Original
ISSN 2413-0516

Using Fungal and Bacterial Bioagent Formula Antagonists to Combat *Fusarium oxysporum* f. sp. *lycopersici* under Greenhouse Conditions

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(Submitted: 10 February 2024 – Revised version received: 30 March 2024 – Accepted: 15 April 2024 – Published online: 26 June 2024)

Abstract
Objectives: This study aimed to test fungal and bacterial bioagents as formula antagonists of *Fusarium oxysporum* f. sp. *lycopersici* under greenhouse conditions.
Methods: The fungal and bacterial formulations' shelf life under different temperatures in growth chamber and greenhouse conditions, as well as their effects on biochemical alterations involved in the induction of plant defense against the causative pathogens, were investigated.
Results: *In-vitro* antagonist screening of bacterial and fungal isolates on radial growth, reduction, and inhibition percentages revealed that *T. harzianum* exhibited 45.6% inhibition, followed by *B. pumilus* with 36.7%. Among the fungal and bacterial bio-agents, the highest effect on disease incidence and efficiency against pathogenic fungus under greenhouse conditions was *B. pumilus* 27 and 69%, respectively followed by *T. harzianum* 33 and 62% when applied before pathogen inoculation. Under greenhouse conditions, bio-fungicides also affected defense enzymes like peroxidase and polyphenol oxidase. The shelf life of the wettable powder formulation of bio-agents under different temperatures was very important factor and established that the most suitable temperature for long-term storage is 25°C for both *B. pumilus* and *T. harzianum*.
Conclusion: Target application of disease control techniques in tomato plants demonstrated the efficacy of *B. pumilus* and *T. harzianum* in reducing Fusarium wilt. These results pave the way for the development of bio-agents that can replace fungicides in agriculture, offering a practical substitute.
Keywords: Biocontrol, seed-borne, soil-borne, wilt diseases

Introduction

The tomato, or *Solanum lycopersicum* L., is also known as *Lycopersicon esculentum* Mill is a staple fruit and vegetable in the human diet. It is grown in fields or as a protected cultural practice in almost every nation. According to Abd-El Kareem et al. (2006)[1], it is among the most significant vegetables in Egypt and other nations. Tomatoes are grown for both food and industrial purposes in Egypt. In 2023, 6.73 million tons of tomatoes were produced there in an area of 150,109 hectares.[2]

Tomato plants can develop serious diseases like root rot and wilt due to a variety of soil-borne fungal pathogens.[3] *Fusarium oxysporum* is a fungal pathogen that infects tomato plants through their roots at all stages of growth, according to El-Khallal (2007).[4] It spreads through seeds, soil, or both. It affects many crop plants, causing necrosis and wilting symptoms that result in large financial losses. Tomato wilt, or *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.), is the primary factor limiting tomato production. Among them, Fusarium wilt is one of the most dangerous illnesses that reduces yield. *Fusarium oxysporum* f. sp. *lycopersici* is the cause of this disease the disease causes a yield loss of 25.14–47.94%. Worldwide, *Fusarium* spp. are well-known soil-borne pathogens that can infect any type of soil. For a considerable amount of time, *Fusarium* species can thrive on organic matter in the soil.

Fungicide treatment is the primary means of controlling such diseases. On the other hand, fungicidal treatments worsen environmental pollution and have negative health effects on people.[5] Furthermore, controlling soil-borne phytopathogenic fungi chemically is expensive and severely harms the beneficial microorganisms that live there. Additionally, it poses a risk to the environment and can trigger several dangerous situations, including the rapid emergence of pathogens resistant to chemicals. All of these facts, combined with the fact that methyl bromide will no longer be used as a soil fumigant worldwide by the year 2015,[6] as well as the fact that other fumigants like carbofuran and metham sodium (metam) allow pathogens to become resistant to chemicals[7] and there is increase need to research non-chemical alternatives.

To provide the nutrients needed to feed the world's population, which is predicted to grow from 7.3 to 9.8 billion people by 2050, new environmentally friendly applications must be found to prevent the spread of plant diseases.[8] Root pathogenic fungus biological control is a sustainable approach that shows promise in managing various agricultural pest issues. It could be accomplished in one of three ways: directly by introducing antagonists that can directly suppress the activity of pathogens; indirectly by adjusting the microbial balance in the soil; or thirdly by inducing, acquiring, or inheriting host resistance.[9] Several microorganisms have been introduced into the soil where pathogens are expected various mechanisms of suppression and ability to use in combination with other biocontrol agents.[10] Because they can produce growth-stimulating factors

or suppress minor pathogens in addition to their antagonistic activity, rhizosphere-competent fungi, and bacteria are potential agents for biocontrol activity.[11] Recent advancements in the commercialization of biological control products have accelerated the application of fungal bioagents and increased their degree of safety and minimal environmental impact.[12] The basis for the natural control of several phytopathogens is the presence of suppressive soils containing a range of biocontrol microorganisms from the genera *Trichoderma, Pseudomonas*, and *Bacillus*.[13] Studies by She-ze et al. (2008)[14] showed the importance of peroxidase and polyphenol oxidase, enzyme activation in biological regulation, and plant resistance to pathogenic invasion.

Compared to gelant or liquid formulations, powder or fine granular carrier systems for biocontrol agents are more practical and work with the farm equipment that is currently in use. Due to their easy handling and low-cost development from agricultural/industrial wastes or by-products, the available powder formulations are less expensive.[15] In the research carried out by Paramasivan et al. (2019)[16], the following carrier materials were used to assess the survival of *T. viride, T. harzianum, P. fluorescens* and *B. subtilis*: talc, gypsum, flyash, talc + gypsum, talc + vermiculate, talc + flyash, talc + neem cake, talc + decomposed coir pith, and talc + farmyard manure (FYM). The findings showed that *T. viride* and *T. harzianum*, followed by neem cake, had the highest population level in talc after 120 days after storage. At the end of the 120th day of storage, the bacterial antagonists of *P. fluorescens* and *B. subtilis* displayed the highest population level in talc, followed by talc + FYM.

Combining the use of two or more biocontrol strains may result in a more effective control strategy since it is expected to more closely resemble the natural environment.[17] Thus, the level and consistency of control are greatly increased by multiple modes of action, a more stable rhizosphere community, and effectiveness over a wider range of environmental conditions.[18] In contrast to the conventional fungicide, Uniform EC (Azoxystrobin + Metalaxyle), the primary objective of this study was to evaluate the biocontrol agents, specifically *T. harzianum, T. viride*, *B. subtilis*, and *B. pumilus*, in a wettable powder formulation as bio-fungicides against the tomato plant disease Fusarium wilt, as well as their effects on biochemical changes that result in the induction of plant defense against the pathogen in greenhouse conditions.

Materials and Methods

Plant Materials

Seeds of tomato (*Lycopersicon esculentum* Mill) Elisa hybrid were obtained from the Department of Vegetables Production Research, Horticultural Research Institute, Agricultural Research Center, Egypt.

Isolation and Identification of the Pathogenic Fungus

From an agricultural field in Qallen, Kafrelshiekh governorate, infected vascular tissues from the stem and root regions of tomato plants exhibiting wilt symptoms were collected separately. Following a three-minute surface sterilization with 3% sodium hypochlorite, tissue bits were sterilized three times using sterile distilled water. After that, they were each placed on a separate potato dextrose agar (PDA) medium and incubated for seven days at 25 ± 2°C. Based on the morphological features of the macroconidia (shape and size), phialids, microconidia, chlamydospores, and colony growth traits, six isolates (F1 to F6) were determined to be *Fusarium oxysporum*.[19,20] An aggressive isolate (F3) was chosen for the current investigation based on the results of the pathogenicity test. Pathogenicity tests were used to determine the forma specials of the causative pathogen.[21] The pathogen was isolated and kept as stock cultures for future research on PDA slants that were kept at 4°C. This was accomplished by transferring the mycelial tip (hyphal tip technique) into new PDA plates.

Pathogenicity Test

The Elisa hybrid tomato seeds were used for the pathogenicity test. The seeds were first sterilized by immersion in 75% ethanol for 7 minutes, then thoroughly rinsed in sterile distilled water.[22] The seeds were then sown in trays (209 holes/tray) and grown at 24–26°C with a 12-hour photoperiod and 70% humidity. After 5 weeks, the plants were transplanted. The medium used was sterilized and contained peat moss (50%) + vermiculite (50%) + NPK (19-19-19) + microelements: 2.5 kg m^{-3}. To verify the pathogenicity of the isolated strain, plants were grown in a greenhouse with daily watering and twice-weekly fertilization using a standard nutrient solution.[23] Five-week-old tomato plants with five or six fully expanded leaves were used in a pathogenicity experiment. At 4°C, the isolates were kept on PDA medium.

According to Singleton et al. (1992)[24] instructions, *F. oxysporum* cultivated on barley grain medium was artificially introduced into the soil. The barley grains were placed in conical flasks and autoclaved at 121°C for 30 minutes after being soaked in a 5% sucrose solution for 12 hours. Three discs of the fungus (1 cm^2) were used to inoculate the flasks, and the barley seeds were manually shaken to distribute the inoculum. The flasks were incubated for 14 days at 25 ± 2°C in a dark, static environment. However, to encourage consistent fungal colonization on barley seeds, the flasks were manually shaken for a few minutes each day. A 5% soil infestation was carried out before seeding and 2.5 kg of autoclaved sandy loam soil was placed into pots measuring 25 cm in diameter. Table 1 presents a summary of the physiochemical properties of the soil experimental site.

A completely randomized experimental design was used to specify five replicate pots for each treatment to test the pathogenicity of each of the six isolates of *F. oxysporum* on tomato plants. The following treatments were a part of the experiment: 1) Soil treated with *F. oxysporum* alone; 2) Soil not infested (control treatment). After four weeks of seedling transplantation, the percentage of infection and healthy survivals in each treatment was calculated using the following formula, which was published by El-Helaly et al. in 1970:[25]

$$\textbf{Infection}\ (\%) = \frac{\text{Total No. of infected seedling}}{\text{Total No. of sown seedling}} x\ 100$$

$$\textbf{Plant survival}\ (\%) = \frac{\text{Total No. of healthy seedling}}{\text{Total No. of sown seedling}} x\ 100$$

The pathogenicity experiment was done to confirm Koch's Postulates. Then, the most virulent isolate was selected for further experiments.

Table 1. **Physico-chemical properties of the soil sample used (sandy loam soil)**

Mechanical properties	**(%)**		
Sand	70.70		
Silt	2.30		
Clay	27.00		
Chemical properties			
Organic carbon %	0.143		
Total nitrogen %	0.020		
Water holding capacity (%)	25.00		
pH	7.75		
EC0	1.150		
$CaCO_3$%	0.600		
Anions and cations	**(meq/L)**	**Anions and cations**	**(meq/L)**
Carbonate	Trace	Calcium	0.64
Bicarbonate	1.13	Magnesium	0.11
Chloride	0.20	Sodium	0.29
Sulphate	0.07	Potassium	1.13

Biocontrol Bacterial Isolation

Using a series dilution technique, bacteria were isolated from the rhizosphere area and root sections of tomato plants. The bacteria were then plated on a variety of general and selective media, including nutrient agar (NA) and King's B medium, to aid in their recovery. The rod-shaped gram-positive colonies were chosen for screening against necrotrophic fungal pathogens by incubating plates at 28 ± 2°C. Based on gelatin liquefaction, arginine dihydrolase, nitrate reduction, and the utilization of different carbon sources, those that were active were further identified to the species level and identified as *Bacillus subtilis* and *B. pumilus*.[26,27] On KMB agar slants, the cultures were kept at 4°C.

Source of Biocontrol Fungal Isolates

Numerous isolates and biocontrol isolates of *T. viride* and *T. harzianum*, among other fungi with known biocontrol activity against soil-borne fungal pathogens, were taken from the Central Laboratory of Bio-Agriculture collection of the Agricultural Research Center in Giza, Egypt. On potato dextrose agar slants, fungal isolates were kept at 4°C.

In-vitro Screening of Antagonist Fungal and Bacterial Isolates

Using dual culture methods, the antagonistic behavior of fungi was assessed against *F. oxysporum* f. sp. *lycopersici in vitro*. Using the reverse side of sterile micropipette tips, five mm mycelial discs of *F. oxysporum* f. sp. *lycopersici* and antagonist fungi, *T. harzianum* and *T. viride*, were cut from the edge of a three-day-old culture. On one side of the plates, one disc containing each antagonist and a disc containing *F. oxysporum* f. sp. *lycopersici* were placed on the solidified PDA medium in opposition to each antagonist. At 28 ± 2°C, plates were incubated. Cells cultivated on NA plates were suspended to create bacterial isolate suspensions. The bacterial suspensions were adjusted to 10^8 cfu/ml. According to Suparman et al. (2002),[28] the bacterial isolate's antagonistic activity against *F. oxysporum* f. sp. *lycopersici* was assessed using the dual culture method as follows: A 9 mm sterile petri dish was filled with 15 ml of PDA medium. A 5 mm diameter mycelial plug of *F. oxysporum* f. sp. *lycopersici* of 4 days old culture was inoculated at the center of the plate. Potential antagonistic bacteria were streaked 3 cm apart from the *F. oxysporum* f. sp. *lycopersici* inoculum and plates were incubated at 28 ± 2°C. Three independent replicates were used. Dishes inoculated centrally with sterilized distilled water (SDW) were used as control.

After 7 days of incubation, the tested pathogen's mycelial radial growth (mm) in treated and untreated control plates was measured. The reduction (%) and inhibition (%) of the causative pathogens were computed using the formula provided by Hmouni et al. (1996).[29]

$$\textbf{Reduction } (\%) = \frac{\text{The mycelial radial growth of the tested pathogen in treated plates}}{\text{The mycelial radial growth of the tested pathogen in nontreated plates}} x\ 100$$

$$\textbf{Inhibition } (\%) = \frac{\left[\text{Growth of pathogen fungus (control)} - \text{Growth of antagonistic agent (treatment)}\right]}{\text{Growth of pathogen fungus (control)}} x\ 100$$

Then, the most bio-aggressive fungal and bacterial isolates were selected for the produce mass/stock culture in a low cost recipe as well as dried wettable powder formulation (bio-fungicide) against the virulent isolate, which identified as *F. oxysporum* f. sp. *lycopersici*.

Creating a Formulation of Biocontrol Agents using Talc

Following the addition of a loop containing *B. pumilus* to the nutrient broth, the mixture was incubated at room temperature (28 ± 2°C) for 72 hours using a rotary shaker set to

150 rpm. After being incubated for 72 hours, the broth containing 9 x 10^8 cfu/ml was used to prepare the talc-based formulation. Using the procedure outlined by Vidhyasekaran and Muthamilan (1999),[30] 400 ml of bacterial suspension, 1 kg of talc powder, 15 g of calcium carbonate (to bring the pH to neutral), and 10 g of carboxy-methyl cellulose (CMC) (as adhesive) were combined under sterile conditions. After the products were shade-dried to a moisture content of 20%, they were sealed and placed inside polypropylene bags. In 250 ml conical flasks, 100 ml of molasses yeast medium was filled with a 9 mm mycelial disc of *T. harzianum*, which was then incubated for 14 days at room temperature. Talc powder and the mycelial mat were combined in a 1:2 ratio after the mat was homogenized. One kilogram of talc and five grams of CMC were combined thoroughly. The materials were heat-sealed, stored at room temperature, and shade-dried before being placed in polypropylene bags.[31]

Efficacy of Bacterial and Fungal Formulations under In-Vitro and In-Vivo Conditions

Inoculum Preparation of Pathogenic Fungi

Pure culture of the more virulent isolate of *F. oxysporum* f. sp. *lycopersici* was subculture on PDA in Petri-Dishes and then incubated the subculture media at 28°C for 7 days, then kept at 4°C till use in the following experiment.

The fungal isolate of *F. oxysporum* f. sp. *lycopersici* was mass cultured on barley grain medium as mentioned before.

A known weight of fungus-colonized barley seeds was added to the sandy loam soil, in 25 cm diameter earthen pots containing 2.5 kg autoclaved soil and compost (3:1). The pots were drenching with water for 10 days for activating the pathogen inoculum till the initializing the experiment under greenhouse condition.

Seed Load of Bio-control Agents and Experiment Design

The formulation (10 weeks of storage at 25°C) was applied to the tomato Elisa hybrid seeds at a rate of 10 g/kg seeds in order to assess the effectiveness of the delivery system to transfer the bio-control agents through seeds. To help the bio-control agent stick to the seeds, a 5% molasses solution was first applied to them. The seeds were then treated with the previously prepared bio-fungicide formula. After thoroughly mixing the seeds to ensure that the bio-control formula was distributed evenly, the pre-coated seeds were allowed to air dry aseptically in the shade for two to three hours.

Another treatment by infested tomato seeds Elisa hybrid with spore suspension (5.0×10^6 spore/ml) of more aggressive isolates of *F. oxysporum* f. sp. *lycopersici* before and after being coated with bio-fungicide.

Chemical treatment of tomato seeds by chemical fungicide Uniform EC (Active ingredient Azoxystrobin + Metalaxyle, at recommended dose 2 $g.kg^{-1}$ seeds.

Effect on Seed Quality Variables in-vitro

Using eight replicates of fifty seeds each, the paper towel method (Kim et al. 2010)[32] was used to test for germination. On a paper towel that had been previously soaked in distilled water, fifty tomato seeds were spread out equally, and another paper towel was placed on top of the first. To keep the paper towels from drying out, they were rolled up and wrapped in polythene before being incubated for ten days at 28 ± 2°C in an incubation chamber. After incubation, paper towels were unrolled, and the quantity of seeds that germinated was counted. For each seedling, the length of the shoot and the root were measured to determine vigor. The vigor index (VI) was calculated using the formula:

$$\mathbf{VI} = [(\text{Mean of root length}) + (\text{Mean shoot length})] \times \text{Germination (\%)}.$$

Effect on Disease Incidence and Defense Enzymes in-vivo

The field experiments were conducted at the greenhouse of the Plant Pathology Research Institute, Agriculture Research Center, Egypt to evaluate the WP formula of active bio-agents by dissolving 20 g/1.0 L water and using as a soil drench At thirty days old, seedlings were planted in a nursery bed, moved, and then mulched. Ten days following transplanting, 20 g L^{-1} of formulation was applied to the soil. For comparison, uniform was applied as a seed treatment at a rate of 2 g kg^{-1} seed and as a soil application at a rate of 2 g pot^{-1} 10 days after transplanting. Included were seeds treated with a wettable powder bio-fungicide formulation at 10 g kg^{-1} seeds, followed by a soil application at 20 g L^{-1} water. The control group consisted of untreated seeds (negative control). Three replicates and a randomized block design were used to set up the experiments. The incidence of the disease was documented as stated.

Thirty-day-old seedlings were transplanted into earthen pots (diameter: 0.25 m) filled with potting soil (5 seedlings/pot). Following the transplant, 50 mL of conidial suspension (1000 microconidia mL^{-1}) of *F. oxysporum* f. sp. *lycopersici* was inoculated per pot seven days after the soil application of bio-fungicide, which consisted of 10 mL/pot of bacterial bio-fungicide suspension (10^8 CFU/mL^{-1}) and 10^6 spore/mL of fungal bio-fungicide. According to Ramamoorthy (2002),[33] wilt incidence was calculated 30 days following transplantation using the following formula:

$$(\%)\ \textbf{Disease Incidence} = \frac{\text{No. of infected plants}}{\text{Total no. of plants}} x\ 100$$

Every treatment had three replicates, and the pots were arranged in a randomized manner.

Collecting Samples for Biochemical Testing

Thirty days after the pathogen inoculation and/or zero time of experiment initialization were the dates on which plants were carefully uprooted (To enable uprooting with maximum root recovery, pots were submerged in water. pots were flooded with water to facilitate uprooting with maximum recovery of roots) without causing any damage to root system. To get rid of any remaining soil particles, the roots were washed with water. The treatments were predetermined by the experimental design, and four plants were separately sampled from each treatment replication and stored for biochemical analysis. Fresh roots were cleaned under running tap water and then homogenized with liquid nitrogen using a pre-chilled mortar and pestle. The homogenized root tissues were kept until they were needed for biochemical analysis in a deep freezer at –70°C.

Assessment of Defense Enzymes

Peroxidase Activity Assay

At 4°C, 1.0 g of root samples were homogenized in 2 mL of 0.1 M phosphate buffer with a pH of 7.0. Centrifuging the homogenate after for 15 minutes at 16,000 g at 4°C, the supernatant was utilized as an enzyme source. 1.5 mL of 0.05 mM pyrogallol, 0.5 mL of enzyme extract, and 0.5 mL of 1% H_2O_2 made up the reaction mixture. The reaction blend was kept under room temperature conditions (28 ± 2°C). For three minutes, every 30 seconds, the absorbance at 420 nm was recorded. The enzyme activity was expressed by changes in the absorbance $min^{-1}mg^{-1}$ protein.[34]

Polyphenol Oxidase Activity Assay

The methodology described by Mayer et al. (1965)[35] was used to measure the polyphenol oxidase (PPO) activity. Following homogenization of 1.0 g of root samples in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5), the samples were centrifuged at 16,000 g for 15 minutes at 4°C. The source of the enzyme was the supernatant. 200 µl of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) made up the reaction mixture. Then, 200 µl of 0.01M catechol was added to initiate the reaction, and the activity was measured as changes in absorbance at 495 nm $min^{-1}mg^{-1}$ protein.

Viability of Bioagent Spores and Cells in Stored Wettable Powder Formulation

The viability of *Trichoderma* spores and Bacillus cells in a wettable powder formulation complex stored at 10, 15, 20, and 25°C were determined at zero time, 8, 16 and 24 weeks of storage. One gram of the preparation was dissolved in 100 milliliters of aseptic sterilized distilled water for every temperature treatment at every interval. Five days of incubation at 25°C were spent spreading one milliliter of the resulting suspension over the PDA medium in plates. Four copies were taken into account. Visible colonies developed were counted and converted to denote the number of spores and cells in one gram of wettable powder formulation.

Data Analysis and Statistics

The data were analyzed using ANOVA, and the significant mean differences between treatments were separated using Duncan's Multiple Range Test.[36] According to Snedecor and Cochran's (1980)[37] instructions, the means were compared using the least significant differences (LSD) at $P \geq 0.05$.

Results

Evaluation of the most Virulent F. oxysporum Isolates under Greenhouse Conditions

Six isolates of the pathogenic fungus (F1 to F6) were identified as *Fusarium oxysporium* based on morphological characteristics of the macroconidia (shape and size), phialids, microconidia, chlamydospores, and colony growth traits. Based on the pathogenicity tests, an aggressive isolate (F3) was selected for the present study. The forma specials of the causal pathogen were identified using pathogenicity tests on tomato, pepper, eggplant, cucumber and common bean plants. The causal agent only infected tomato plants. Six isolates of *Fusarium oxysporum* were evaluated (as soil infestation) to select the most aggressiveness one caused vascular wilt using tomato Elisa hybrid plants, in addition using inocula-free seeds as comparison control.

Table 2 displays data indicating that tomato seedlings infested with *F. oxysporum* (isolate F3) have a significantly higher damping-off infection (60%) and a significantly lower survival rate (40%) compared to the 100% untreated control.

Examination of the Antagonistic Properties of Bacterial and Fungal Strains in vitro

On Petri dishes containing PDA medium, the antagonistic effects of strains of *T. harzianum, T. viride, B. subtilis* and *B. pumilus* against *F. oxysporum* f. sp. *lycopersici* were assessed. Table 3 demonstrates that the bio-agent strains were successful in stopping *F. oxysporum* f. sp. *lycopersici* radial growth.

Trichoderma harzianum was more active than *T. viride* for reducing the radial growth of *F. oxysporum* f. sp. *lycopersici* being 4.9 and 6.2 cm respectively. Moreover, *B. pumilus* inhibited the over growth of *F. oxysporum* f. sp. *lycopersici*, comparing with *B. subtilis*. In comparison to the control, the growth percentage of the *B. pumilus* and *B. subtilis* strains was decreased by 36.7 and 33.3%, respectively (Table 3). This behavior is a crucial strategy for managing tomato plant vascular welt disease. The strains utilized have the potential to secrete hydrolytic enzymes or metabolites that are antifungal.

Table 2. **Evaluation of the most virulent *F. oxysporum* isolates individually against tomato seedling infection and survival (%) under greenhouse conditions**

Treatment (Infested isolate)	Infection (%)	Survival seedling (%)
Control (Non-infested)	00.0	100.0^{a}
F1	49.7	50.3^{d}
F2	39.5	60.5^{c}
F3	60.0	40.0^{e}
F4	16.5	83.5^{b}
F5	33.8	66.2^{c}
F6	19.3	80.7^{b}
L.S.D. (0.05)		***6.31

The results are the mean of five replicates, Values within a column followed by different alphabets are significantly different at $P \leq 0.05$.

Table 3. ***In-vitro* screening of antagonist fungal and bacterial isolates on the radial growth, reduction (%) and inhibition (%) as bio-agents against *F. oxysporum* f. sp. *lycopersici***

Treatment (Bioagent strain)	Radial growth (cm)	Inhibition (%)
Control (Pathogen only)	9.0^{a}	0.0^{d}
T. harzianum	4.9^{c}	45.6^{a}
T. viride	6.2^{b}	31.1^{c}
Bacillus subtilis	6.0^{b}	33.3^{c}
Bacillus pumilus	5.7^{b}	36.7^{b}

Each value represents the mean of three replicates, Values within a column followed by different alphabets are significantly different at $P \leq 0.05$.

Efficacy of Bio-fungicide Formulations

Effect on Seed Quality Variables in-vitro

The plants' root and/or shoot growth, germination percentage, and vigor as seedlings were all enhanced by treatment with a biofungicide. *T. harzianum* and *B. pumilus* isolates were chosen for formulation experiments based on the findings of the antagonism study and the impact of biofungicide formulation on seed quality (Table 4). In comparison with the treatment in control, the tomato seed germination and seedling vigor were effectively increased by the biofungicide isolates under evaluation. Before and after pathogen inoculation, treated seeds treated with *B. pumilus* formulation showed a significant increase in germination of up to 90.00 and 92.67%, respectively. 90.67 and 88.33%, respectively, of the positive and negative controls germinated. In a similar vein, seedlings treated with *B. pumilus* isolate formulation had the highest Vigor index values (VI = 2038.74) when compared to the positive control (VI = 1752.65).

Under greenhouse conditions, it was discovered that the sawdust-soil molasses mixture-based formulation of *B. pumilus* was efficient for the control's seed treatment effective for seed treatment on the control of Fusarium wilt of tomato, increasing seed quality variables upon seed treatment. This might have resulted from their capacity to suppress *F. oxysporum* growth in the rhizosphere, as demonstrated by the results of the *in-vitro* antagonist assay.

Effect on Disease Incidence and Defense Enzymes In-Vivo

Plants treated with biofungicide formulations under field conditions showed protection against wilt, particularly the sawdust–soil molasses mixture based formulation containing *T. harzianum* and *B. pumilus*. Tomato plants were not protected against wilt by talc powder alone (Tables 5 and 6).

Viability of T. harzianum and B. pumilus in Wettable Powder Preparation

Trichoderma harzianum and *B. pumilus* prepared in the form of wettable powder stored at 10, 15, 20, and 25°C for 24 weeks, were allowed to grow again on PDB and NB, respectively. Recorded spore numbers of *T. harzianum* at 10°C at zero time, 8, 16 and 24 weeks' storage were 8.2, 4.7, 3.5 and 2.4 x 10^6 spore/g, respectively. On the other hand, at 25°C was recorded 8.2, 5.5, 4.6 and 3.5 x 10^6 cfu/g at zero time, 8, 16 and 24 weeks respectively (Fig. 1). Similar trend could be observed for bacteria stored at the tested temperatures. Moreover, *B. pumilus* at the same inoculum density gave the same general trend of decrease by time up to 24 weeks. *B. pumilus* at 10°C at zero time, 8, 16 and 24 weeks' storage were 6.4, 4.5, 3.2 and 2.2 x 10^8 cfu/g, respectively, while, it was observed that the increase in temperature led to an increase in the number of cells in *B. pumilus*. Where it was found at the temperature 25°C 6.4, 5.1, 4.0 and 3.2 x 10^8 cfu/g, at 10, 15, 20, and 25°C for 24 weeks, respectively (Fig. 1).

Discussion

This study has demonstrated the efficacy of *B. pumilus* and *T. harzianum* in greenhouse conditions for the management of *F. oxysporum* f. sp. *lycopersis* related diseases. It's crucial to remember that *T. harzianum* and *B. pumilus* symbionts may have different symbiotic associations that are more successful at boosting resistance or tolerance. They most likely use a variety of different mechanisms to function. *B. pumilus* controls competition to provide protection. The total amount of *B. pumilus* control in the plants was significantly increased by the presence of fungi and *T. harzianum*. Moreover, a higher intensity of *B. pumilus* was caused by the pathogens and *T. harzianum*. According to a study, AMF eliminates potentially harmful strains or species by controlling the competitive balance.

Getha et al. (2005)[38] found that *T, harzianum* and *B. subtilis* were potent antagonists against *F. oxysporum*, and they reported similar outcomes. The main root colonizers are plant growth-promoting rhizobacteria (PGPR), like *Bacillus* sp.[39] PGPR can also strengthen plant defenses.[40] Numerous mechanisms have been documented to explain their implementation, including the synthesis of antibiotics, the development of plants that produce indole acetic acid (IAA), competition for space and nutrients, the inactivation of pathogen enzymes like protease, the induction of resistance, and the improvement of root systems.[41] Additionally, several investigations have been conducted on the genus *Trichoderma* spp. in relation to biological control against numerous pathogens, including *F. culmorum, Fusarium oxysporum*, and *Pythium aphanidermatum*.[42]

Table 4. **Effect of biofungicide formulations against *Fusarium* wilt of tomato on seed quality variables (*in-vitro*)**

Treatment (Bioagent isolate formulation)	Germination (%)	Root length (cm)	Shoot length (cm)	Vigor index (VI)
Un-treated control (Negative)	88.33^{a,b}	5.67^{b}	14.33^{b}	1174.95^{d}
Control (Pathogen only)	75.67^{c}	4.00^{c}	8.67^{d}	958.74^{e}
Chemical control (Positive)	90.67^{a}	6.00^{a}	13.33^{b}	1752.65^{b}
T. harzianum (Before artificial inoculation)	89.67^{a}	5.33^{b}	14.33^{b}	1762.91^{b}
T. harzianum (After artificial inoculation)	84.67^{b}	5.33^{b}	11.00^{c}	1382.66^{c}
Bacillus pumilus (Before artificial inoculation)	92.67^{a}	6.33^{a}	15.67^{a}	2038.74^{a}
Bacillus pumilus (After artificial inoculation)	90.00^{a}	5.67^{b}	13.67^{b}	1740.60^{b}
L.S.D. $_{(0.05)}$	**3.721**	**0.325**	**1.141**	**56.231**

Each value represents the mean of three replicates, Values within a column followed by different alphabets are significantly different at $P \leq 0.05$.

Table 5. **Effect on disease incidence as affected by bio-fungicide against *Fusarium* wilt of tomato under greenhouse conditions (*in-vivo*)**

Treatment (Bioagent isolate formulation)	Disease incidence (%)	Efficiency (%)
Un-treated control (Negative)	20[e]	–
Control (Pathogen only)	87[a]	–
Chemical control (Positive)	20[e]	77
T. harzianum (Before artificial inoculation)	33[c]	62
T. harzianum (After artificial inoculation)	40[b]	54
Bacillus pumilus (Before artificial inoculation)	27[d]	69
Bacillus pumilus (After artificial inoculation)	33[c]	62
L.S.D. $_{(0.05)}$	**3.548**	

Each value represents the mean of three replicates, Values within a column followed by different alphabets are significantly different at $P \leq 0.05$.

Table 6. **Effect of defense enzymes as affected by bio-fungicide against *Fusarium* wilt of tomato under greenhouse conditions (*in-vivo*)**

Treatment (Bioagent isolate formulation)	Enzyme assessment (min^{-1} mg^{-1} protein)	
	Peroxidase	Polyphenol Oxidase
Un-treated Control (Negative)	0.204	0.050
Control (Pathogen only)	0.104	0.048
Chemical control (Positive)	0.233	0.053
T. harzianum (Before artificial inoculation)	0.312	0.163
T. harzianum (After artificial inoculation)	0.209	0.100
Bacillus pumilus (Before artificial inoculation)	0.339	0.178
Bacillus pumilus (After artificial inoculation)	0.220	0.101
L.S.D. $_{(0.05)}$	**0.092**	**0.034**

The present findings align with the findings of Montealegre et al. (2005)[43], who discovered the presence of chitinase and β 1,3 glucanase in supernatants derived from *Trichoderma* spp. Furthermore, studies by Montealegre et al. (2005)[43] showed that the cell free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, spore germination, and germ-tube length of *F. oxysporum*. Furthermore, *B. subtilis* has been shown by Alippi and Monaco (1994)[44] to secrete a number of antifungal metabolites that have an inhibitory effect on certain fungal pathogens, including subtilin, bacitracin, bacillin, and bacillomycin.

The study conducted by Vinale et al. (2014)[45] concentrated on the primary and secondary metabolites that are produced by certain beneficial fungal species, like *Trichoderma* spp., and their function in both plants and pathogens. Long before

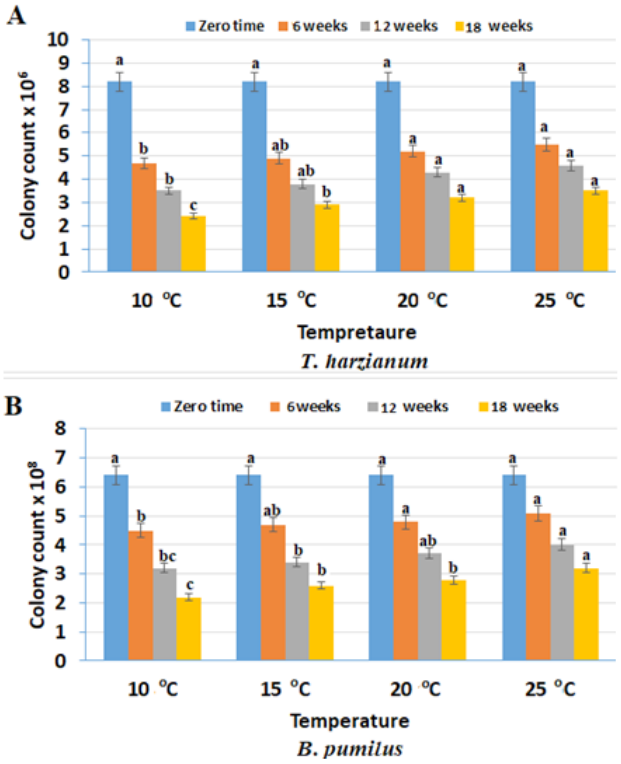

Fig. 1 **Effect of storage period on *T. harzianum* and *B. pumilus* in wettable powder at different temperatures.**

lovastatin and compact in (antihypercholesterolemic agents), cephalosporin and penicillin, and the immunosuppressant cyclosporine were discovered, fungal secondary metabolites were employed in human medicine.[46] The majority of other bacteria and fungi are poisoned by these types of secondary metabolites. Numerous SMs that were isolated, purified, and produced from *Trichoderma* species demonstrated a notable antifungal effect.

This is consistent with the findings of Jaderson and Park (2020)[47], who claimed that temperature and other storage conditions have a significant impact on the growth of fungi. Furthermore, if stored at the right temperature, the fungi's survival may be prolonged.

Furthermore, Kumar et al. (2013)[48] demonstrated that talk powder and charcoal-based formulations retained a sufficient number of viable *T. viride* spores after 120 days of storage. Additionally, Sarode et al. (1998)[49] used FYM, peat soil, charcoal powder, talc powder, and neem powder as carriers among five different substrates. For the longest period of time, the best carriers for *Trichoderma* spp. were talc, FYM, and charcoal. The survival of *T. viride, T. harzianum, P. fluorescens,* and *B. subtilis* was evaluated in the studies of Paramasivan et al. (2019)[16] using the following carrier materials: talc, gypsum, vermiculate, flyash, talc + gypsum, talc + flyash, talc + neem cake, talc + decomposed coir pith, and talc + farmyard manure (FYM).

At the end of the 120th day of storage, the bacterial antagonists of *P. fluorescens* and *B. subtilis* displayed the highest population level in talc, followed by talc + FYM. To guard against a range of crop diseases, farming systems have applied *Bacillus* species, such as *B. subtilis*, *B. amyloliquefaciens*, *B. laterosporus,* and *B. cereus*, which are all environmentally friendly bacteria and frequently used as biocontrol agents in commercial forms. The pathogens' competition for

nutrients and space, the pathogens' bacteriolysis, the induction of plant resistance leading to enhanced plant growth, and the secondary metabolites functioning as antibiotic compounds are just a few of the numerous biological control mechanisms of *Bacillus* that have been identified.[50] The study has shown that, even after being stored for two to twelve weeks at room temperature or at 25°C, the biocontrol agents in the formulation that were made using the novel technique of incubating a portion of the stock culture of biocontrol fungi and bacteria (sawdust–soil molasses mixture) remained viable without any appreciable microbial contamination. According to Khan et al. (2011)[51], the formulations effectively controlled Fusarium wilt and root-knot on tomato seeds when applied at a rate of 10 g/kg.

Antimicrobial proteins produced by Bacillus, including fengycin, surfactin, and iturin, can effectively fend against infections that cause wheat head blight and stop the growth and accumulation of toxins associated with the condition.[52] Applying two or more biocontrol strains together is likely to more closely resemble the natural environment and, as a result, could be a more effective control method. Both *Trichoderma* and *Bacillus* species are well-known biocontrol agents. However, they differ in a few ways that allow them to work together as biocontrol agents against pathogens. For example, *Trichoderma* has a stronger mycoparasitism and resistance induction than *Bacillus* species. Bacillus, on the other hand, is more resilient to stress and has a stronger antibiotic effect. In this instance, using both microbes together is an optional way to improve the biocontrol effect by utilizing their benefits and synergistic effects.

Conclusion

According to the results of this study, wilt in tomato plants caused by *F. oxysporum* f. sp. *lycopersici* could be considerably reduced by using bioagents such as *B. pumilus* and *T. harzianum* in comparison to a control group. On the other hand, defense related enzyme upregulation (PPO and POX) may be responsible for the bioagent. Additionally, the bio agent extended the wettable powder formulation's shelf life at various temperatures, which was a crucial finding that determined the ideal temperature for long-term storage.

Conflict of Interest

None.

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