

Application of biofungicides in control of damping disease off in greenhouse crops as a possible substitute to synthetic fungicides

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ABSTRACT: The purpose of this study was to evaluate the effectiveness of a commercial biofungicide such as Trichomix-HV in controlling damping-off disease in cucumber seedlings of greenhouses. In this regard, 504 fungal isolates were collected from greenhouses at 31 districts in city of Jiroft in Iran. *Pythium aphanidermatum*, *P. ultimum*, *P. irregulare*, *Phytophthora drechsleri*, and *Ph. melonis* accounted for 9.9 %, 8.3 %, 4.5 %, 4.9 %, and 21 % of total isolates collected, respectively. Isolates of *P. aphanidermatum* obtained from commercial cucumber in greenhouses were tested in vitro and under greenhouse conditions for sensitivity to chemical and biological treatments. To this aim, Trichomix-HV a commercial formulation of *Trichoderma harzianum* strain T969 and the fungicides Metalaxyl and Metalaxyl MZ were amended into the culture medium as well as into sterilized or non-sterilized greenhouse soils inoculated by the pathogen and containing plants at the seedling stage. Trichomix-HV significantly ($P < 0.001$) reduced seedling infection at a rate of 82 % when applied into soil medium at a concentration of 10^7 conidia ml/L and reduced vegetative growth of *Pythium aphanidermatum* in vitro. The result from this study shows that Trichomix-HV can be effectively used as a biocontrol agent for controlling damping-off cucumber seedlings and having the potential to replace chemical fungicides as a mean of disease control.

Keywords: Biocontrol; Greenhouse; Synthetic Fungicides; *Trichoderma harzianum*

INTRODUCTION

Production of cucumbers (*Cucumis sativus*) in Iran has expanded consistently over the last 10 years and making cucumber as one of the fastest growing agricultural products in Iran. City of Jiroft is ranked fourth in the production of cucumber in Iran, especially in soil-based greenhouse cucumbers (Mubli and Pirasteh 1995.; Yedidia, et al.; 1999). Different species of Oomycetes, belonging to *Phytophthora* and *Pythium* cause very similar damage to cucumber seed and seedlings, being virtually indistinguishable without laboratory tests. These pathogens damage seed root, pre-emergence damping-off, and post-emergence damping-off, often associated with tan-brown, soft, rotted tissue (Igwe et al., 2008; Malakootian et al., 2009). They can cause serious

damage under favorable environmental conditions (Farr et al., 1989; Weller, et al.; 2000; Siddiqui, et al., 2000). Damping-off in cucumber is a worldwide problem in greenhouses. Stanghellini and Phillips (1975) reported up to 87 % mortality of cucumber seedlings in untreated greenhouse soil in Abu Dhabi. In Canada, damping-off has been reported as a serious disease of several vegetable crops including cucumber (Martin and Loper, 1999). Although other fungal pathogens such as *Rhizoctonia solani* and *Fusarium* spp. have been associated with damping-off (Lida, et al., 1983; Abbasi, et al., 2004; Vinodhini and Narayanan, 2008), *Pythium* spp. was shown to be the most frequent cause of damping-off in cucumber (Stanghellini and Phillips, 1975; Gubler and Davis, 1996; Deadman, et al., 2002). The continuous production of cucumbers in monoculture has been accompanied by increase in post-

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emergence damping-off disease, which has become the main limiting biotic factor to profitable production of cucumbers in greenhouses all over the world (Farr et al., 1989). One of the most common means to control plant diseases in the field, greenhouse and storage is the use of chemical compounds. Currently, several fungicides belonging to different classes of chemicals are used to protect plants from *Pythium* spp., including azoxystrobin (strobilurin), fosetyl-aluminum (alkyl phosphonate), mefenoxam and metalaxyl (acylanilide), etridiazole (triadiazole), and propamocarb (carbamate) (Brantner and Windels, 1998); (Goodwin and McGrath, 1995). These compounds are chemical pesticides, and the majority of them, especially fungicides and nematocides are substances potentially dangerous for environment and can be extremely toxic for consumers (Bandyopadhyay and Chattopadhyay, 2007; Cuthbertson et al., 2009a and b; Cuthbertson and Brown, 2009; Igbinosa and Okoh, 2009; Cuthbertson and Murchie, 2010). Some of them could also have adverse genetic effects, cause morphological and physiological abnormalities in test animals (Cook, 1993). For this reason, a number of restrictions are imposed in the licensing, registering and using of each chemical (Cuthbertson et al., 2010). In this regard, biological disease control is a promising strategy for managing soilborne or foliar diseases in a wide range of crops. Several commercial formulations of the biological control agents *Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces griseoviridis*, and *Gliocladium virens* have been reported previously for application against plant fungal pathogens in the field and greenhouse (Cook, 1993; Sutton and Peng, 1993; Lo et al., 1996; Elad and Kapat 1999; Yedidia et al., 2001; Sultana et al., 2005.). Since spread of active growing mycelium through potting mix is an important means of spread of *Pythium* and *Phytophthora* species, disease control strategies were targeted to prevent seedling infection and reduce pathogen growth in the soil. (Altomare et al., 1999) The main objective of the present study was to evaluate *in vitro* and *in vivo* the effect of the commercially available biofungicide Trichomix-HV, based on the biocontrol agent *Trichoderma harzianum*, against the soilborne pathogen *Pythium aphanidermatum*. (Obire and Anyanwu 2009). In different trials on cucumber seedlings under greenhouse condition, the efficacy of Trichomix-HV was compared with that of two synthetic fungicides (Metalaxyl and Metalaxyl MZ) commonly

applied on this vegetal crop. Present research was carried during 2006 to 2008 in the province of Kerman, Iran.

MATERIALS AND METHODS

Collection and identification of Pythium isolates

A survey during 2006 and 2008 was made in different greenhouses in the 31 districts of Jiroft where more than 87 % of all greenhouses are located. *P. aphanidermatum* was isolated from roots and crowns of cucumber seedlings showing damping-off symptoms including leaf yellowing, leaf wilting and rotting in the collar and crown parts. Isolates were recovered from the crown and roots of infected cucumber plants grown in commercial greenhouses. The sample size varied between times of the year and district. Isolations were carried out by washing soil debris from roots and crowns, which were surface-sterilized in 70 % ethanol for 30s. Seedling tissue was then disinfected with sodium hypochloride (1% active chlorine) for 2 min, washed in sterile distilled water for 1 min and dried on sterile filter paper (Tsao, 1983; Shafizadeh, 2003). Tissue was plated onto water agar medium in 100 × 15 mm Petri dishes. Dishes were incubated under ambient laboratory conditions (20 to 23°C), under fluorescent lights (12-h photoperiod) for approximately 10 days, after which time the pathogen was transferred to 1.7 % corn meal agar (CMA) amended with 5µg pimarinic (Davet and Rouxel, 2000), as well as on 2.5 % potato dextrose agar (PDA), for 1–3 days. Monocultures were obtained by excising hyphal tips and *Pythium* cultures were maintained on CMA slants at 8°C. To induce formation of asexual and sexual reproductive structures, one plug (5mm diameter) from a colony growing on amended or unamended CMA was placed on a fresh water agar (WA) plates. The inoculated plates were incubated in the dark at 22-25°C. Sporangia, oogonia, antheridia and oospores were observed during 4 to 10 days of incubation. Identification of species was based on the monograph of Plaats-Niterink (1981). Reference isolates of *Pythium* species served as standards to confirm the identification.

Evaluating the antifungal activity of Metalaxyl and Metalaxyl-Mancozeb

Potato dextrose agar (PDA) at 45 °C was amended with final concentration 0, 0.01, 0.1, 1, 10, 50 and 100 ppm Metalaxyl (Previcur Energy, Bayer) and Metalaxyl-Mancozeb (wetable powders, 72 %, Previcur Energy,

Bayer) and dispensed in 9-cm diameter plastic Petri plate. A 6-mm-diameter agar plug disk, taken by using a sterilized cork borer from the margin of a 5-day-old *Pythium* CMA culture, was transferred into the center of a new Petri dish (10 replicate plates per medium and per each isolate). Dishes were incubated in the dark at $21 \pm 1^\circ\text{C}$ using a randomized block design with time as the blocking factor. Every 48 h, during a period of 4 days, the linear growth of fungal colonies was measured.

Testing of *Trichoderma harzianum* antagonist

In order to determine the antagonistic potential of the commercial biofungicide Trichomix-HV (containing in its formulation the biocontrol agent *T. harzianum*, strain T969) two different types of assay including simultaneous and non-simultaneous *in vitro* dual culture tests were developed. According to Dennis and Webster (1971), the efficacy of Trichomix-HV against *P. aphanidermatum* by simultaneous dual culture technique on PDA medium was done as follows. A mycelial agar plug of the pathogen (6 mm diameter) was placed at one end of a pre-sterilized Petri plate and at the opposite end, a mycelial agar plug (6 mm diameter) of the antagonistic fungus *T. harzianum* strain T969 was placed aseptically. After 2, 3, 4 and 5 days of incubation, the radial growth of pathogen isolates was measured and the average percentage of radial growth inhibition, with respect to fungal growth in controls, was calculated as follows: $L = [(C - T)/C] \times 100$, where L is inhibition of radial mycelial growth; C is radial growth of the pathogen in the control; T is radial growth of the pathogen in the presence of *Trichoderma* isolates (Edington et al., 1971). Since *T. harzianum* grows faster than *P. aphanidermatum*, non-simultaneous dual cultures were also performed in which a 6 mm diameter of *P. aphanidermatum* agar plugs were taken from the growing margin of fresh cultures and cultured on PDA for 2 days before inoculating *T. harzianum*. Agar plugs of *T. harzianum* were placed 6 cm apart on the surface of PDA in a Petri dish. Plates were incubated at room temperature and the mycelial growth of the fungus was monitored. Percentage of mycelia growth inhibition of the commercial product Trichomix-HV against *P. aphanidermatum* was measured and calculated as described above for comparison with the treatment based on the pure culture of *T. harzianum*.

Evaluation of the biofungicide Trichomix-HV and chemical fungicides under greenhouse conditions

The efficacy of the biofungicide Trichomix-HV and two chemical fungicides (Metalaxyl and

Metalaxyl MZ) were compared under glasshouse condition by using a sterilized soil in comparison with *P. aphanidermatum* infected soils collected from different glasshouses. For the first set of experiments, the pot mixture was sterilized at 15 psi for two hrs at 121°C for two consecutive days. The sterilized pot mixture was filled in half size pots. The pathogen, *P. aphanidermatum* was mass multiplied on sand-maize medium and inoculated into the sterilized pot mixture at a rate of 5 % (w/w). In the second set of experiments, *P. aphanidermatum*-infected soils collected randomly from glasshouses were mixed and used directly for the experiments. Pots were arranged in a randomized block design (five replicates per treatment) in a greenhouse at $25 \pm 3^\circ\text{C}$ and a 12-h photoperiod for 4 weeks. Stand counts for pathogen verification were done daily and the percentage reduction in seedling number was determined with respect to the number of seedlings in uninfected sterilized soil (control) after 30 days. The Trichomix-HV biocontrol material was used directly for seed treating or soil inoculating. For seed treatment, cucumber seeds (*Cucumis sativus* L. cv. Royal), were pre-soaked in water for overnight and surface sterilized with 70 % (v/v) ethanol for 1 min and air dried in a flow hood. Trichomix-HV was incorporated into seeds at the amount of 10 g of biocontrol formulate per Kg of cucumber seeds or incorporated into soils at the amount of 50 g of biocontrol formulate per m^2 of soil. Trichomix-HV treated and non-treated seeds were sown in pathogen contaminated soil at a density of 10 seeds per pot. In each experiment non-treated seeds and soils were considered as the control. All experiments were performed at least twice. For studying the control effect of fungicides, cucumber seedlings at the 2-3 leaf stage were subjected to treatments with the chemical fungicides Metalaxyl (5 % WG) or Metalaxyl MZ (72 % WT) at the concentrations of 0.5 % and 0.25%, respectively. Data collection and analysis were as described for Trichomix-HV. Non-treated infested soil and those immersed in water were also used for growing the cucumber seedlings to serve as control. The design used for this experiment was a complete randomized design (CRD) with four replications. Experiments were analyzed by analysis of variance (SAS Institute, Cary, NC), and mean separations were according to Duncan multiple range test, $P < 0.001$.

RESULTS AND DISCUSSION

Pythium and *Phytophthora* species were isolated from samples taken from diseased cucumber plants. Of the 504 isolates collected during the survey, *P. aphanidermatum*, *P. ultimum*, *P. irregulare*, *Phytophthora drechsleri*, and *Ph. melonis* accounted for 9.9 %, 8.3 %, 4.5 %, 4.9 %, and 21 % of total isolates respectively. These species were identified by comparison of cultural and morphological characteristics to literature and reference isolates (Plaats-Niterink, 1981). Among 504 isolates from decayed roots and crowns, 50 (9.9 %) were identified as *P. aphanidermatum*. This pathogen was detected in 30% of the visited greenhouses of Jiroft.

Activity of synthetic fungicides and the biofungicide versus P. aphanidermatum

Fungicides used during the present study produced a gradual decline in growth of *P. aphanidermatum* by increasing concentration in the medium (Table 1). Metalaxyl was found to be the most effective, providing 85 % reduction in growth of *P. aphanidermatum* at a concentration of 0.5 ppm (Table 2). Metalaxyl MZ was the second most effective product with a 78 % reduction in the 0.5 ppm treatment,

however, its efficacy was not significantly different from that of Metalaxyl.

For testing the inhibitory effect of Trichomix-HV biocontrol commercial product on mycelia growth of *P. aphanidermatum* isolates, *T. harzianum* strain T969 was used as the main effective ingredient of Trichomix-HV biological product directly on media because of its high contaminating effect on medium cultures. Results showed that the inhibition of radial mycelia growth of tested *Pythium* isolates was not greater than that provided by the compared fungicides when *T. harzianum* strain T969 was used versus the pathogen on medium 5 days after treatment (Table 3).

In studying *T. harzianum* strain T969 and the pathogen species in simultaneous and non-simultaneous dual cultures, *Trichoderma* had a marked inhibitory effect on the pathogen growth compared with the control, however, with different efficiency in the *in vitro* test. *Trichoderma* was most effective in pathogen mycelial growth inhibition when it was cultured before inoculating *Pythium* on the medium (Table 3). Compared to fungicides, *T. harzianum* showed a lower inhibitory effect on *Pythium* mycelial growth on PDA.

Table 1: Reduction in growth of *Pythium aphanidermatum* on potato dextrose agar containing different concentration of the fungicides

Fungicide (common name)	Control*	Doses (ppm)							
		0.01	0.1	0.5	1	5	10	50	100
Metalaxyl (5%, WG)	5.6 ^a	3.3	1.7	0.8	0	0	0	0	0
Metalaxyl MZ (72%, WT)	5.6	3.6	2.3	1.2	0	0	0	0	0

Each value is an average of 10 replicates combined from four experiments (10 seeds planted per replicate). * Water used as the control. ^a Numbers within a column are stated as cm

Table 2: Percentage inhibition of radial mycelia growth of *P. aphanidermatum*

Fungicide (common name)	Control*	Doses (ppm)							
		0.01	0.1	0.5	1	5	10	50	100
Metalaxyl (5%, WG)	0	41	69	85	100	100	100	100	100
Metalaxyl MZ (72%, WT)	0	35	58	78	100	100	100	100	100

Each value is an average of 10 replicates combined from four experiments (10 seeds planted per replicate). * Water used as control

Table 3: Percentage inhibition of radial mycelial growth of *P. aphanidermatum* in simultaneous and non-simultaneous dual culture assays

DAI	simultaneous dual culture				non-simultaneous dual culture			
	2	3	4	5	2	3	4	5
<i>Trichoderma harzianum</i> strain T969	28.44 ^a	34.11	39.33	55.12	64.69	67.37	69.35	74.48

DAI = Days after inoculation. ^a Numbers within a column are mean value of 8 replicates per treatment

Evaluation of the biofungicide Trichomix-HV and chemical fungicides under greenhouse conditions

Control of damping-off provided by the fungicides Metalaxyl and Metalaxyl MZ was high in non-sterilized greenhouse soils, with inhibition rates of 77 % and 17 %, respectively compared to Trichomix-HV with an inhibition rate of 68.6 % (Fig. 2). A significant increase of damping-off control was obtained when Trichomix-HV was applied in pre-sterilized greenhouse soil, resulting in efficient control of damping-off up to 82 %, compared to Metalaxyl (73.7 %) and Metalaxyl MZ (17 %) (Fig. 1). When water, as a control, was added into pre-sterilized and non-sterilized greenhouse soils, no damping-off inhibition was observed.

The results of this study showed that most of the visited greenhouses in Jiroft are infected with at least one species of the fungal-like oomycete causing damping-off on cucumber plants. It was also observed that *P. aphanidermatum* was the most prevalent *Pythium* species in the region of interest. It is important for growers to know which *Pythium* species occurs in their cropping soils and the overall levels of inoculum that they have to deal with to decide when to use biocontrol agents or fungicides for maximum impact. Soilborne plant pathogens such as bacteria, fungi and nematodes annually create major economic losses in many important crops. Some chemical compounds have been successfully used to control soilborne plant

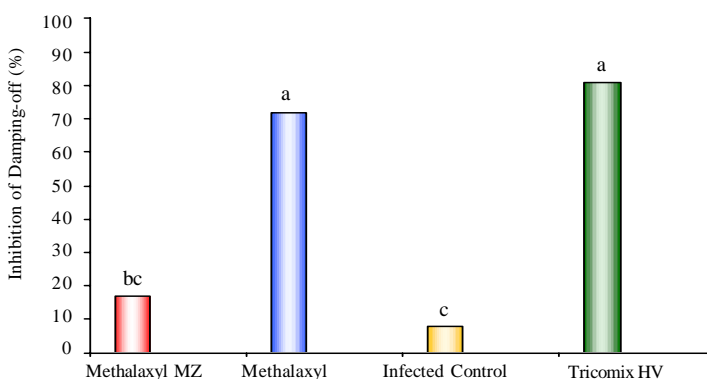


Fig. 1: Efficacy of the commercial biofungicide Trichomix-HV, in comparison with two chemical fungicides. Bars followed by common letters do not differ significantly according to Duncan multiple range test (Pd" 0.001)

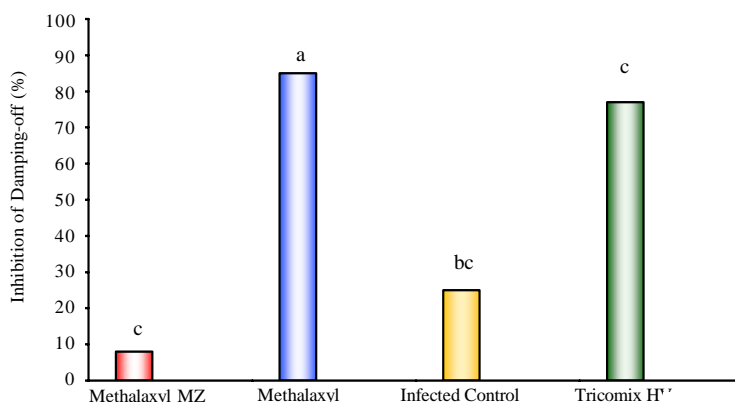


Fig. 2: Efficacy of the commercial biofungicide Trichomix-HV, in comparison with two chemical fungicides. Bars followed by common letters do not differ significantly according to Duncan multiple range test (Pd" 0.001)

pathogens. In this study, the inhibitory effect of Metalaxyl and Metalaxyl MZ fungicides on *P. aphanidermatum* was evaluated. The results indicate a high inhibitory effect of these fungicides on *Pythium* under *in vitro* and greenhouse conditions. Combined treatments with *Trichoderma* spp. and fungicides to control plant diseases has been attempted with other crops, using either natural strains or strains selected for fungicide resistance (Howel, 2007). Previous studies have demonstrated the lack of inhibitory effect of Metalaxyl and Metalaxyl MZ on *Trichoderma* (Singh et al., 1995). This result indicates that mixed applications of Metalaxyl or Metalaxyl MZ with *Trichoderma* spp. can be possible and then this strategy could be also adopted in Jiroft to lead higher inhibitory effects versus *Pythium* and lower application rates of the fungicides. In many cases, fungicides appear to be the most economical and efficient means of controlling plant pathogens, however, these products require a variety of other considerations such as evaluation of their environmental impact and toxicological risks. Global policy barring use of active chemicals in the process of agricultural production represents a burden for agricultural farmers dependent on chemical fungicides.

Biological control is a promising strategy for managing soilborne or foliar plant diseases in a wide range of crops (Stack, et al., 1988; Lo et al., 1996). In this research, the efficacy of a new biocontrol product; Trichomix-HV containing in its formulate *T. harzianum* was evaluated as the main active biologic ingredient exerting a strong inhibitory effect against *P. aphanidermatum* on cucumber plants under greenhouse conditions. Results showed that Trichomix-HV is an efficient biocontrol product controlling *P. aphanidermatum* when applied in medium cultures (Tables 1 and 2). However more growth inhibitory effects were obtained when Trichomix-HV was applied in non-simultaneous dual culture under *in vitro* conditions (Table 2). This suggests that for optimal results Trichomix-HV should be preventively applied before seedling plantation. It agrees with other studies indicating that effective antagonists must become established in cucumber ecosystems and remains active versus target pathogens during periods favorable for plant infection (Nelson et al., 1994).

CONCLUSION

The antagonistic activity (hyperparasitism, mainly) of the genus *Trichoderma* versus *Pythium* species has been widely demonstrated (Chao, et al., 1986; Yedidia, et al.; 2001). Applications of granules of this fungus resulted in establishment of stable and effective populations in several plant species (Lo, et al., 1996). To be effective, biological disease control depends not only on suitable biocontrol organisms but also on methods and strategies for introducing and maintaining high population level and activity of these organisms in association with crop and plant (Stack, et al., 1988; Jin, et al., 1991). Regardless of the quality of the biocontrol agent, the methods used to produce, formulate, and deliver the active organism may profoundly influence its efficacy (Cook and Baker, 1983; Jin, et al., 1991). For this reason, *Pythium* inhibitory activity of the commercial biocontrol product Trichomix-HV has been evaluated, in which all of mentioned parameters were considered to be most effective against a pathogen in field. The results confirmed a good antagonistic effect of this product in greenhouse soils (Figs. 1 and 2). No significant difference was found between percentage of damping-off inhibition due to metalaxyl application and Trichomix-HV application in sterilized or non-sterilized soils, supporting the premise that Trichomix-HV is a good substitute for Metalaxyl versus *Pythium* on cucumber crops under greenhouse conditions. Previously a commercial formulation of *T. harzianum* T39 named Trichodex was tested for its ability to protect potato and tomato plants from disease caused by *P. erythroseptica* in greenhouses in Iran, which was highly effective on tomato plants (Etebarian, et al., 2000). Here, the efficacy of a new commercial formulation of *T. harzianum*, Trichomix-HV, versus *P. aphanidermatum* under greenhouse condition on cucumber plants has been demonstrated. The results of this study encourage further investigations in order to better understand Trichomix-HV stability and duration in the soil and its interaction with environmental conditions on cucumber plants.

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