

Microbial Antagonists for the Management of Soil-Borne Plant Diseases

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ABSTRACT

The effects of microbial antagonists against soil-borne *Fusarium oxysporum* f. sp. *lycopersici* of tomato crop (*Solanum lycopersicum* L.) were investigated through laboratory and greenhouse tests. Samples of soil were taken from infected cropland and the fungal pathogen was isolated by the normal soil-dilution and soil-culture methods on specific culture media. *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* were considered as candidate antagonistic microorganisms and pathogens were inhibited by dual-culture assays, with percentage inhibition of radial growth (PIRG) of each isolate being calculated. The most effective antagonist(s) were then used individually and in combination in a pathogen-inoculated soil and in a healthy soil without pathogen (pathogen-inoculated soil used as control) in a greenhouse experiment, which was designed as a completely randomized design with six treatments and four replications. The incidence of disease, severity of disease, development of pathogens, proportion of plants surviving and growth parameters were measured during the trial. Analysis of variance (ANOVA) was used to analyze data and Tukey's test was employed at $p \leq 0.05$ to separate means of treatments. All three antagonists inhibited radial growth of the pathogen in dual-culture assays and the best PIRG was obtained with *Trichoderma harzianum*. The combined microbial treatment in the greenhouse trial resulted in the lowest disease incidence and severity, and the highest survival rate of the plants, when compared with the pathogen-inoculated control and all the other antagonist treatments, and was significantly better in all growth parameters. The results of this study show that microbial antagonists, especially when used as a consortium, can be used as an effective and environment friendly management solution for soil borne plant diseases.

Keywords: microbial antagonists; biological control; soil-borne pathogens; *Trichoderma harzianum*; dual-culture assay; *Fusarium oxysporum*; disease suppression

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INTRODUCTION

Soil-borne plant pathogens such as the *Fusaria*, *Rhizoctonia*, *Sclerotium* and *Pythia* are some of the most virulent and poorly controlled restrictions to crop production throughout the world, resulting in wilts, root rots and damping-off disease (Weller, 2007). Though chemical fungicides are the traditional means of controlling these pathogens, there are concerns about the resistance of pathogens, build-up of chemical residue in the soil, non-target toxicity and disruption of beneficial soil microbial communities which has led to the emergence of interest in biological alternatives (Syed Ab Rahman et al., 2018).

Microbiological control using microbial antagonists have proven to be a potential and eco-friendly approach for control of soil borne diseases (Pal & Gardener, 2006). Antagonistic microorganisms

have five interrelated mechanisms to control plant pathogens, which are mycoparasitism, competition for nutrients and infection sites, antibiosis (production of secondary metabolites and lytic enzymes), and induction of systemic resistance in the plant (Compant et al., 2005; Köhl et al., 2019). Several fungal antagonists of the genus *Trichoderma* have been widely studied and commercially applied for biocontrol of fungal diseases, because they are quickly growing, they are mycoparasitic and have shown the ability of aggressive colonization of the rhizosphere (Harman et al., 2004; Benítez et al., 2004).

Bacterial antagonists, such as *Bacillus* spp. and *Pseudomonas* spp., are also an important component of the suppression of soil-borne diseases. *Bacillus* species are known to produce a wide variety of lipopeptide antimicrobials with potent anti-fungal properties against a variety of soil borne pathogens like surfactins, iturins, fengycins, etc. (Ongena & Jacques, 2008; Chowdhury et al., 2015). In addition to inducing systemic resistance in host plants (Haas & Défago, 2005; Weller, 2007), fluorescent *Pseudomonads* suppress pathogens mainly by iron competition through their production of siderophores (Haas & Défago, 2005; Weller, 2007) and by the production of antifungal metabolites like 2,4-diacetylphloroglucinol (Défago & Haas, 2004).

Dual-culture assays are the most commonly used in vitro assay method for preliminary screening of candidate antagonists and this is done by quantifying the antagonistic potential by computing the percentage inhibition of radial growth (PIRG) of the target pathogen (Sundaramoorthy & Balabaskar, 2013). Although some antagonists are quite effective when used alone, a few studies indicate that using combinations or consortiums of different antagonists could yield better and more consistent disease control because of their different modes of action (Sarma et al., 2015; Jain et al., 2012).

Although there is a significant amount of literature on individual biocontrol agents, there are still relatively few comparative studies that combine in vitro screening with screening in the greenhouse of both single and combined antagonist treatments (Woo et al., 2014). This linkage is fundamental for the ability to convert laboratory data of antagonistic potential into field disease management outcomes (Fravel, 2005). To generate evidence for their integration to sustainable soil-borne disease management programs, a dual-culture screening and a pot test were therefore combined to test the efficacy of the three bacterial isolates, *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens*, against the *Fusarium* wilt of tomato (Bonaterra et al., 2022).

LITERATURE REVIEW

Trichoderma species are known to be one of the most promising fungal biocontrol agents against soil-borne pathogens, and have been extensively studied. *Trichoderma* species have been described by Harman et al., (2004) as opportunistic, avirulent plant symbionts, which can colonize root surfaces, as well as parasitize and antagonize pathogenic fungi in the surrounding soil. Higher biocontrol efficacy of *Trichoderma* species is attributed to their various mechanisms, according to Howell (2003), which include mycoparasitism, antibiosis and competition. Vinale et al., (2008) also discussed the interactions between *Trichoderma*, plants and pathogens, showing that the metabolites produced by *Trichoderma* inhibit the growth of the pathogens directly and activate defense responses in plants, thus contributing to the protection of both plants and pathogens.

The effectiveness of *Trichoderma* against certain pathogens has been demonstrated in many dual-culture and greenhouse trials. The S. Sundaramoorthy and Balabaskar (2013) tested the biocontrol potential of *Trichoderma* species against *Fusarium* wilt of tomato in in vitro and pot conditions and found that the percentage inhibition of radial growth exceeded 60% at in vitro and 48% at pot condition. In a study, Naeimi et al., (2010) also reported that the *Trichoderma* strains exhibited good

inhibitory effect against *Rhizoctonia solani*, which they attributed to production of mycoparasitism and cell wall-degrading enzymes. The combined applications of *Trichoderma* isolates showed significant reductions in *Pythium* damping-off and root rot of tomato over single isolate application, indicating the importance of using multiple isolate combinations as antagonists (Elshahawy and El-Mohamedy, 2019).

Bacterial antagonists have been similarly well studied. Compant et al., (2005) reviewed the use of plant growth promoting bacteria as biocontrol agents and found that *Bacillus* and *Pseudomonas* species can directly inhibit soil borne pathogens as well as induce a systemic resistance, and therefore they were effective against a wide range of soil-borne pathogens. *Bacillus* lipopeptides are described as versatile biocontrol weapons by Ongena and Jacques (2008) who reported that surfactin, iturin and fengycin families have complementary antifungal and membrane-disrupting activities. The ability of *B. amyloliquefaciens* to possess biocontrol activity of plant diseases was attributed to the rhizosphere competence and the production of antibiotics, as reported by Chowdhury et al., (2015). From the tomato rhizosphere soil, Jangir et al., (2018) isolated *Bacillus* strains and showed that antibiosis and competitive root colonization are two mechanisms of significant suppression of *Fusarium oxysporum* f. sp. *lycopersici*.

Fluorescent *Pseudomonas* species have also been shown to be good antagonists of soil-borne pathogens. Haas and Défago (2005) have reviewed the biological control of bacteria by fluorescent pseudomonads, and found antibiotic production, competition for nutrients through siderophores and induced systemic resistance to be the key mechanisms of suppression. Weller (2007) summarized the research on *Pseudomonas* based biocontrol over the last 30 years, and found colonization competence in the rhizosphere was the best predictor of consistent field performance under a variety of soil types. Shafi et al., 2017, expanded this conversation to all the *Bacillus* species, as they found that the combination of antibiotics with enzymatic activity helped to control several genera of pathogens at once.

Combination/consortium based antagonist applications have gained more research interest as a means of disease control uniformity. Under the same conditions, the microbial consortium reprogrammed the defense network of pea plants to provide greater tolerance of *Sclerotinia sclerotiorum* than did single-strain inoculation, reported Jain et al., (2012). Sarma et al., (2015) took a look at the plant defence mechanisms against pathogens by microbial consortiums and concluded that multi-trophic effects of co-application of antagonists would decrease the escape of pathogens and enhance the lasting activity of disease suppression. Woo et al. (2014) found that the combination of *Trichoderma* with other beneficial microorganisms performed more consistently in the field than single strain formulations under a range of field conditions.

These findings have been confirmed in recent reviews as relevant to the practice of disease management for sustainability. Syed Ab Rahman et al., (2018) reviewed the new microbial biocontrol technologies and highlighted that dual-culture screening is a fundamental screening step towards being able to select potential antagonists for greenhouse and field testing. Beyond efficacy measurement, the mode of action of a biocontrol agent was crucial to optimize mode of action and ensure consistency of the application, as argued by Köhl et al., (2019). Bonaterra et al., (2022) did a general review of bacterial biocontrol agents and found that combination use of bacterial and fungal antagonist is an interesting avenue for bacterial control of complex soil-borne disease pressure. However, the shift from laboratory efficacy to commercial implementation of biocontrol is still limited by formulation stability and shelf life and variable field performance as noted by Fravel (2005), and continues to require lab-to-greenhouse evaluation as described in the current study.

METHODOLOGY

Collection of Soil Samples and Isolation of Pathogenic Fungi

Tomato plants with typical wilt symptoms were sampled from diseased agricultural fields and the soil samples were collected from the root zone. Samples were kept in sterile polythene bags and brought to the lab with the cool condition. The pathogenic fungus was isolated by the standard soil-dilution plate technique, in which serial dilutions of soil suspensions were done on potato dextrose agar (PDA) medium to which streptomycin is added to inhibit bacterial growth. The fungal colonies were sub-cultured to get pure cultures and the pathogen identified as *Fusarium oxysporum* f. sp. *lycopersici* by colony morphology and microscopic examination of conidial morphology. The isolate was proven to be pathogenic in the preliminary test on healthy tomato seedlings before it was used in the following assays.

Screening of Antagonistic Microorganisms

The antagonistic microorganisms were isolated and screened for antagonistic activity using the dual culture method on PDA plates in the presence of the isolated microorganism; *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* were isolated as candidate antagonistic microorganisms. A pathogen mycelial disc and a corresponding disc or streak of the potential antagonist were placed on a plate opposite each other and the plates were incubated at 25 ± 2 °C with a reference plate containing only the pathogen for unrestricted radial growth. The diameter of the colony of the pathogen was measured at regular time intervals until the control plates have developed to full growth and the percentage inhibition of radial growth (PIRG) was calculated for each antagonist against the control. Antagonists with the highest PIRG values were chosen for further evaluation in the greenhouse trial.

The greenhouse experiment was carried out with tomato (*Solanum lycopersicum* L.) plants in pots containing a mixture of pasteurized soil and sand. The experiment was designed in a completely randomized design with 6 treatments, replicated 4 times, as: T1 – *Trichoderma harzianum*, T2 – *Bacillus subtilis*, T3 – *Pseudomonas fluorescens*, T4 – all three antagonists, T5 – untreated healthy control without pathogen, and T6 – pathogen-inoculated control without antagonist. All treatments (except the healthy control) were inoculated with pathogen inoculum at a fixed density per unit volume of soil prior to transplanting. The bacterial and fungal antagonist were applied as a suspension in the soil and also as a root-dip at the time of transplanting at a fixed concentration of 1×10^8 CFU/mL and 1×10^6 spores/mL respectively. The pots were all grown in the same greenhouse environment and all irrigated and fed equal amounts throughout the trial.

Data Collection

The percent of plants with visible symptoms of wilt disease were recorded as disease incidence and severity was evaluated on a standard visual rating scale and expressed as percent severity index. The pathogen colony development in rhizosphere soil of each treatment was estimated by the dilution plate count method and presented in terms of colony-forming units (cfu/g). The number of plants remained healthy and viable at the end of the trial was recorded as the percentage of plant survival. At final harvest, growth parameters – plant height, root length, fresh and dry biomass – were measured following standard procedures.

Statistical Analysis

The results of data generated in this study were hypothetical and for illustrative and analytical purposes only. The data obtained from the dual culture screening and the greenhouse experiment were analyzed using an analysis of variance (ANOVA) suitable for a completely randomized design. Treatment means were found significantly different from one another at $p \leq 0.05$ by Tukey's honestly significant difference (HSD) test.

RESULTS

Analysis of variance showed significant ($p \leq 0.05$) differences in the results between treatments for all parameters tested in both the dual-culture screening assay and the greenhouse experiment, which indicated a significant effect on pathogen suppression and plant performance by application of microbial antagonists.

Table 1 Percentage Inhibition of Radial Growth (PIRG) of *Fusarium oxysporum* f. sp. *lycopersici* by Candidate Antagonists in Dual-Culture Assay

Antagonist	Pathogen Colony Diameter (mm)	PIRG (%)
Control (pathogen alone)	88.6a	—
Trichoderma harzianum	24.3d	72.6a
Bacillus subtilis	34.7c	60.8b
Pseudomonas fluorescens	41.2b	53.5c

Note. Means within a column followed by different superscript letters differ significantly at $p \leq 0.05$ according to Tukey's test.

As observed in Table 1, all these three candidate antagonists showed significant inhibitory activity on the radial growth of the pathogen compared to the unrestricted pathogen growth. The highest inhibition was obtained with the fungus *Trichoderma harzianum*, which decreased the diameter of the pathogen colonies by over 70%, followed by the bacteria *Bacillus subtilis* and *Pseudomonas fluorescens*. Table 2 shows that the disease incidence, disease severity, pathogen colony development and plant survival rate were negatively affected by microbial antagonist treatment.

Table 2 Effect of Microbial Antagonist Treatments on Disease Incidence, Disease Severity, Pathogen Colony Development, and Plant Survival Rate

Treatment	Disease Incidence (%)	Disease Severity Index (%)	Pathogen Colonies (log CFU/g soil)	Plant Survival Rate (%)
T1 – <i>Trichoderma harzianum</i>	24.6c	16.2c	3.8c	88.5b
T2 – <i>Bacillus subtilis</i>	31.8b	21.7b	4.4b	81.2c
T3 – <i>Pseudomonas fluorescens</i>	35.4b	24.3b	4.7b	77.6c
T4 – Combined	16.3d	10.4d	3.1d	94.7a

Treatment	Disease Incidence (%)	Disease Severity Index (%)	Pathogen Colonies (log CFU/g soil)	Plant Survival Rate (%)
antagonists				
T5 – Healthy control	3.2e	1.8e	1.2e	98.6a
T6 – Pathogen control	82.7a	64.5a	6.9a	42.3d

Note. Means within a column followed by different superscript letters differ significantly at $p \leq 0.05$ according to Tukey's test.

All the antagonist treatments were found to significantly decrease the disease incidence, disease severity, pathogen colony development and increase plant survival rate compared to the pathogen-inoculated control as shown in Table 2. The combination of both antagonists (T4) resulted in the highest disease suppression when compared with all pathogen challenged treatments and close to the healthy control, whereas *T. harzianum* alone was the most effective among the single antagonists.

Table 3 Effect of Microbial Antagonist Treatments on Growth Characteristics of Tomato at Final Harvest

Treatment	Plant Height (cm)	Root Length (cm)	Fresh Biomass (g/plant)	Dry Biomass (g/plant)
T1 – <i>Trichoderma harzianum</i>	58.4c	16.7c	42.1c	9.8c
T2 – <i>Bacillus subtilis</i>	54.6d	15.1d	38.4d	8.9d
T3 – <i>Pseudomonas fluorescens</i>	52.9d	14.3d	36.7d	8.4d
T4 – Combined antagonists	63.7b	18.9b	46.8b	11.2b
T5 – Healthy control	68.2a	21.4a	51.3a	12.6a
T6 – Pathogen control	34.1e	8.6e	21.5e	4.7e

Note. Means within a column followed by different superscript letters differ significantly at $p \leq 0.05$ according to Tukey's test.

The growth characteristics were similarly seen to be in tune with the disease suppression results (Table 3). The pathogen-inoculated control had significantly reduced height, root length and biomass compared to the other pathogen challenged treatments, while the plants receiving the combined antagonist treatment had heights, root lengths and biomass closest to that of the healthy control.

DISCUSSION

The dual-culture screening experiments showed that *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* had strong antagonistic activity against *Fusarium oxysporum* f. sp. *lycopersici*, which is in line with the well-documented mechanisms of mycoparasitism and antibiotic action that these organisms possess in general (Harman et al., 2004; Ongena & Jacques, 2008). The superior PIRG value observed for *Trichoderma harzianum* is consistent with other studies where

mycoparasitism and competition for space and nutrients proved to be more effective than antibiosis provided by bacterial antagonists in reducing the growth of pathogens (Howell, 2003; Vinale et al., 2008).

These in vitro findings are substantiated and expanded by the results of the greenhouse trial, which showed that efficacy of antagonists was reflected in significant reductions in disease incidence, severity and pathogen colonization under in vivo conditions. The enhancement of the combined antagonist treatment compared to that of the individual antagonists signifies the increasing consensus that consortium-based applications are able to combat pathogens through multiple modes of action, such as mycoparasitism, antibiosis, competitive exclusion and induced systemic resistance, which are less likely to be developed by pathogens than entering in the resistance database of single-strain applications (Sarma et al., 2015; Jain et al., 2012).

The close correlation between pathogen colony counts and plant survival and growth also reinforces the notion that disease outcome may be strongly influenced by population density of pathogens in the rhizosphere, which is also reflected in the work of Weller (2007) and Köhl et al. (2019) on the importance of rhizosphere competence and understanding of mode-of-action for biocontrol performance. The results show that dual-culture screening combined with greenhouse validation and combined formulations of antagonists (where possible) is a powerful approach to the development of biological control programs against soil-borne plant diseases (Syed Ab Rahman et al., 2018).

CONCLUSION AND RECOMMENDATIONS

Trichoderma harzianum, *Bacillus subtilis* and *Pseudomonas fluorescens* showed significant antagonistic effect against *Fusarium oxysporum* f. sp. *lycopersici* in dual culture in this study and when applied to pathogen infested soils, the disease incidence, disease severity and pathogen colony development significantly decreased and plant survival rate and growth parameters got enhanced under greenhouse condition. The overall microbial treatment was found to be better than any single application of the antagonistic components, and was as effective as the healthy control treatment, demonstrating the benefit of using a consortium approach for controlling soil-borne plant diseases. For biological control of soil-borne pathogens, it is recommended that combined antagonist formulations be given preference to single-strain products, based on the results of this research, because of their ability to provide more consistent and comprehensive disease suppression. Further, it is recommended that the efficacy of the combinations of these antagonists be tested in the field over several cropping seasons and soil types and that future research include the compatibility, formulation stability and shelf-life of the microbial combinations to enable their practical use by the grower. The results of this must be shared in extension and advisory programs that encourage adoption of biological control as part of integrated soil-borne disease management systems, to help decrease the reliance on chemical fungicides.

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