for both foliar and soil treatment by transferring the mycelial discs of the pre grown fungal pathogen onto the sand- maize media for the soil Sand-maize media was prepared in the ratio of 3:1 respectively with an addition of 4 drops distilled water to maintain the required moisture within the media [21]. The media was sterilized twice before the inoculum discs were transferred. The prepared inoculum was incubated for 7 days at 25 ℃. On the other hand, Potato Dextrose Broth (PDA) was prepared, sterilized, and the pure culture of *Fusarium oxysporum* (NFCCI-3426) was transferred into the media, which was kept for incubation for 7 days at 25 ℃ for foliar application. Meanwhile, the CFU values were checked until the expected growth of *Fusarium oxysporum* (NFCCI-3426) within the prepared inocula was achieved, *i.e.*, 10^6 CFU/g before their amendment on the decided treatments (Table 1).

2.4.3 In-Situ Test for Pathogenicity

The prepared inoculum of *Fusarium oxysporum* (NFCCI-3426) in sand-maize media was mixed well for soil application with the pre-sterilized potting mixture of each pot in the ratio of 3:1 (the soil-to-inoculum ratio specified for this experiment), and 4 kg each of pre-sterilized potting mixture was then transferred to F6, F7, F8, F9, and F10. The prepared inoculum of *Fusarium oxysporum* (NFCCI-3426) in PDA was sprayed on the 15-day-old juvenile seedlings of F1, F2, F3, F4, and F5. The soil and inoculum were both tested and validated for pathogen CFU count 10^6 [22]. The seedlings were then sown in the respective treatments accordingly (Table 1) along with the control C1, C2, C3, C4, and C5. The treatments were kept under observation until symptoms appeared for the wilt disease in tomato plants of both foliar and soil application. After 4-7 days post inoculation, according to the visual observation, the score for the disease severity was given from 1-4 as a degree of damage done to the plants by pathogenic wilt caused by *Fusarium oxysporum* (NFCCI-3426) (Table 2). The disease incidence was calculated[7] by the given formula;

$$Disease\ Incidence = (Total\ no.of\ diseased\ plants / Total\ no.of\ tested\ plants)\ x\ 100\% \quad (1)$$

2.5 Evaluation of the Efficacy of Biocontrol Capabilities

2.5.1 Biocontrol Inoculum Preparation

The pure cultures of *Bacillus subtilis* (JUB2024-001), *Bacillus amyloliquefaciens* (JUB2024-002), and *Pseudomonas fluorescens* (JUB2024-003) were inoculated in the prepared pre-sterilized Nutrient Broth (NB) media and incubated at 37 ℃ for 24 h.

Table 1. Distribution of the experimental layout of the pathogenicity test

Treatment	Pathogen	Application	No. ofpots	No. of plants/pot
C1, C2, C3, C4, C5	No pathogen	Control	5	1
F1, F2, F3, F4, F5	*Fusarium oxysporum*	Foliar	5	1
F6, F7, F8, F9, F10	*Fusarium oxysporum*	Soil	5	1

Table 2. Five-category (0–4) scale to score for the disease severity based on symptoms.

Degree of severity	Symptoms	Reference
0	No symptoms	General 0–4 scoring approach: Soleha *et al.* (2022) [24]. Root and shoot symptom descriptions are specific to the present tomato study.
1	Slightly brown tap root and adventitious root, and the vegetative part shows no symptoms of wilt	
2	Brown taproot, with visible damage on the adventitious roots, vegetative part showing slight wilting withlimited growth	
3	Completely brown taproot with poorly developedappendages, the stem growth is only half of the length, with wilt symptoms	
4	Dark brown tap root with necrosis, no adventitious root, the plant is completely withered due to wilt	

The bacterial culture's growth was observed until the desired OD and CFU/ml were obtained. Similarly, pure culture of *Trichoderma harzianum* (JUF2024-006) was inoculated on pre-sterilized Potato Dextrose Broth (PDB) media and incubated at 25 ℃ for 7 days [23]. The fungal growth was observed until the desired spore count of CFU was obtained. All the inocula of biocontrol were grown in duplicates, and samples were drawn regularly to check the cell morphology and purity under the compound microscope objective lens 60 and 100X oil immersion for fungus and bacteria, respectively, to avoid cross-contamination [19].

2.6 Pot Experiment

According to Díaz-Gutiérrez (2021) [25], the pot experiment layout was designed and distributed. The treatments were divided into two major groups: prophylactic (preventive) and Curative. The plant growing bags (15x15 dim.) were first surface sterilized with 70% ethanol twice, and the potting mixture was also sterilized twice by moist sterilization to remove the undesirable microflora, *i.e.*, primarily present in the soil in abundance [8] .The seedlings of tomato were then transferred into the prepared pots containing 8kg of pre-sterilized potting mixture according to the treatment distribution (Table 3). In prophylactic treatments, the soil was first drenched with 0.2 g/plant of the dosage formulation of biocontrol from the prepared standard dosage of biocontrol, *i.e.*, 2 kg/ac or 2 L/ac formulation [26] containing 10^9 CFU/ml inoculum.

To achieve the desired establishment of biocontrol within the treatments, the soil samples were withdrawn every day for 5 days until values of 10^6 CFU/g were obtained. After the prophylactic treatment of biocontrol, the prepared inoculum of *Fusarium oxysporum* (NFCCI-3426) in sand-maize medium was added in the prescribed ratio (40 g/kg) [27] containing 10^6 CFU/ml in both prophylactic and curative treatments except controls (Table 3). After 4-7 days post-inoculation, the disease symptoms appeared in both curative and prophylactic treatments. After the disease incidence, the curative treatments were soil drenched with 0.2 ml /plant of the dosage formulation of biocontrol from the prepared standard dosage of biocontrol, i.e., 2 Kg/ac or 2 L/ac formulation [28]. The treatment plan was followed the same for prophylactic and curative tests for the *in-situ* experiment until 30 days described by [25]. The biocontrol efficiency was calculated according to Yang *et al.* (2009).

2.7 Statistical Analysis

Analysis of variance (ANOVA) was performed on all parameters to compare treatment effects on the indigenous microorganisms. Means were separated by using Duncan's Multiple Range Test (DMRT) (IBM SPSS 20 version).

3. Results and Discussion

3.1 Cultural and Morphological Characteristics of the Retrieved Cultures

The retrieval of *Fusarium oxysporum* (NFCCI-3426) on PDA plates after incubation for 7 days showed characteristic pink-white colour mycelium with concentric zones of progressive growth, which later turned to purplish-pink reverse after 10 days of incubation. The plate of *Alternaria alternata* (NFCCI-3474) after 5 days of incubation showed vigorous growth of thick white-grey raised cottony mycelium. The plate of *Trichoderma harzianum* (JUF2024-006) after 7 days of incubation showed white-green, moderately compact flat mycelial. The retrieved bacterial cultures of *Bacillus subtilis* (JUB2024-001) and *Bacillus amyloliquefaciens* (JUB2024-002) after 24 h of incubation showed a clearly visible growth on NA plates with white colour and dry texture with medium-

sized isolated colonies. Alongside *Pseudomonas fluorescens* (JUB 2024-003), after 48 h of incubation showed a visible growth of white colour and smooth texture with small-sized isolated colonies on the NA plate (Figure 1).

Table 3. Treatment layout of the pot trial for efficacy test as prophylactic and curative

Treatment	No. of Bags	No. of Plants/bag	Biocontrol dose/plant
T1 *Pseudomonas fluorescens* (JUB 2024 003)	1	5	0.2 ml
T2 *Bacillus amyloliquefaciens* (JUB 2024 002)			
T3 *Bacillus subtilis* (JUB 2024 001)			
T4 *Trichoderma harzianum* (JUF 2024 006)			
T1 + T2			
T3 + T4			
T2 + T3			
T4 + T1			
T2 + T4			
T1 + T3			
Control (Untreated)			0.0 ml
Positive control (Carbendazim 50%)			0.2 ml
F0 (Negative control) *Fusarium oxysporum* (NFCCI-3426)			0.0 g

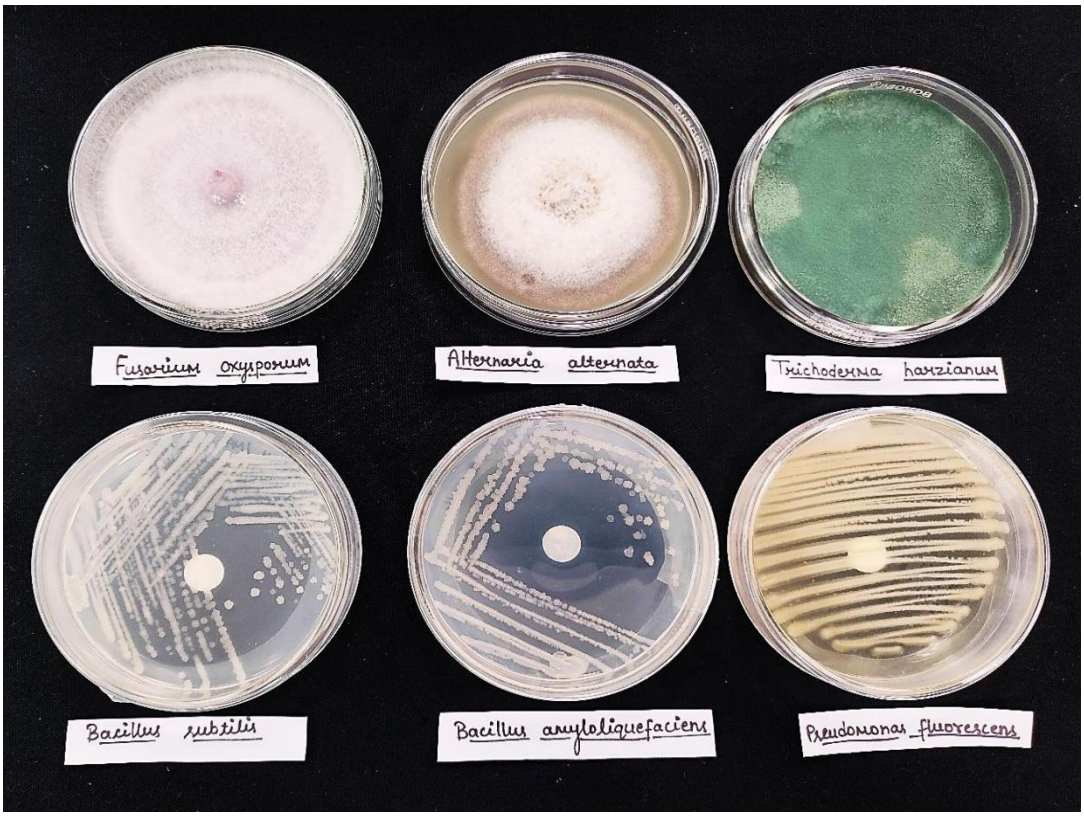

Figure 1. Showing cultural morphology of the revived cultures, top left: *Fusarium oxysporum* (NFCCI-3426), top middle: *Alternaria alternata* (NFCCI-3474), top right: *Trichoderma harzianum* (JUF2024-006), bottom left*: Bacillus subtilis* (JUB2024-001), bottom middle: *Bacillus amyloliquefaciens* (JUB2024-002), and bottom right: *Pseudomonas fluorescens* (JUB2024-003).

3.2 Microscopic Identification of the Retrieved Cultures

The LPCB mount staining of recovered fungal cultures under a 60X objective lens revealed a segregated hyphal network along with their respective structured spore body. The microscopic investigation of *Fusarium oxysporum* (NFCCI-3426) showed germinated vertical and horizontal vegetative hyphae with micro-conidia slightly curved at the end (Figure 2a), *Alternaria alternata* (NFCCI-3474) showed septate hyphae, with long conidial chains (Figure 2b), *Trichoderma harzianum* (JUF2024-006) with round clustered spores (Figure 2c). The gram staining performed for the bacterial cultures revealed their cell shape and gram's reaction where, *Bacillus subtilis* (JUB2024-001) and *Bacillus amyloliquefaciens* (JUB2024-002) retained only crystal violet *i.e*., primary stain which is a clear indication of gram-positive reaction with rod shape cells (Figure 3a and Figure 3b) whereas, *Pseudomonas fluorescens* (JUB2024-003) retained counter stain only *i.e*., safranin which is a clear indication of gram-negative reaction with short rods (Figure 3c).

3.2.1 In-Vitro Antagonistic Activity against Phytopathogen

The bacterial culture *Bacillus amyloliquefaciens* (JUB2024-002) has shown more biocontrol activity in terms of percentage control against phyto-pathogen *Fusarium oxysporum* (NFCCI-3426) culture, followed by *Bacillus subtilis* (JUB2024-001) and *Trichoderma harzianum* (JUF 2024 006), whereas *Pseudomonas fluorescens* (JUB 2024 003) has shown moderate control over *Fusarium oxysporum* (NFCCI-3426) as compared to the control plate (Figure 4).

3.2.2 In-Situ Pathogenicity Effect of Fusarium oxysporum (NFCCI-3426) on tomato

The treatments (F1, F2, F3, F4, and F5) with foliar application induced wilt disease symptoms on the score of 2, 3, and 4, *i.e*., limited growth, vegetative part showing wilt with visible damage to adventitious roots that turned dark brown in colour. On the other hand, the treatments (F6, F7, F8, F9, and F10) with induced soil application developed more severe symptoms on a range of 3, and 4 where, stem growth was half of the control with poorly developed root system and the plant was completely withered due to higher infectivity of wilt as compared to control treatment (C1, C2, C3, C4, and C5) containing lush green foliage with well branched root system (Figure 5).

3.2.3 In-Situ effect of Biocontrol Activity against Fusarium oxysporum (NFCCI-3426)

The percentage control in preventive (prophylactic) treatment T2 *Bacillus amyloliquefaciens* (JUB2024-002) along with T3 *Bacillus subtilis* (JUB2024-001), and T2 *Bacillus amyloliquefaciens* (JUB2024-002) along with T4 *Trichoderma harzianum* (JUF2024-006) displayed a significant biocontrol response against *Fusarium oxysporum* (NFCCI-3426), negative control, *i.e*., 83.33 and 77.78 % respectively.

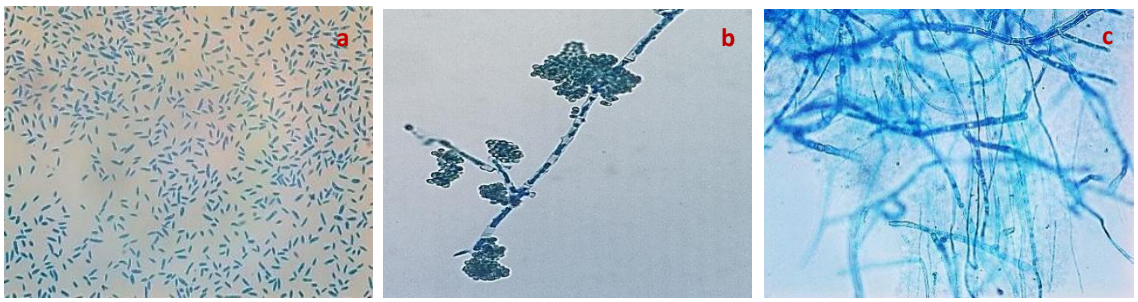

Figure 2. Microscopic appearance of fungus; **(a)** *Fusarium oxysporum* (NFCCI-3426), **(b)** *Trichoderma harzianum* (JUF2024-006), **(c)** *Alternaria alternata* (NFCCI-3474) (60X).

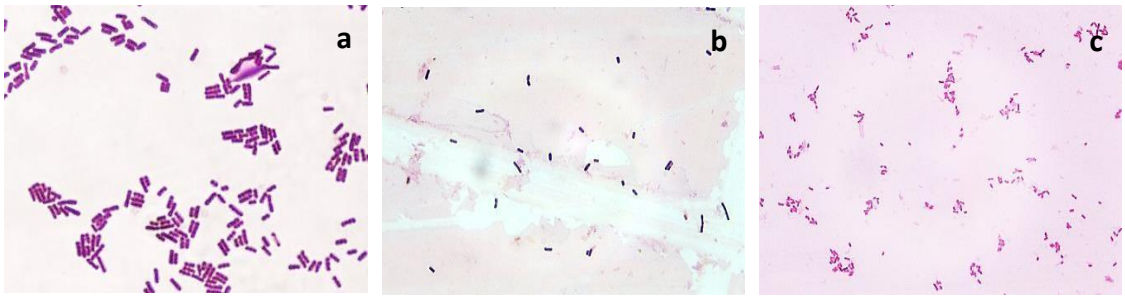

Figure 3. Microscopic appearance of bacteria; **(a)** *Bacillus subtilis* (JUB2024-001), **(b)** *Bacillus amyloliquefaciens* (JUB2024-002), **(c)** *Pseudomonas fluorescens* (JUB2024-003) (100X).

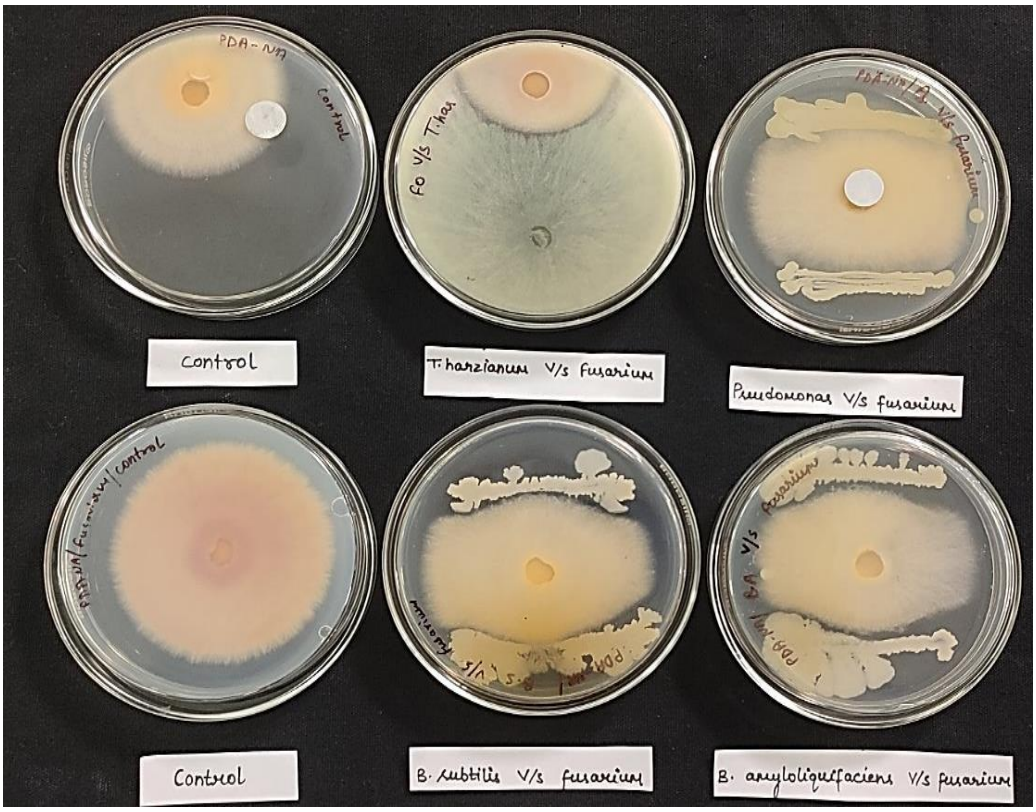

Figure 4. Antagonistic activity of fungus and bacteria (*Trichoderma harzianum* (JUF 2024 006), *Pseudomonas fluorescens* (JUB2024-003), *Bacillus subtilis* (JUB2024-001), and *Bacillus amyloliquefaciens* (JUB2024-002) against phytopathogen *Fusarium oxysporum* (NFCCI-3426).



Figure 5. Pathogenicity response of *Fusarium oxysporum* (NFCCI-3426) on tomato (*Solanum lycopersicum*); **(a)** in the root region (left-control (untreated), middle-soil, and right-foliar, **(b)** shoot and foliage (left row-control (untreated)) (C1, C2, C3, C4, and C5), middle-foliar treatment (F1, F2, F3, F4, and F5), and right-soil treatment (F6, F7, F8, F9, and F10*).*

The Treatment T2 *Bacillus amyloliquefaciens* (JUB2024-002) along with T3 *Bacillus subtilis* (JUB.S01012024)) with 83% control efficiency is at par with positive control of Carbendazim 50 % with the percentage control of 94.4% (Table 4 and Figure 6). Whereas, the percentage control of *Fusarium oxysporum* (NFCCI-3426) in curative treatment of T2 *Bacillus amyloliquefaciens* (JUB2024-002) along with T3 *Bacillus subtilis* (JUB2024-001) recorded with the efficacy of 50% *i.e.*, on par with positive control of Carbendazim 50% with the recorded efficacy of 68.75% followed by the combination of treatments T1:T3, T1:T4, and T1:T2 *i.e.*, 43.75% were, T1 was *Pseudomonas fluorescens* (JUB 2024003).

Table 4. Biocontrol (%) of *Fusarium oxysporum* (NFCCI-3426) in tomato plant through prophylactic (preventive) treatment.

S.No.	Treatment details	R1	R2	R3	R4	R5	Avg.	% PDC over negative control
1	T1 Pseudomonas fluorescens (JU B 2024 003)	2	1	2	1	2	1.600	55.56
2	T2 Bacillus amyloliquefaciens (JU B 2024 002)	2	1	1	0	1	1.000	72.22
3	T3 Bacillus subtilis (JU B 2024 001)	2	0	2	2	1	1.400	61.11
4	T4 Trichoderma harzianum (JU F 2024 006)	1	1	0	1	2	1.000	72.22
5	T1 + T2	2	1	2	1	1	1.400	61.11
6	T3 + T4	1	1	2	2	2	1.600	55.56
7	T2 +T3	0	1	1	0	1	0.600	83.33
8	T4 + T1	2	1	2	1	0	1.200	66.67
9	T2 + T4	1	1	1	0	1	0.800	77.78
10	T1 + T3	1	1	1	2	1	1.200	66.67
11	Control (Untreated)	0	0	0	0	0	0.000	100.00
12	Carbendazim 50% (positive control)	0	0	0	1	0	0.200	94.44
13	FO (Negative control)	3	3	4	4	4	3.600	0.00



Figure 6. Biocontrol response against wilt pathogen *Fusarium oxysporum* (NFCCI-3426) on tomato plant prophylactic treatment; **(a)** untreated control, positive control, and FO (negative control), **(b)** control and treated (T2:T3 and T2:T4).

Table 5. Biocontrol of *Fusarium oxysporum* (NFCCI-3426) in Tomato (*Solanum lycopersicum*) through curative treatment

S.No.	Treatment details	R1	R2	R3	R4	R5	Avg.	% PDC over negative control
1	*T1 Pseudomonas fluorescens (JU B 2024 003)*	3	2	2	1	2	2.000	37.50
2	*T2 Bacillus amyloliquefaciens (JU B 2024 002)*	2	3	1	2	3	2.200	31.25
3	*T3 Bacillus subtilis (JU B 2024 001)*	2	3	2	2	3	2.400	25.00
4	*T4 Trichoderma harzianum (JU F 2024 006)*	3	1	2	3	2	2.200	31.25
5	T1 + T2	2	1	2	2	2	1.800	43.75
6	T3 + T4	3	1	2	2	2	2.000	37.50
7	T2 +T3	1	2	2	1	2	1.600	50.00
8	T4 + T1	2	1	3	1	2	1.800	43.75
9	T2 + T4	2	3	1	3	1	2.000	37.50
10	T1 + T3	3	1	1	2	2	1.800	43.75
11	Control (Untreated)	1	0	1	0	0	0.400	87.50
12	Carbendazim 50% (positive control)	1	0	1	2	1	1.000	68.75
13	FO (Negative control)	3	3	4	3	3	3.200	0.00



Figure 7. Biocontrol response against wilt pathogen *Fusarium oxysporum* (NFCCI-3426) on tomato plant through curative treatment; **(a)** untreated control, positive control, and FO (negative control), **(b)** control and treated (T2:T3 and T2:T4).

Treatment T2 *Bacillus amyloliquefaciens* (JUB 2024 002), along with T4 *Trichoderma harzianum* (JUF2024-006) displayed less efficiency, *i.e.*, 37.5% against *Fusarium oxysporum* (NFCCI-3426) as compared to the positive control of Carbendazim 50 % *i.e.*, 68.75 % (Table 5 and Figure 7). The values were recorded periodically at a 7-day interval, and final observation taken after 30 days of prophylactic and curative treatment.

4. Discussion

The cultural and morphological characteristics of the obtained fungal and bacterial cultures were consistent with their known identities. For instance, the growth of *Fusarium oxysporum* (NFCCI-3426) on PDA plates showed characteristic pink-white mycelium with concentric zones of progressive growth, which later turned purplish-pink after 10 days of incubation. This is in line with previous descriptions of *Fusarium oxysporum* cultures. The LCB mount staining revealed segregated hyphal networks and spore structures for fungal cultures. For example, *Fusarium oxysporum* showed germinated vertical and horizontal vegetative hyphae with micro-conidia slightly curved at the end, aligning with previous microscopic descriptions [1, 21].

Similarly, *Bacillus subtilis* and = *Bacillus amyloliquefaciens* exhibited white colonies with dry texture on Nutrient Agar (NA) plates, typical for these species [29]. Gram staining of bacterial cultures confirmed their Gram-positive or Gram-negative nature. Both *Bacillus subtilis* and *Bacillus amyloliquefaciens* retained crystal violet stain, indicating they are gram-positive rods [30]. *Pseudomonas fluorescens* showed white colonies with smooth texture, consistent with its known morphology; further, it retained safranin counterstain, indicating it is gram-negative [3].

The *in-vitro* dual assay demonstrated the antagonistic properties of biocontrol agents against *Fusarium oxysporum*. *Bacillus amyloliquefaciens* (JUB2024-002) showed the highest biocontrol activity against *Fusarium oxysporum*, followed by *Bacillus subtilis* (JUB2024-001) and *Trichoderma harzianum* (JUF2024-006). *Pseudomonas fluorescens* (JUB2024-003) exhibited moderate control compared to the control plate. This finding is supported by previous studies that have shown the efficacy of these biocontrol agents in controlling fungal pathogens. For instance, *Bacillus amyloliquefaciens* has been reported to produce antibiotics and other secondary metabolites that inhibit fungal growth [31, 32]. Similarly, *Trichoderma harzianum* is known for its myco-parasitic activity against various fungal pathogens [6, 33].

The pathogenicity test revealed that soil application of *Fusarium oxysporum* induced more severe symptoms compared to foliar application. This is consistent with the nature of Fusarium wilt disease, which typically enters plants through the roots [34]. The disease severity scores ranged from 3 to 4 in soil-applied treatments, indicating significant damage to the root system and overall plant health.

The prophylactic (preventive) treatment with biocontrol agents significantly reduced the severity of Fusarium wilt disease. *Bacillus amyloliquefaciens* (JUB2024-002) alone or in combination with other biocontrol agents showed high efficacy in controlling the disease. For example, the combination of *Bacillus amyloliquefaciens* and *Bacillus subtilis* achieved an 83% control efficiency, comparable to the positive control using Carbendazim 50% (94.4%). This high efficacy can be attributed to several mechanisms, such as production of antibiotics, competition for nutrients, and induction of systemic resistance in plants [35-37].

Table 6. Changes in recorded disease-response measurements over seven days following foliar and soil inoculation with Fusarium oxysporum (NFCCI-3426), compared with the uninoculated control.

Days	Control	Foliar	Soil
Day 1	0.00	# 0.00 ± 0.00g	0.00 ± 0.00
Day 2	0.00	1.07 ± 0.00f	2.20 ± 0.00
Day 3	0.40	3.00 ± 0.00e	4.02 ± 0.00
Day 4	0.40	3.49 ± 0.00d	5.46 ± 0.00
Day 5	0.40	4.80 ± 0.05c	5.50 ± 0.04
Day 6	0.40	7.27 ± 0.03b	8.54 ± 0.05
Day 7	0.40	9.46 ± 0.03a	11.35 ± 0.08
F6,14		1033.905***	787.218***

Each value is a mean of three replicates ±SE (standard error). This means that a column followed by the same superscript (P>0.05) differs according to Duncan's multiple range test. *** Significant at P<0.001.

Table 7. Comparison of recorded treatment-response measurements under prophylactic and curative applications of individual and combined biocontrol agents against Fusarium wilt in tomato (Solanum lycopersicum L.) cv. Pusa Ruby.

Treatment	Prophylactic application	Curative Application
T1	165.36 ± 0.22h	112.20 ± 0.37e
T2	214.37 ± 0.64e	94.30 ± 0.20f
T3	183.96 ± 0.37g	75.45 ± 0.08g
T4	216.46 ± 0.18e	94.65 ± 0.42f
T1+T2	184.70 ± 0.46g	129.55 ± 0.28d
T3+T4	165.56 ± 0.23h	112.60 ± 0.31e
T2+T3	248.41 ± 0.40c	150.40 ± 0.18c
T1+T4	199.37 ± 0.38f	129.83 ± 0.25d
T2+T4	231.38 ± 0.36d	112.60 ± 0.09e
T1+T3	200.22 ± 0.05f	129.85 ± 0.23d
Untreated control	299.00 ± 0.33a	261.30 ± 0.20a
PC	276.02 ± 1.20b	204.05 ± 0.51b
NC	0.00 ± 0.00i	0.00 ± 0.00h
F12,26	2488.751***	5287.099***

Each value is a mean of three replicates ±SE (standard error). This means that a column followed by the same superscript (P>0.05) differs according to Duncan's multiple range test. *** Significant at P<0.001.

The curative treatments also showed significant control but were less effective than prophylactic treatments, highlighting the importance of preventive measures in disease management.

In the present greenhouse study conducted to evaluate the efficacy of biocontrol application as it is preventive and curative, against fusarium wilt disease on tomato susceptible variety of Pusa Ruby demonstrated the disease severity of fusarium wilt in tomato plants when infection occur through soil containing phyto-pathogen *Fusarium oxysporum* (NFCCI-3426) an outcome in pathogenicity test. *In-vitro* dual assay revealed the presence of antagonistic nature of biocontrol agents of *Bacillus subtilis* (JUB2024-001), *Bacillus amyloliquefaciens* (JUB2024-002), *Pseudomonas fluorescens* (JUB2024-003) and *Trichoderma harzianum* (JUF2024-006).

The prophylactic and curative greenhouse study provided the possible outcome of wilt disease control in tomato plants through the applied dosage of 2 L/ac of biocontrol both as individual treatment and combined treatment. The combined treatment that included *Bacillus amyloliquefaciens* (JUB2024-002)) showed promising results in controlling disease at an applied dosage. The additional disease-observation data and the summary of prophylactic and curative treatments are presented in Tables 6 and 7, respectively.

5. Conclusion

This greenhouse study evaluated the potential of four microbial biocontrol agents to manage Fusarium wilt in the susceptible tomato cultivar Pusa Ruby. The pathogenicity experiment indicated that soil inoculation with Fusarium oxysporum (NFCCI-3426) caused more severe wilting than foliar inoculation, emphasizing the importance of managing soilborne infection. In vitro observations showed antagonistic activity by Bacillus amyloliquefaciens, Bacillus subtilis, Trichoderma harzianum, and Pseudomonas fluorescens, with B. amyloliquefaciens showing the strongest qualitative response. Under greenhouse conditions, preventive applications generally provided greater disease suppression than treatments applied after symptoms developed. The combination of B. amyloliquefaciens (JUB2024-002) and B. subtilis (JUB2024-001) achieved 83.33% disease control in the prophylactic trial and 50.00% in the curative trial, compared with 94.44% and 68.75%, respectively, for the carbendazim positive control. These findings suggest that combining compatible bacterial antagonists may be a useful preventive strategy within an integrated Fusarium wilt management programme. However, the results were obtained under controlled greenhouse conditions and should not be interpreted as evidence of consistent field performance. Further studies should validate the reported formulation rate, examine dose–response relationships and microbial compatibility, and evaluate disease reduction, plant growth, and yield across different soils, seasons, and locations before practical recommendations are established. Through replicated field experiments.

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Yes.

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