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Article

Biological Management of Basal Rot of Onion by *Trichoderma harzianum* and *Withania somnifera*

Manejo Biológico da Podridão Basal de Cebola por ***Trichoderma harzianum*** *e* ***Withania somnifera***

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ABSTRACT - Onion is attacked by destructive soil-borne fungal plant pathogen *Fusarium oxysporum* f. sp. *cepae*, resulting in basal rot disease. In the present study, three *Trichoderma* species (*T. pseudokoningii, T. harzianum* and *T. reesei*) and leaves of solanaceous weed *Withania somnifera* were used for management of this disease. The *in vitro* interaction study revealed *T. harzianum* as the most effective biocontrol agent against the pathogen. In a pot trial, dried leaf material of *W. somnifera* (1%, 2% and 3% w/w) and inoculum of *T. harzianum* were mixed in the pot soil previously inoculated with the pathogen. The highest incidence of the disease (87%) was found in positive control (pathogen inoculation without any amendment). Different rates of dry leaf material reduced the incidence of the disease to 41-66%. *T. harzianum* in combination with leaf material reduced the incidence of the disease to 20-53%. In a laboratory bioassay, the dry leaf extract of *W. somnifera* was prepared in methanol and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The highest concentration (200 mg mL^{-1}) of all except for the *n*-butanol fraction significantly decreased fungal biomass over control. This study concludes that basal rot of onion can be controlled by combined application of *W. somnifera* dry leaf material and biological control agent *T. harzianum*.

Keywords: basal plate rot, biological control, weed, *Withania somnifera.*

RESUMO - A cebola é atacada por um patógeno fúngico no solo, ***Fusarium oxysporum****, que resulta na doença da podridão basal. No presente estudo, três espécies de Trichoderma (****T. pseudokoningii****,* ***T. harzianum*** *e* ***T. reesei****) e as folhas da planta daninha solanácea* ***Withania somnifera*** *foram utilizadas para o manejo dessa doença. O estudo de interação in vitro revelou que* ***T. harzianum*** *é o agente de biocontrole mais eficaz contra o patógeno. Em um ensaio em vaso, misturou-se material de folha seca de* ***W. somnifera*** *(1%, 2% e 3% p/p) e o inóculo de* ***T. harzianum*** *no substrato já inoculado com o patógeno. A maior incidência da doença (87%) foi observada em controle positivo (inoculação de patógenos sem qualquer alteração). Diferentes doses do material da folha seca reduziram a incidência da doença para 41-66%. Em combinação com o material foliar,* ***T. harzianum*** *reduziu a incidência da doença para 20-53%. Em um bioensaio laboratorial, foi preparado um extrato de folhas secas de* ***W. somnifera*** *em metanol e particionado com n-hexano, clorofórmio, acetato de etilo e n-butanol. A concentração mais elevada (200 mg* mL^{-1}*) de todas as frações, exceto a de n-butanol, diminuiu significativamente a biomassa fúngica em relação ao controle. Este estudo conclui que a podridão basal da cebola pode ser controlada pela aplicação combinada de material de folha seca de* ***W. somnifera*** *e o agente de controle biológico* ***T. harzianum****.*

Palavras-chave: podridão basal, controle biológico, planta daninha, *Withania somnifera.*

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INTRODUCTION

Onion (*Allium cepa*), family Alliaceae, is an economically important horticulture crop extensively cultivated worldwide (Griffiths et al., 2002). It is an important source of phytoconstituents and also famous for having good flavor. Apart from being used as food, it also has medicinal values. It performs a number of biological activities, e.g. anti-inflammatory, antioxidant, anticancer, antiplatelet aggregation, antidiabetic, antimicrobial, neuroprotective and immunomodulatory properties (Bora and Sharmam, 2009). Onion is susceptible to many foliar, bulb and root fungal pathogens that reduce its yield and quality. One of the important fungal diseases of onion is basal rot disease caused by *Fusarium oxysporum* f. sp. *cepae* (Javaid and Rauf, 2015; Javaid et al., 2017a), which leads to serious crop failure and storage losses (Southwood et al., 2015).

Synthetic fungicides are known to markedly control basal rot of onion. Application of Granosan 200 (benomyl 15% + mancozeb 60%) declined this disease by 77%, consequently onion yield was increased by 106% (Naik and Burden, 1981). Recently, Behrani et al. (2015) found carbendazim and antracol very effective in controlling basal rot of onion. Application of these fungicides at 10,000 ppm resulted in 100% emergence of seedlings. However, synthetic fungicides have adverse effects on the environment and also affect the beneficial microorganisms present in the field soil (Muñoz-Leoz et al., 2013; Mimbs et al., 2016). Management of plant pathogens by biological means is considered an alternative environment friendly strategy for sustainable agriculture (Al-Naemi et al., 2016; Youssef et al., 2016). *Trichoderma* is among the most commonly used biocontrol agents and is an effective antagonist against plant pathogenic fungi (Kumar and Mukerji, 1996; Stocco et al., 2016). Recent studies have shown that *Trichoderma* spp., especially *T. harzianum*, can control diseases caused by *Rhizoctonia solani*, *Macrophomina phaseolina* and *Ceratocystis radicicola* (Al-Naemi et al., 2016; Javaid et al., 2017b; Youssef et al., 2016).

The use of biodegradable material such as fresh plant extracts and dry biomass of certain plant species is gaining importance in the management of fungal diseases of plants. Numerous recent studies have shown that extracts of many plant species such as *Imperata cylindrica*, *Raphanus sativus* and *Acacia nilotica* have antifungal potential against fungal pathogens, namely *Macrophomina phaseolina*, *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotium rolfsii* (Javaid and Bashir, 2015; Sana et al., 2016; Banaras et al., 2017). Likewise, soil amendment with dry biomass of various plant species, namely *Coronopus didymus*, *Chenopodium album* and *Melia azedarach*, gave remarkable results in management of collar rot of bell pepper, basal rot of onion and collar rot of chickpea (Javaid and Iqbal, 2014; Javaid and Rauf, 2015; Javaid and Khan, 2016). Recently, Javaid et al. (2017b) reported that the combined use of dry biomass of *Sisymbrium irio* and *T. harzianum* gave better results in the management of charcoal rot of mungbean than the application of either alone. *Withania somnifera* is a perennial shrubby weed of the family Solanaceae; it has various withanolides which are important for their biological activities (Marie, 2006). In the present study, dry leaf biomass of *W. somnifera* was used in combination with *Trichoderma harzianum* to control basal rot disease of onion.

MATERIALS AND METHODS

Interaction of *Trichoderma* spp. and *F. oxysporum* f. sp. *cepae*: The test fungus, namely *F. oxysporum* f. sp. *cepae* (FCBP 1114) and three species of *Trichoderma*, namely *T. pseudokoningii* (FCBP 0213), *T. harzianum* (FCBP 1277) and *T. reesei* (FCBP 0271), were obtained from the Fungal Culture Bank of Pakistan. The fungi were sub-cultured on 2% malt extract agar (MEA) medium to maintain their pure cultures.

Autoclaved MEA was poured in 9 cm Petri plates and allowed to solidify. Plugs (5 mm) of the fungal pathogen and three species of *Trichoderma* were placed in 9 cm Petri plates at a distance of 2 cm. Petri plates were incubated at 27 ^{o}C for 5 days. A monoculture treatment of the pathogen was included as control. Four replicates were made of each treatment. Radial growth of the pathogen was recorded in mono- and dual culture plates using a scale after 5 days' incubation. Fungal colony diameter in each Petri plate was measured at three points and averaged. Percentage of inhibition in the growth of the fungal pathogen by *Trichoderma* spp. was calculated according to the formula given by Rini and Sulochana (2007).

$$\text{Growth inhibition (\%)} = \frac{\text{Control - Treatment}}{\text{Control}} \times 100$$

Pot Experiment: A pot trial was carried out for biological control of basal rot of onion by amending the soil with *T. harzianum* and dried leaves of *W. somnifera* following the procedure of Javaid and Rauf (2015) with some modifications. For preparation of inocula of *F. oxysporum* f. sp. *cepae* and *T. harzianum*, boiled and autoclaved chickpea seeds were inoculated with actively growing mycelial discs of these fungi followed by incubation at 28 ^{o}C for 10 days.

The experiment was conducted in plastic pots each containing 450 g sandy loam soil. All pots except for the negative treatment were inoculated with *F. oxysporum* f. sp. *cepae* by thoroughly mixing 10 g chickpea based inoculum in each pot. After irrigation with tap water, the pots were left under natural environmental conditions for one week. Thereafter, dried and crushed leaves of *W. somnifera* were mixed in the soil at 1%, 2% and 3% either with or without the inoculum of *T. harzianum*. The experiment was conducted in a completely randomized design. The incidence of the disease was calculated after 60 days of sowing by calculating the percentage of diseased plants out of the total amount of plants sown in the pots.

Laboratory bioassays

Dried and thoroughly crushed *W. somnifera* leaves were extracted with methanol for 14 days. The materials were filtered and the solvent was evaporated on a rotary evaporator. The remaining material was mixed in 200 mL water and extracted with *n*-hexane, chloroform, ethyl acetate and *n*-butanol using a separating funnel. After evaporation of solvents, 9.1 g, 24.3 g, 1.9 g and 6.2 g *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions were obtained (Javaid and Akhtar, 2015).

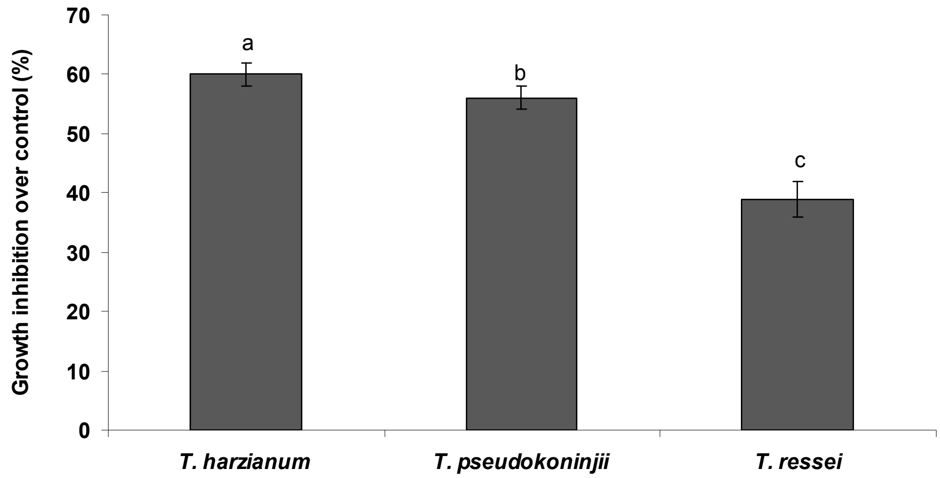
An amount of 1.2 g of each fraction of methanolic extract was dissolved in 1 mL DMSO and 5 mL malt extract broth was added to it. This stock solution (200 mg mL^{-1} concentration) was serially double diluted by adding malt extract broth to prepare 100, 50, 25, 12.5, 6.25, and 3.125 mg mL^{-1} concentrations. A series of control treatments was prepared by dissolving 1 mL of DMSO in 5 mL malt extract broth followed by serial double dilution. Bioassays were carried out in glass test tubes (10 mL) each containing 1 mL of the growth medium. For preparation of *F. oxysporum* f. sp. *cepae* inoculum, mature fungal colony was suspended in sterilized distilled water and passed through cheese cloth, of which 15 ìL was added to each test tube. The experiment was conducted in triplicate using a completely randomized design. After 7 days of incubation at room temperature, fungal biomass in each test tube was filtered, dried and weighed (Javaid et al., 2015).

Statistical analysis

Data of the laboratory bioassays and the pot trial were analyzed by ANOVA. The treatments' means were separated by Duncan's Multiple Range tests at $P \leq 0.05$ (Steel and Torrie, 1997).

RESULTS AND DISCUSSION

***In vitro* antagonistic activity of *Trichoderma*:** All the three *Trichoderma* species (*T. harzianum*, *T. pseudokoningii* and *T. reesei*) tested *in vitro* markedly reduced the growth of *F. oxysporum* f. sp. *cepae*. Among the three *Trichoderma* species, maximum inhibitory effect (60%) on growth of the fungal pathogen was due to *T. harzianum*. *T. pseudokoningii* was the second best antagonistic fungus resulting in 56% inhibition in growth of the target fungal pathogen. Least inhibition of 39% in growth of the pathogen was due to *T. reesei* (Figure 1). Earlier in similar studies, *T. harzianum* and *T. pseudokoningii* were the most effective against *R. solani*, and *T. viride* against *F. oxysporum* (Rini and Sulochana, 2007; Akrami et al., 2011). Important factors responsible for antagonistic activity are production of anti-microbial metabolites, faster metabolic rates and physiological conformation by *Trichoderma* species. Various mechanisms involved in antagonism

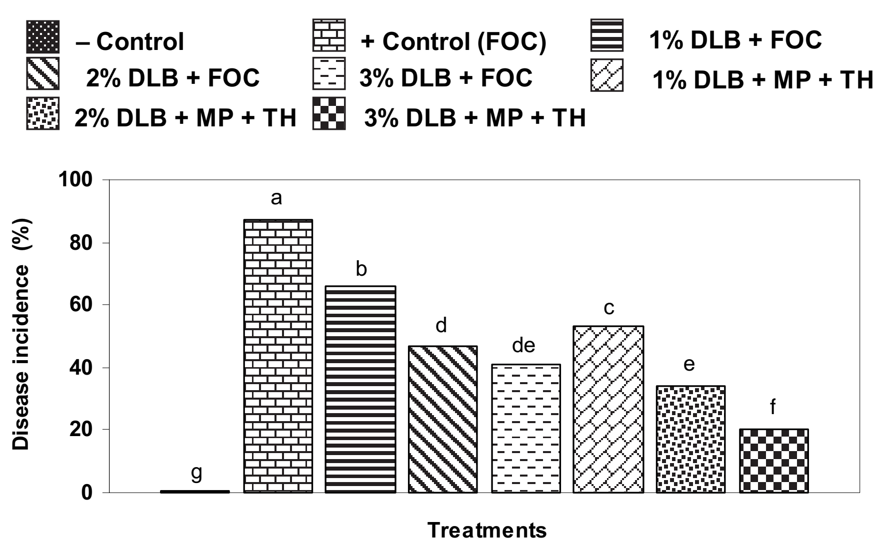


Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

Figure 1 - Growth inhibition of *Fusarium oxysporum* f. sp. *cepae* as a result of interactions with different *Trichoderma* species.

of *Trichoderma* with fungal pathogens also include spatial and nutrient competition, mycoparasitism, and antibiosis by enzymes and secondary metabolites. (Mausam et al., 2007).

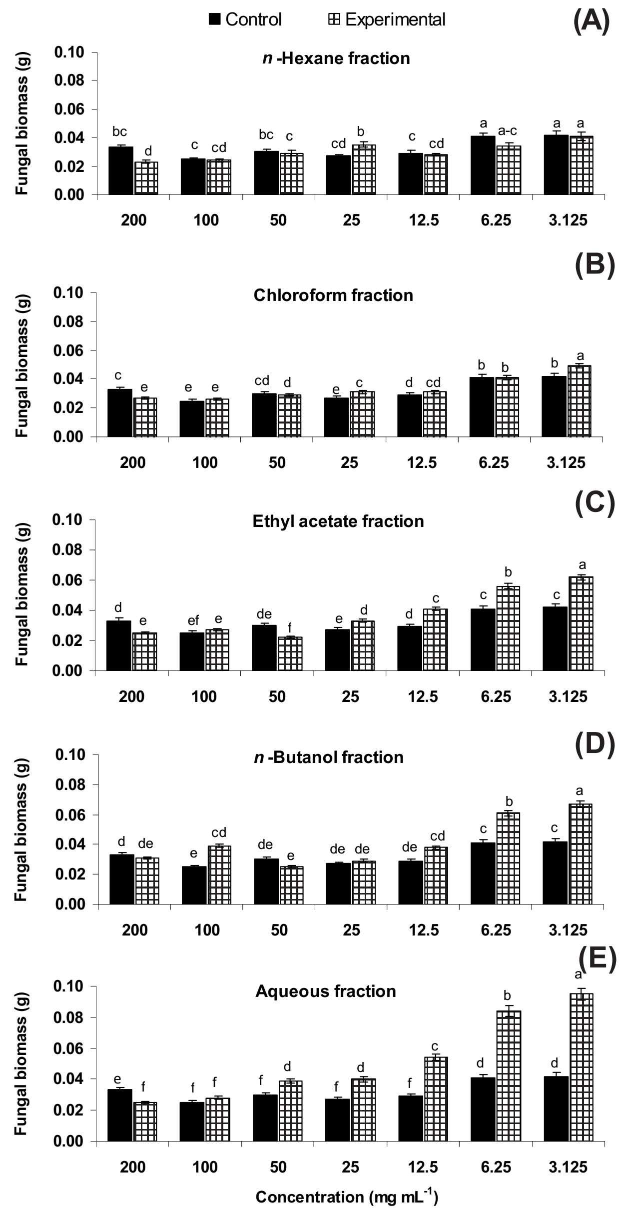
Pot trial: No disease symptoms were found on onion plants in the negative control. In the positive control where inoculum of the fungal pathogen was applied without any soil amendment, onion bulbs showed 87% disease incidence. Application of various rates of leaf manure as soil amendment markedly decreased the basal rot disease. Disease incidence decreased significantly by application of leaf material of *W. somnifera* and there was 41-66% disease incidence in 1% to 3% application of leaf material treatments (Figure 2). In the treatments where leaf material was used in combination with *T. harzianum*, the effect was more pronounced in comparison with the positive control as well as the treatments in which *T. harzianum* was not used. Disease incidence in these treatments was 20-53%. The lowest disease incidence (20%) was recorded in the treatment where 3% dry leaf material was used in combination with *T. harzianum* (Figure 2). Earlier studies have shown that *Trichoderma* spp. are very beneficial fungi and have the ability



Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

Figure 2 - Effect of soil amendment with dry leaf biomass (DLB) of *W. somnifera* and *T. harzianum* (TH) on incidence of basal rot of onion caused by *Fusarium oxysporum* f. sp. *cepae* (FOC).

to control many pathogens (Fontenelle et al., 2011; Tchameni et al., 2011). Application of culture filtrates and spore suspension of *T. harzianum* reduced the size of necrotic spots of black scorch disease of date palm caused by *Ceratocystis radicicola* (Al-Naemi et al., 2016). *T. harzianum* is also known to reduce severity of leaf blotch of wheat caused by *Zymoseptoria tritici* (Stocco et al., 2016). The modes of action of *Trichoderma* are competition, antagonism and production of certain antifungal compounds (Nederhoff, 2001). Both physical and chemical interactions are involved in growth suppression of pathogenic fungi by *Trichoderma* species. A variety of chemicals are produced by *Trichoderma* spp. which induce localized or systemic resistance in the host plants (Gary et al., 2004). Recently, Youssef et al. (2016) reported that *T. harzianum* mediated protection in tomato against *Rhizoctonia solani*, which may be linked with alleviation of oxidative burst in host cells. There was an increase in guaiacol peroxidase, superoxide dismutase, catalase and ascorbate peroxidase activities in response to pathogen attack.



Bars with different letters at their top show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

Vertical bars show standard errors of means of three replicates.

Figure 3 - Effect of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and the aqueous fraction of methanolic leaf extract of *Withania somnifera* on growth of *Fusarium oxysporum* f. sp. *cepae*.

Antifungal activity of different fractions of methanolic leaf extract: A concentration of 200 mg mL^{-1} of *n*-hexane fraction significantly reduced the fungal biomass by 30%. The rest of the concentrations either showed insignificant effect or enhanced fungal biomass. Similarly, the same concentration of chloroform fraction caused significant reduction of 18% in the biomass of *F. oxysporum* f. sp. *cepae*. Lower concentrations (6.25 to 100 mg mL^{-1}) generally showed no effect on fungal growth. In contrast, the lowest concentration (3.125 mg mL^{-1}) significantly increased fungal biomass by 17% (Figure 3A, B). A highly variable antifungal activity of ethyl acetate fraction was recorded with respect to extract concentration. The highest concentration (200 mg mL^{-1}) significantly suppressed fungal biomass by 24%. Similarly, 50 mg mL^{-1} also declined fungal biomass by 27% over control. Conversely, 3.125 to 25 mg mL^{-1} concentrations significantly stimulated growth of the pathogen. There was 22 to 52% increase in fungal biomass as a result of various concentrations from 3.125 to 25 mg mL^{-1} (Figure 3C). In general, 12.5-200 mg mL^{-1} concentrations of *n*-butanol fraction did not show any pronounced effect on fungal biomass. By contrast, the lower concentrations (3.125 and 6.25 mg mL^{-1}) significantly stimulated the fungal biomass. The highest stimulatory effect was recorded due to 3.125 mg mL^{-1} concentration where 60% increase in fungal biomass was noted (Figure 3D). There was a significant decrease of 24% in fungal biomass because of 200 mg mL^{-1} concentration of the aqueous fraction. As the concentration of the leaf extract decreased, fungal growth was stimulated. In 3.125 mg mL^{-1} concentration, fungal biomass was increased by 95% followed by a 90% increase because of 6.25 mg mL^{-1} (Figure 3E). In general, the concentration of 200 mg mL^{-1} of all the fractions except *n*-butanol significantly reduced fungal biomass. Major compounds of *W. somnifera* are withanolides. Withaferin A is the most important one that might be responsible for antifungal activity against *F. oxysporum* f. sp. *cepae*. Antifungal activity might also be due to other metabolites in *W. somnifera*, namely phenolics, glycowithanoldies, flavonol glycosides and sterols (Kandil et al., 1994; Kannan and Kulandaivelu, 2007).

In conclusion, a 3% rate of dry leaf biomass of *W. somnifera* as soil amendment is highly effective in the management of basal rot disease. Antifungal activity of the leaf material can be further enhanced by using it in combination with *T. harzianum.*

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