IN VITRO EVALUATION OF Trichoderma spp. AGAINST Phytophthora nicotianae

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ABSTRACT


The Blank sink disease of tobacco caused by Phytophthora nicotianae results in considerable loss of the crop. This lab study aimed to investigate antagonistic activity of Trichoderma spp isolates against P. nicotianae. Three isolates of Trichoderma harzianum and one of T. viridae were evaluated in vitro for their efficacy suppressing the growth of P. nicotianae. The results showed considerable antagonistic potential for the biocontrol of P. nicotianae isolate by used all of Trichoderma species. T. harzianum (0034H) was found highly inhibitory to P. nicotianae in dual culture followed by T. harzianum (0034M) and T. harzianum (0034W). T. viridae (0034S) showed least effective to inhibit the mycelial growth of P. nicotianae. Microscopical observations indicate that the inhibitory effect was caused by an interaction that took place in close contact with the host hypha, causing lysing, swelling and coiling mycelia resulting potentially reduced of mycelial growth of P. nicotianae over control treatment.

Key words: evaluation, in vitro, Trichoderma spp., Phytophthora nicotianae

INTRODUCTION

Black shank (Phytophthora nicotianae Breda de Haan) of Tobacco (Nicotiana tabacum) is among the most destructive and widespread of all tobacco-cultivated area (Prinsloo 1994). The Pathogen damage the pith (the water transport system of the plant) of a plant resulted wilting, stunting, yellowing leaves and finally plant dies (Lucas 1975). Black shank first appeared in the United States in 1915 and was first reported in North Carolina in Forsyth County in 1931. The disease can be managed in many ways. For effective black shank control, growers use a combination of crop rotation, cultivar resistance and fungicide applications (Melton 1998). Fungicides such as metalaxyl, are however expensive and growers tend to use lower than optimum dosages (Csoinos & Bertrand, 1994). Furthermore, the use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens (Bruin & Edgington, 1980). It is therefore necessary to develop alternative ways of control. One such alternative is biological control, in which microorganisms are selected for their ability to antagonize pathogens. 

Trichoderma spp. have been widely used in biological control studies against Rhizoctonia solani (Beagle- Ristaino et al. 1995) among others. However, little attention has been paid to their ability to control Phytophthora spp. in general, and P. nicotianae in particular, although their inhibitory effect on some pathogenic fungi that form zoospores suggests that they may have a role to play (Smith et al. 1990).

Literature reports mycoparasitic fungi displaying lethal mechanisms against phytopathogenic fungi that could be of importance for plant disease biological control (Benhamou & Chet, 1996; Bélager et al. 1995). Isolates of the genera Trichoderma has been the focal point of a number of studies concerning their ability to control plant pathogens. Strains of the species T. harzianum has shown effectiveness when used in disease control caused by several fungi, including Sclerotium rolfsii, a widely distributed and highly destructive plant pathogen (Benhamou and Chet, 1996), and Sclerotinia sclerotiorum affecting runner beans (Inbar et al. 1996). In a similar fashion, strains of T. harzianum, T. koningii, and T. longibrachiatum have been effective in Armillaria control in tea (Osando and Waudo, 1994), while T. koningii has been used for Fusarium solani corn rot control (McAllister et al. 1994), T. viride and Gliocladium virens have been effective in the control of Phytophthora spp. causing cotton root disease (Heller and Theiler-Hedtrich, 1994). G. virens and Trichoderma sp. have been used to control Fusarium oxysporum and Fusarium solani (Zhang et al. 1996), and Rhizoctonia solani (Askew and Waudo, 1994). T. harzianum produces antibiotics active against Botrytis cinerea (Belager et al. 1995); and T. viride, T. harzianum and Trichoderma sp. were able to stunt Sclerotium cepivorum growth the causative agent of white onion root rot (Kay & Stewart, 1994). Furthermore, Trichoderma spp. produce metabolites, volatile substances and enzymes which display antifungal activity against the basidiomycetes causing wood rot Pycnoporus coccineus, Tyromyces palustris (Doi et al. 1994a), Pholiota nameko and Lentinus edodes (Doi et al. 1994b). Bell et al. (1982) demonstrated that Trichoderma spp were effectively potential to antagonize the P. nicotianae in vitro. The present experiment was undertaken to evaluate the efficacy of the mycelial growth inhibition of P. nicotianae by Trichoderma isolates and investigating the mechanisms involved in inhibition under microscopic observation that could be used in the future for P. nicotianae control.
MATERIALS AND METHODS

Collection, identification and purification of test pathogen and antagonists

The test pathogen, *P. nicotianae* and antagonists were collected from the plant pathology lab, Woolly building and soil microbiology lab, Ross St. Building, University of Sydney, respectively. Earlier, the pathogenicity of *P. nicotianae* was confirmed in inoculating tobacco seedlings properly. *P. nicotianae* and *Trichoderma* spp. were identified by distinctive characters mentioned by Dhingra & Sinclair (1995) and Barnett (1980), respectively. Selective media that is Oat Meal Agar contains Oatmeal (25gm/litre), agar (13 gm/litre) and water was used to grow *Phytophthora nicotianae* and commercially available Potato Dextrose Agar (PDA) for *Trichoderma* spp. Then sub culturing, purification and multiplication of the above fungi following hyphal tip technique (Tuite 1969) were carried out on combined PDA and OMA (1:1) media.

Setting Dual Culture

In this study, the reduction in growth and inhibition zone in the dual culture test were used as the criteria to evaluate the in vitro antagonistic property of three isolates of *T. harzianum* (0034H, 0034M &0034W) and one isolate of *T. viridae* (0034S). The dual culture media was prepared with mixture of 1:1 PDA and OMA as both the isolates have specific growth nature to nutrient media so equal growing opportunity given to both of the isolates during duel culture technique. The in vitro evaluation consisted of placing 4mm diameter discs of the pathogen and antagonists taken from the peripheries of expanding colonies grown on mixed media. The dual culture was set in 3 different ways, firstly, the isolates of antagonist and pathogen were placed on opposite sides of the Petri dish secondly, antagonist placed in the middle and 3 discs of pathogen in the periphery of the Petri dish and thirdly, 1 disc of pathogen placed in middle and 3 discs of antagonist placed in the periphery of Petri plates. This mix cultures were incubated same time at ambient room temperature. Untreated/ control i.e., without placing the disc of the antagonist only pathogen was kept for comparison. Radial mycelial growth was measured when pathogen’s mycelial growth covered whole 9cm Petri places (at seven days after incubation).

Types of interactions were studied in dual culture on seventh day. After both the fungi came in contact with each other, the contact/inhibition zone cut using sharp blade. It was gently washed with water, mounted under lecithinol-cotton-blue over a clean glass slide and observations were made under light microscope. The reduction in mycelial growth was recorded and the percentage of inhibition over control for each treatment was calculated in this dual plate culture test as given in Table 1. The following formula was used for calculation the percentage reduction in growth, which is,

\[
\text{\% Reduction in growth} = \frac{(X-Y) \times 100}{X}
\]

Where,

- \(X\) = Growth of pathogen alone without antagonist (control)
- \(Y\) = Growth of pathogen along with the antagonist

Statistical Analysis

The laboratory experiment consisted of 5 treatments including control with five replications. The design was completely randomizes (CRD) which is used for statistical analysis. Data from the *P. nicotianae* mycelial growth inhibition were analyzed by ANOVA. Percent data were transformed arcsin/square root where necessary. Differences within the means were compared by using Fisher’s LSD (Least Significant Difference) test.

RESULTS

Mycelial growth inhibition of *P. nicotianae*

Differential action of the biocontrol agents was noticed on mycelial growth of the *P. nicotianae*. A reduction in the growth of *P. nicotianae* was evidenced when it was paired with antagonists. Among all the treatments, fast mycelial growth (6.1 cm) was recorded from the isolate-0034H of *T. harzianum* followed by isolate 0034M (6.1 cm), 0034W (4.3 cm) and 0034S (3.5 cm) (Fig.1 B,C). The results indicate that among the four species of *Trichoderma* tested for their in vitro antagonism, the *T. harzianum* (0034H) was consistently found to be the most effective with 61% reduction in radial growth of pathogen over control and was significantly superior to all other isolates. *T. harzianum* (0034M) is the next best and it reduced the growth of pathogen to 60% followed by *T. harzianum* (0034W). *T. viridae* was the least effective with 32% reduction to the test pathogen (Table 1).

Micoparasitism assays

In dual cultures of *P. nicotianae* and the antagonists several morphological changes were seen when inhibition zone were analyzed under light microscope. *T. harzianum* hyphae coiled around those of *P. nicotianae* (Fig.2/E). Under
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In the microscope, toxin like substances on the hyphal wall of strain 0034H of T. harzianum was observed (Fig.2/A). Lysis of hyphae of P. nicotianae with close contact of T. harzianum (0034H) hyphae was observed (Fig.2/B). Swelling of P. nicotianae hyphae with the mycelium of T. harzianum (0034) was identified (Fig.2/C, D). Although, penetration of Trichoderma hyphae was not observed in this study. All the observations were compared with the control plate study of P. nicotianae (Fig. E).

Table 1. In-vitro antagonism of Trichoderma harzianum against Phytophthora nicotianae

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mean mycelial growth of P. nicotiana (cm)</th>
<th>Mean mycelial growth of Trichoderma spp.</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. harzianum (0034M)</td>
<td>3.0</td>
<td>6.0</td>
<td>60</td>
</tr>
<tr>
<td>T. harzianum (0034H)</td>
<td>2.9</td>
<td>6.1</td>
<td>61</td>
</tr>
<tr>
<td>T. harzianum (0034W)</td>
<td>4.7</td>
<td>4.3</td>
<td>42</td>
</tr>
<tr>
<td>T. Viridae (0033S)</td>
<td>5.5</td>
<td>3.5</td>
<td>32</td>
</tr>
<tr>
<td>Control (no antagonist)</td>
<td>9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD at 5% level</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Dual culture of Antagonist and pathogen. A. P. nicotianae (control: without antagonist), B. Trichoderma viridae (0034S) and P. nicotianae/least suppression, C. T. harzianum (0034H) and P nicotianae (middle) /highest suppression.

Figure 2. Trichoderma harzianum (0034H) undergoes with some mechanisms inhibiting the mycelial growth of P. nicotianae. A - Toxin like substances on the mycelial wall of T. harzianum, B - Lysis of hyphae of P. nicotianae, C & D - Bulging/swelling of P. nicotianae hyphae, E - Mycelial coiling of Trichoderma and P. nicotianae. F - P. nicotianae mycelium (control culture)
DISCUSSION

Differences found in P. nicotianae mycelial growth inhibition caused by the antagonistic strains of Trichoderma spp. indicates that among these isolates there are physiological differences, and these variations could be due to the mechanism involved in the antagonistic activity by differential secretion of antifungal substances. Highest rapid growth of the antagonist isolate-0034H (6 cm) compared with slow growth of the P. nicotianae isolate (3cm) could have caused nutrient depletion, and therefore mycelial growth inhibition; this strategy is the simplest action mechanism used by some antagonists, since the inhibition was observed the probable mechanism of action involved production of toxic substances like enzymes, metabolites, antibiotics, volatile and non-volatile substances, released by the antagonist, as has been shown in other studies with phytopathogenic fungi (Askew & Laing, 1994; Kay & Stewart, 1994; Elias & Arcos, 1984). In our microscopic observation, a few drops of liquid substances at the mycelial wall of Trichoderma might be volatiles toxin substances and may involve in the antagonistic mechanisms. Although in this study, we did not test the nature of the liquid substances.

The in vitro culture of P. nicotianae and T. harzianum together led to a variety of interactions. P. nicotiana growth was generally inhibited, the hyphae lysed on dual culture media and hyphae were intensely parasitized by T. harzianum. Similar reactions were reported previously by Barnett & Binder, (1973) and Elad et al. (1983) who noticed inhibition of growth, lysis and parasitism by Trichoderma spp. of some species of Phytophthora, but not of P. nicotianae. T. harzianum produces various toxic and antibiotic metabolites (Dennis & Webster, 1971a, b; Claydon et al. 1987; Lorito et al. 1994) and enzymes (Lorito et al. 1993) which are involved in the inhibition and lysis of pathogenic fungi. In vitro and in vivo studies of the behaviour of Phytophthora spp. in the presence of antagonistic fungi such as T. harzianum, Gliocladium spp. (Smith et al. 1990) or Pythium numm and Penicillium funiculosum (Fang & Tsao, 1995a, b) have shown the biocontrol capacity of these fungi on Phytophthora spp. under controlled conditions. Salem (2005) showed that the produced antibiotics by Trichoderma that inhibited the mycelial growth of P. nicotianae induced swelling and plasmolysis of affected cell. P. nicotianae was highly antagonized by one or more isolates of Trichoderma in vitro investigated by Bell et al. (1982). Partial greenhouse control of Phytophthora infestans using commercial Trichoderma formulations was performed by Lozoya-Saldana et al. (2006).

According to Papavizas & Lumsden (1980) the mechanisms involved in the control of pathogens by Trichoderma spp. are probably: antibiosis, lysis, competition and mycoparasitism. However, Ayers & Adams (1981) indicated that interactions observed in vitro do not necessarily confirm their operation for the decrease in pathogen populations and reduction in diseases observed in natural conditions. Further investigations are needed in order to characterize the antagonist-host interactions observed during these studies.

The in vitro screening with our arbitrary system of bio-antagonists effective against soil borne pathogens is a simplistic approach to understand a small sector of biological system in diseases control. Therefore, it may be more prudent to search for biological antagonists against specific pathogen and evaluate blends of antagonists for wider applications (Baker and Cook, 1974). Our results show that although considerable success in biocontrol is achieved under laboratory conditions the outcome is not proportionate under field conditions. Hence, work is needed towards a better understanding and development of technologies that allow the biocontrol agent to spread and proliferate in soil. In addition as suggested by Papavizas (1985) research should be directed towards the improvement of strains of biological agents that are more capable of becoming established and surviving under adverse field conditions. Thus, it is obvious that biological control offers, durable environmentally safe and cost effective alternative to chemical for the efficient management of plant disease.

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