The in vitro Efficacy of Trichoderma Isolates Against
Pythium aphanidermatum, the Causal Agent of Sugar Beet Root Rot

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ABSTRACT

Application of botanical and biological methods in plant disease controls are unique techniques in reducing environmental pollutions. Trichoderma species are effective agents in biocontrol strategies and identifying their useful isolates for further studies seems to be helpful. The purpose of this study was to assess the in-vitro biocontrol efficacy of some native Trichoderma isolates against Pythium aphanidermatum an important causal agent of sugar beet root rot disease in North Iran. Evaluations were conducted in Shahrood Agricultural Research Center, North Iran during 2007. Collected Isolates of Trichoderma belonging to four species (T. harzianum, T. longibrachatum, T. erinaceum and T. koningii), among which T. erinaceum is a new fungus for Iran's mycoflora. Results of lab tests including dual culture technique, assessment of volatile and non-volatile effects on pathogen' colony growth indicated the effectiveness of all ten isolates against P. aphanidermatum. In dual culture T. harzianum-2733 and T. longibrachatum-2734 were best in reducing the colony growth of P. aphanidermatum by 57% compared to control. Volatile antibiotic compounds of T. longibrachatum-2737 and non-volatile antibiotic compounds of T. harzianum-2739 were best among Trichoderma isolates and controlled the colony growth of the pathogen by 66 and 87% respectively.

Keywords: Trichoderma isolates, Pythium aphanidermatum, Biocontrol, Sugar beet

INTRODUCTION

The potential of Trichoderma species as biocontrol agents in plant disease control was first recognized in the early 1930s (Weindling, 1932). Subsequently, they were applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003). In view of difficulties and problems associated with chemical control of soil born plant diseases, such as poor
penetration of fungicides into the soil, their rapid leaching and degradation, high cost and environmental pollution, application of biocontrol agents for plant disease control is considered as a good alternative. Control of plant diseases with Trichoderma depends on its metabolic versatility, capability to occupy and degrade organic substrates and tolerance to microbial inhibitors in the soil (Papavizas, 1985). The diversity and operation of these characteristics is, in turn, dependent on the particular isolate of the antagonist. Control might be achieved by competition, production of antibiotics or by mycoparasitism (Campbell, 1989). During recent 2-3 decades, attentions have been paid to this group of fungi. Trichoderma species have been studied for the control of many soil born diseases including Fusarium wilts under lab, green house and field conditions and in developed countries. Several Trichoderma species have been formulated as safe formulations for plant disease control (Sivan et al., 1984; Inbar et al., 1994; Papavizas, 1985; Chet and Inbar, 1994). Several superior strains have also been identified and formulated into commercial bio-pesticides.

*T. harzianum* is sold as F-Stop for the control of several soil-borne diseases, while a mixture of *T. harzianum* and *T. polysporum* is available as BINAB-T for the control of wood decays (Agrios, 2005). Siameto et al. (2010) in Kenya tested antagonistic effect of several isolates of *Trichoderma harzianum* against five soil borne phytopathogenic fungi (*Rhizoctonia solani*, *Pythium* sp., *Fusarium graminearum*, *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *Lycopersici*) using dual culture assay and through production of nonvolatile inhibitors and found that all *T. harzianum* isolates had considerable antagonistic effect on mycelial growth of the pathogens in dual cultures compared to the controls. Maximum inhibitions occurred in *Pythium* sp.-055E interactions (73%). *Pythium* sp. was inhibited the most compared to other pathogens. Tran (2010) in Vietnam reported that all *Trichoderma* tested species had the ability of suppressing fungal growth of *Phytophthora palmivora*, *Rhizoctonia solani*, *Fusarium* spp., *Sclerotium rolfsii* and *Pythium* spp. under lab condition. He claimed that the efficacy of *Trichoderma* species on these soil borne fungal pathogens was higher than fungicides. Mishra (2010) screened ten strains of *Trichoderma* species against *Pythium aphanidermatum* using dual culture method and among the tested strains, *T. viride* 1433 was found to be most effective against *P. aphanidermatum*.

Yang et al. (2004) collected over 400 *Trichoderma* strains from the rhizosphere of vegetable crops in which plants had survived *Pythium* damping-off. In co-culture trials in the laboratory, 24 isolates of *Trichoderma* spp. displayed the ability to steadily colonize and aggressively attack mycelia of *P. ultimum* and to produce numerous conidia on *Pythium* colonies. Hajieghrari et al. (2008) studied the antagonistic potential of some selected Iranian isolates of *Trichoderma*, (*T. hamatum* T614, *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969 and *T. virens* T523) against four isolates of soil born pathogenic fungi (*Phytophthora cacturnum*, *Fusarium graminearum*, *Rhizoctonia solani* and *Macrophomina phaseolina*) and found that all antagonists had inhibitory effect against the colony growth of these pathogens in dual culture and volatile metabolite method evaluations compared to controls. Maximum inhibitions occurred in *F. graminearum*-*T. hamatum* (T614) interaction. Significant pathogen colony growth inhibitions were observed when culture of *Trichoderma*-*F. graminearum* were exposed to the trapped atmosphere. *F. graminearum* was most susceptible to the volatile inhibitors produced by *T. hamatum* T612 (48.65%).
Ashrafizadeh et al. (2005) studied the potential of Trichoderma species in controlling Fusarium wilt of melon and found T. virens (DAR-7429) to have best control of the disease. The aim of present study was to find out effective isolates of Trichoderma against P. aphanidermatum for further studies.

**MATERIALS AND METHODS**

*Preparation of pathogenic isolate of the disease*

Several sugar beet roots showing soft rotting symptoms were collected from different fields of Shahrood in north Iran during autumn. Using P5ARP selective medium (Jeffers and Martin, 1986), Pythium aphanidermatum was isolated from majority of samples and after pathogenicity tests an isolate showing proper performance was selected for further studies.

*Preparation of Trichoderma isolates*

*Isolation of Trichoderma isolates from soil*

For isolation of Trichoderma from soil, the technique used by Rifai (1969) was followed. Different soil samples were collected from sugar beet rhizosphere (20 cm. deep) of different fields. 20 g of each sample were thoroughly mixed with 500 ml distilled water containing 0.02% citric acid. 5 ml of prepared solutions were then added to petri plates containing 15 ml water agar at 50°C and shacked gently to mix properly. After cooling, 5 cm solidified water agar discs were then transferred to plates containing Davet selective media (Davet, 1979) and were incubated at 25°C. After that, Isolates were purified and identified after proper growth (Rifai 1969).

*Inhibitory mechanism of Trichoderma isolates against Pythium aphanidermatum, Macroscopic and microscopic studies using dual culture method*

Ten Trichoderma isolates were used for dual culture study (Skidmore and Dickinson 1976). Five mm plugs of seven days old cultures of P. aphanidermatum and Trichoderma were placed in opposite sides of plates containing PDA. In case of control instead of Trichoderma plugs, PDA discs were placed. Plates were then incubated at 25°C and were daily checked in respect of Trichoderma reactions against P. aphanidermatum colonies, their growth speed and their sporulation on its mycelium. Daily measurement of radial growth of the pathogen was done but final measurements for calculations were obtained after 96 hours.

For microscopic observation of Trichoderma reaction on mycelia of the pathogen such as penetration and twisting, when PDA of plates cooled down up to 50°C, sterilized microscopic slides were kept in between plugs of Trichoderma and P. aphanidermatum inside plates in such a way that a tiny layer of the media covered the microscopic slides. After 5 days, mycelia of both fungi grew on microscopic slides; they were removed and observed under microscope for their mode of interactions (Burgess and Hepworth, 1996).

*Effect of volatile inhibitors of Trichoderma isolates on mycelial growth of P. aphanidermatum*

In this test, the technique used by Dennis and Webster (1971b) was followed. 5 mm plugs of seven days old cultures of P. aphanidermatum and Trichoderma isolates were kept in the middle of petri
plates containing PDA. The plates containing P. aphanidermatum were inverted and were kept over plates containing Trichoderma isolates. The two plates were sealed together with parafilm under sterilized condition and were incubated at 26°C. The radial growths of P. aphanidermatum were measured after 24, 48, 72 and 96 hours.

Effect of non-volatile inhibitors of Trichoderma isolates on mycelial growth of P. aphanidermatum

In order to study the effect of liquid materials produced by Trichoderma isolates in controlling mycelial growth of the pathogen, cellophane layers were used (Dennis and Webster 1971a). Cellophane layers (9 cm dia.) were covered in between layers of batman filter paper and sterilized. Cellophane layers were then placed on PDA inside plates. 5 mm plugs of seven days old Trichoderma cultures were placed over the cellophane layers. For control, only discs of PDA were placed in middle of cellophane layers. After 24 and 48 hours cellophane layers along with Trichoderma plugs were replaced with 5 mm plugs of P. aphanidermatum. All data of lab tests were calculated by the following formula:

\[
\% \text{ of colony growth inhibition} = \frac{\text{Radial growth of colony in control} - \text{Radial growth of colony in treatment}}{\text{Radial growth of colony in control}} \times 100
\]

RESULTS AND DISCUSSION

Dual culture studies

Our results indicated different preventive mechanisms of Trichoderma isolates against P. aphanidermatum under lab condition. Macroscopic observations in dual culture method showed faster growth of Trichoderma isolates and preventing the growth of P. aphanidermatum and growing on its mycelium (Figure 1 and 2) and there was significant differences in all treatments compared to control (P=0.05). Reduction of T. harzianum-2733 and T. longibrachiatum-2734 with 58% colony had highest effects against the pathogen and T. koningii-2731 with 37% colony reduction had lowest effect in this regard, respectively. Yang et al. (2004) also reported that 24 isolates of Trichoderma spp obtained from the rhizosphere of vegetable crops in which plants had survived Pythium damping-off displayed the ability to steadily colonize and aggressively attack mycelia of P. ultimum and to produce numerous conidia on Pythium colonies. Our results indicated high food consumption capacity of these isolates against the pathogen and is in agreement with results of Etebarian et al. (2000), Behboodi et al. (2005) and Bazgeer and Okhovat (1996) who reported high food consumption capacity and antibiosis mechanisms employed by Trichoderma isolates in their findings. Garrett (2011) in his book mentioned that, rhizosphere is rich in organic matter and due to better food competition of antagonists, their faster growth and sporulation on other fungi. they can suppress soil born fungal diseases and this lead to their aggressiveness. During microscopic observations of dual culture, it was noticed that mycelia of Trichoderma grew toward mycelia of P. aphanidermatum which might be due to positive tropism or materials available in pathogen's cell wall. It was also noticed that in some parts Trichoderma's mycelia twisted around mycelia of the pathogen. Trichoderma's mycelia grow alongside the mycelia of the Pythium sticking on them by producing smaller branches and penetrating into their cells by producing several historian. In other observations, twisting of Trichoderma mycelia were
observed around mycelia of *Pythium* which in some cases resulted their disruption (fig 3). Others have also reported that this attraction may be a chemical tropism due to materials or lignin present in pathogen's cell wall or it may be due to production of appressoria, penetrating organs or traps (Chet et al., 1981; Inbar et al., 1994).

**Effect of non-volatile inhibitors of Trichoderma isolates against P. aphanidermatum**

As it is obvious from table 1, all non-volatile inhibitors of *Trichoderma* isolates also had significant differences with control in reducing the colony growth of *P. aphanidermatum* (*P*= 0.05). In this connection, *T. harzianum*-2739 presented 87% colony reduction of the pathogen. *T. koningii*-2731 with 64% colony reduction also presented good ability in this respect but four *Trichoderma* isolates (*T. harzianum*-2733, *T. harzianum*-27310, *T. longibrachiatum*-2732 and *T. longibrachiatum*-2738) with 39% colony reduction of the pathogen performed lowest effects in this regard. In our study, it was observed that non-volatile inhibitors were increases by time and therefore preventory ability of *Trichoderma* isolates against the pathogen. In some cases, checking the growth progress of *Pythium* and in some other cases weakness or narrowing of pathogen's mycelia were observed. Mishra (2010) also tested extracellular enzymatic activity of *Trichoderma* isolates and among the strains tested, *T. viride* 1433 was found most effective against *P. aphanidermatum*. Dennis and Webster (1971a) reported that different *Trichoderma* species and even isolates have various inhibitory materials, which make different inhibitory patterns.

**Effect of volatile inhibitors of Trichoderma isolates against *P. aphanidermatum***

Volatile inhibitors of all *Trichoderma* isolates had significant differences with control and reduced colony growth of the pathogen (*P*=0.05). After 48 hours, *T. longibrachiatum*-2737 and *T. harzianum*-2739 with 66 and 22% colony reduction had highest and lowest effects against *P. aphanidermatum*. *T. harzianum*-2736 showed 60% colony reduction of the pathogen when *P. aphanidermatum* were exposed against *Trichoderma* isolates after 96 hours. Several works confirmed the production of volatile compounds such as trichodermin, paracellicine, trichotoxin, gliotoxin and viridian by *Trichoderma* isolates which control the pathogen's colony growth (Papavizas, 1985; Elad et al., 1982a). Dennis and Webster (1971b) introduced staldeid as the main volatile antibiotic compound of *Trichoderma*. Cook and baker (1983) in their study found that volatile and non-volatile antibiotic compounds move in between soil particles and act on pathogenic agents even without direct contact with them. They also concluded that ethanol and carbon dioxide produced by *Trichoderma* culture had antagonistic effect on pathogens. Siameto et al. (2010) also found that among several pathogenic fungi, *Pythium* sp. was most prevented by volatile antibiotic compounds of isolates of *T. harzianum* by 73%. It is reported that the quality and quantity of volatile antibiotic compounds produced by *Trichoderma* greatly depend on its isolate and the media in which it is grown (Zeppa et al., 1991).
Figure 1. Preventive effects of *T. harzianum* isolates against *P. aphanidermatum* in dual culture after 4 days (Top left: T-2733, Top right: T-2736, Bottom left: T-27310, and Bottom right: T-2739)
Figure 2. Preventive effects of *T. longibrachiatum* isolates against *P. aphanidermatum* in dual culture after 4 days (Top left: T-2734, Top right: T-2732, Bottom left: T-2737, and Bottom right: T-2738)

![Image of dual culture showing prevention effects](image)

Figure 3. Twisting of *Trichoderma* mycelia around mycelium of *P. aphanidermatum* (*Trichoderma* mycelia are thinner)

![Image of twisting mycelia](image)

Table 1. Inhibitory effects of *Trichoderma* isolates on *P. aphanidermatum* in comparison to control

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>% Inhibition in dual culture</th>
<th>% Inhibition due to volatile</th>
<th>% Inhibition due to non-volatile</th>
<th>Isolate</th>
<th>96 hours</th>
<th>48 hours</th>
<th>96 hours</th>
<th>72 hours</th>
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<td>52cde</td>
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<td>22b</td>
<td>33b</td>
<td>87c</td>
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Values of a column followed by the same letters have no significant difference at 5% (LSD test)

REFERENCES


