In vitro and in vivo Suppression of Pythium ultimum the Causal Agent of the Cucumber Damping-Off by Some Compost Fungi

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Abstract: Seven fungi isolated from an animal manure compost are tested for their in vitro and in vivo antagonistic activity against Pythium ultimum, the causal agent of the cucumber damping-off. In vitro, dual culture experiments, observed after incubation at 25°C on PDA during 4 days, showed that six fungi inhibited by 18 to 61% the mycelial growth of P. ultimum, whereas one fungus showed no activity. Competition for media was the most remarkable mechanism of action noted on PDA. In vivo experiments, sterilized peat individually treated with Aspergillus sp., Trichoderma viride (strain 1 and strain 2), at a rate of 0.5 g L⁻¹ and infested with P. ultimum was evaluated for its suppressive effect of the cucumber (Cucumis sativus L. Morogoui) damping-off. Results showed the tested fungi, incorporated to the culture substrates, decreased the in vivo development of P. ultimum, but at variable degrees, comparatively to the untreated control. Damping-off was lower for cucumber plants treated with T. viride (strain 2) than those treated with Aspergillus sp. and with T. viride (strain 1). Aspergillus sp. isolated from compost, which was inactive in vitro, suppressed the cucumber damping-off by 69.4%.

Key words: Biocontrol, Aspergillus sp., Trichoderma sp., inhibition, disease severity

INTRODUCTION

Pythium ultimum is a phytopathogenic fungus of a recognized importance in nurseries production. Once introduced, infection can reach a higher level because of pathogen development and spreading to the whole cultural system. Some methods are now available to prevent infection such as the use of resistant cultivars and fungicides application. However, resistant cultivars against Pythium sp. do not exist. In the other side, few fungicides are registered against this pathogen (Le Floch et al., 2003). Consequently, other alternatives for this pathogen control are required (Rankin and Paulitz, 1994).

Composts prepared from heterogeneous organic wastes showed the potential of biological control for several plant diseases, caused by soilborne pathogens (Hotink et al., 1991, 1997). Phytopathogenic fungi such as Phytophthora sp. (Hotink and Boehm, 1999; Aryantha et al., 2000), Rhizoctonia solani (Nelson and Hotink, 1983; Chung and Hotink, 1990) and Fusarium sp. (Chef et al., 1983; Cottarrera et al., 2002) were successfully suppressed by composts. Numerous reports of the suppressive effects of composts against damping-off, caused by Pythium sp., were also published (Chen et al., 1987; Chen et al., 1988; Mandelbaum and Hadar, 1990; Zhang et al., 1996; Diab et al., 2003).

Beneficial microorganisms present in composts are implicated in various suppressive activities (Hotink et al., 1991) and strains of Trichoderma sp. (Cottarrera et al., 2002) and strains of Bacillus subtilis (Phae et al., 1990) isolated from composts were effective antagonists against several

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Pathogens. Strains of *T. harzianum* suppressed *Fusarium oxysporum* (Triallle-Gay et al., 1986). Isolates of *Penicillium* sp. and of *Aspergillus* sp. were suppressive to *F. solani, F. graminearum*, *F. sambucinum* and *F. oxysporum* F.sp. *tuberosum* (Daami-Remadi et al., 2005). Several microorganisms have been reported to be biocontrol agents for suppression of *Pythium* damping-off (Harman and Hadar, 1983). Daami-Remadi (2001) also showed an antagonistic activity of *T. harzianum* against *P. aphanidermatum* and *P. ultimum*. Antagonistic interactions with other fungi and mechanism of biological control were based on antibiosis, parasitism, induced resistance and competition for space and limited resources (Hoitink et al., 1993).

Preliminary dual culture of some compost extracts with *Pythium ultimum*, showed inhibition of the pathogen mycelial growth (Kerkeni et al., 2007). The aim of this study is to test individually, some fungi isolated from compost, against *P. ultimum* and those showing higher inhibitory effect, will be evaluated for their suppressive ability of the cucumber damping-off.

**MATERIALS AND METHODS**

**Pathogen**

*P. ultimum*, was isolated from potato tuber showing typical leak symptoms. This pathogen was cultured on PDA at 25°C for one week and stored at 4°C for long preservation.

**Compost Fungi**

A mature compost (>12 months), composed of 40% cattle manure, 40% sheep manure and 20% vegetable wastes and produced on 2006 at the composting-unit of the Technical Center of Organic Agriculture of Chott Mariem-Tunisia, was used for antagonistic fungi isolations.

Potato Dextrose Agar (PDA; Sigma) supplemented with 5 mg L⁻¹ Penicillium-G was used for fungal isolation. A sample of 10 g of solid compost was suspended in 90 mL of sterilized distilled water in 250 mL bottle. The sample was stirred for 1 h at 200 rpm. A serial dilution up to 10⁻¹ was carried out and then 100 µL aliquots of this dilution were spread onto PDA medium plates. After incubation at 25°C for 5 days, formed fungal colonies were individually transferred on PDA medium. The same procedure was repeated until having a purified fungal culture. A total of seven fungi were isolated.

Selected compost fungi were identified on the basis of their macroscopic and microscopic characteristics (El-Masry et al., 2002). They were sustained on PDA at 25°C.

**In vitro Bioassay of the Antagonistic Activity of the Compost Fungi**

The antifungal activity of each tested compost fungi against *P. ultimum* was studied via the dual culture technique in the Laboratory of Phytopathology of the Regional Center of Research in Horticulture and Organic Agriculture, Chott Mariem (Tunisia). The method consists of placing an active mycelial disc (6 mm in diameter) of the pathogen, 1 cm from the edge of a 9 cm Petri plate containing freshly prepared PDA medium. Another disc (6 mm) of the antagonist fungi was deposited in a diametrically opposed position 1 cm away from the other set of the plate. For untreated plates, an agar disc of *P. ultimum* was placed at the center of the Petri dish. All plates were then incubated at 25°C and evaluated for pathogen growth inhibition after 4 days of incubation. Three replicates were used per elementary treatment.

To determine the inhibition rate of *P. ultimum* by each of the tested compost fungi, the fungal growth of *P. ultimum* was recorded by measuring the colony diameters (average of the two perpendicular diameters). These diameters (control and treated) served for the calculation of the inhibition rate of the fungal growth. This rate is calculated according to the following formula used by Hibar et al. (2005):
Inhibition rate (%) = \frac{1 \cdot \text{Average diameter of the treated}}{\text{Average diameter of the control}} \times 100

**In vivo Bioassay of the Antagonistic Activity of the Compost Fungi**

**Plant Material**

Disease suppressiveness of substrates individually amended with the tested compost fungi, against *P. ultimum* was tested using cucumber (*Cucumis sativus* L. Monnegui) (Tunisian cultivar) as host plant. Seeds of cucumber were surface-disinfected in 1% sodium hypochlorite for 30 min and rinsed several times with sterile distilled water.

**Preparation of Inoculum**

*P. ultimum* was grown at 25°C on Potato Dextrose Agar (PDA). Three fungal plugs with active pathogen mycelium (0.6 cm) were used to inoculate rice grains (25 g). This substrate was already sterilized for two consecutive days (Diab et al., 2003). Inoculated rice was then incubated in the dark for 10 days at 20°C (Fuchs, 1993). After incubation, the pathogen-colonized rice was ground aseptically with a spatula and used as inoculum in this experiment.

Three fungal plugs with active mycelium of each of the tested compost fungi (0.6 cm) were also used to inoculate rice grains (25 g) and the same method was adopted for the preparation of the inoculum of the compost fungi. They were grown at 25°C.

**Bioassay**

Among the seven compost fungi tested *in vitro*, three fungi C2-4, C2-5 and C2-6, identified as *Aspergillus* sp., *Trichoderma viride* (strain 2) and *T. viride* (strain 1), respectively, were selected for the *in vivo* evaluation during 2006, in the greenhouse of the high Agronomic Institute of Chott Mariem, in Tunisia. The remaining fungi were not identified.

Sterilized peat was mixed with rice grains colonized at the rate of 0.5 g L⁻¹ of *P. ultimum* and 0.5 g L⁻¹ of each tested compost fungi. For an uniform distribution of the inoculum in the substrate, 0.5 g of rice cultures were mixed with 50 g of sterilized sand. The mixture was then homogenized with the substrate (peat) and was distributed equally into 10 cm-diameter plastic pots. Eight cucumber seeds (*Cucumis sativus* L. Monnegui) were sown at equal circular spacing in each pot.

Bioassay was conducted under greenhouse conditions at 25°C and under a photoperiod of 12 h dark/12 h light. The plants were watered regularly. Five replicate pots of each elementary were used and the whole bioassay was repeated twice.

**Disease Assessment**

Final evaluations of the damping-off suppressiveness were made after 14 days. The disease intensity was estimated on treated and inoculated plants comparatively to the untreated and non inoculated controls. The Disease Suppressiveness (DS) of the substrates amended with compost fungi, against *P. ultimum* was calculated as:

\[ \text{DS} = \frac{D_{\text{treated}} - D_{\text{untreated}}} {D_{\text{treated}}} \times 100(\%) \]

Where, the disease intensity in the substrates without compost fungi (\(D_{\text{untreated}}\)) was calculated as:

\[ D_{\text{untreated}} = \frac{H_{\text{untreated}} - H_{\text{control}}} {H_{\text{control}}} \times 100(\%) \]

And the disease intensity in the substrates supplemented with compost fungi (\(D_{\text{treated}}\)) was calculated as:
\[ DI_d = \frac{HP_{d,0} - HP_d}{HP_{d,0}} \times 100(\%) \]

Where,
- \( HP_{d,0} \) = No. of healthy plants for the control b.
- \( HP_{d} \) = No. of healthy plants for the control a.
- \( HP_{d,0} \) = No. of healthy plants for the substrates with either of the three tested compost fungi and non inoculated with \( P. \ ultimum \) (0).
- \( HP_{d} \) = No. of healthy plants for the substrates inoculated with \( P. \ ultimum \) (i) and with either of the three tested compost fungi.

At the end of the bioassay, the mean shoot and root fresh weights of plants per elementary treatment were also determined.

**Detection of Cellulase**

The cellulase enzyme is a lysogenic enzyme that could have a possible role in fungal degradation. It was analysed in the substrates amended with either of the three tested fungi, according to the method of Alef and Nannipieri (1995). Cellulase activity was expressed as micrograms of hydrolyzed glucose per gram dry weight of substrate per 24 h.

**RESULTS**

**In vitro Inhibition of the \( P. \ ultimum \) Growth by the Tested Compost Fungi**

The results in Table 1 showed that, of the seven compost fungi tested, six fungi inhibited the mycelial growth of \( P. \ ultimum \) by more than 18%, comparatively to the untreated control. The most effective fungus was C2-5, where the pathogen development was of about 61%. Whereas, the fungal isolate C2-4 showed no inhibitory effect. The compost fungi C2-6 inhibited \( P. \ ultimum \) growth by 33% (Table 1).

Competition, traded by overlapping of the pathogen colonies by tested fungal isolates, was the main mechanism, employed by the most effective antagonist to \( P. \ ultimum \).

**In vivo Inhibition of the \( P. \ ultimum \) Growth by Some Compost Fungi**

The two isolates C2-5 and C2-6, showing an inhibition rate of more than 30% *in vitro*, were identified as \( T. viride \) strain 2 and strain 1, respectively. The isolate C2-4 showing no *in vitro* inhibitory effect against \( P. \ ultimum \) was identified as *Aspergillus sp.* Those three fungi were selected for their *in vivo* antagonistic activity evaluation. Results showed that those compost fungi were suppressive to the damping-off caused by \( P. \ ultimum \) (Table 2). Suppression percentage of \( P. \ ultimum \) was significantly higher in substrate amended individually with \( T. viride \) strain 2 (C2-5) and

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Inhibition rate (%)</th>
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<tbody>
<tr>
<td>C2-1</td>
<td>20c</td>
</tr>
<tr>
<td>C2-2</td>
<td>18c</td>
</tr>
<tr>
<td>C2-3</td>
<td>30b</td>
</tr>
<tr>
<td>C2-4</td>
<td>0d</td>
</tr>
<tr>
<td>C2-5</td>
<td>61a</td>
</tr>
<tr>
<td>C2-6</td>
<td>33b</td>
</tr>
<tr>
<td>C2-7</td>
<td>24c</td>
</tr>
<tr>
<td>Control</td>
<td>0d</td>
</tr>
</tbody>
</table>

Compost isolates C2-1, C2-2, C2-3 and C2-7 were not identified; isolates C2-4, C2-5 and C2-6 were identified as *Aspergillus sp.*, *Trichoderma viride* (strain 2) and *T. viride* (strain 1), respectively. They were selected for *in vivo* evaluation. Each value represents the mean of 3 values. Different letter(s) within columns represent values that are significantly different at \( p = 0.05 \) based on ANOVA and Duncan test.
Table 2: Suppression percentage of cucumber damping-off, caused by *P. ultimum*, by some compost fungi

<table>
<thead>
<tr>
<th>Antagonists</th>
<th><em>Trichoderma viride</em> str 2 (C2-5)</th>
<th>Aspergillus sp.</th>
<th><em>Trichoderma viride</em> str 1 (C2-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppression (%)</td>
<td>87.1a</td>
<td>69.4b</td>
<td>10.4c</td>
</tr>
</tbody>
</table>

Each value represents the mean of 5 values. Different letters within columns represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test.

Table 3: Cellulase activity noted in the three substrates amended with tested compost fungi and inoculated with *Pseudomonas ultimum*

<table>
<thead>
<tr>
<th>Treatments (μg Glucose/g/24 h)</th>
<th><em>T. viride</em> (strain 2)</th>
<th>Aspergillus sp.</th>
<th><em>T. viride</em> (strain 1)</th>
<th>Control a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td>136.9b</td>
<td>131.3b</td>
<td>94.58c</td>
<td>164.32a</td>
</tr>
</tbody>
</table>

Each value represents the mean of 3 values. Different letter(s) within columns represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test.

Fig. 1: Shoot and root fresh weights of 14 days old cucumber seedlings. Control a: Substrates with *P. ultimum* alone. Control b: Substrates non inoculated. Each value represents the mean of 5 values. Different letter(s) within columns represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test.

Aspergillus sp. (C2-4). Contrarily to the in vitro results, Aspergillus sp. which found to be inefficient, showed an important suppression rate (69.4%) in vivo. Suppressive activity induced by *T. viride* strain 1 (C2-6) was lower than that noted in in vitro tests (10.4%).

Shoot and Root Fresh Weights

Figure 1 and 2 showed that the pathogen presence in the substrates resulted in a pronounced decrease in the cucumber shoot and root fresh weights, compared to the non inoculated control (control b) and to substrates individually amended with the tested fungi. Presence of *P. ultimum* alone in the substrate decreased significantly the fresh weight of cucumber plants in comparison to substrates with the two most effective compost fungi Aspergillus sp. and *T. viride* strain 2. The reduction in shoot and root fresh weights amounted to 51.67 and 50%, respectively in the control a, inoculated with *P. ultimum* alone. No shoot fresh weight reduction was noted in the presence of *T. viride* strain 2 (Fig. 1). Whereas, root fresh weight reduction for this treatment, was not very important compared to the non inoculated control (control b).

Cellulase Activity

The results showed that cellulase enzyme was present in the tested substrates. Table 3 indicates that the highest cellulase activity was detected in the culture substrate amended with *T. viride* (strain 2) (136 μg Glucose/g/24 h) and in that supplemented with *Aspergillus* sp. (131.3 μg Glucose/g/24 h). Whereas, the least cellulase activity (94.58 μg Glucose/g/24 h) was obtained in the substrate amended with *T. viride* (strain 1).
DISCUSSION

These results showed for the first time in Tunisia that fungi isolated from an animal manure compost are suppressive against the phytopathogenic fungus *Pythium ultimum*. In a previous study, Daami-Remadi (2001) reported a suppressive effect against *P. ultimum* induced by a local *T. harzianum* isolated from soil.

The current study showed that some of the tested compost fungi were able of inhibiting the mycelial growth of *P. ultimum* and suppressing the cucumber damping-off. This results joined that obtained by Phuc et al. (1990), Zhang et al. (1998) and Bess (2000), showing that compost contain microorganisms suppressive to plant pathogens. The effectiveness of compost fungi against plant diseases as biocontrol agents was previously found by Daami-Remadi et al. (2006), where an antagonistic effect of some filamentous fungi, isolated from compost, was noted against the *Fusarium* sp. complex, causing dry rot of potato tubers. El Masry et al. (2002) and Muhammad and Amusa (2003) isolated from compost, several fungal microorganisms such as *Aspergillus niger*, *Rhizopus* sp., *Drechlera* sp. and *Trichoderma harzianum*, showing an inhibitory effect against *Pythium aphaniidermatum*, *Fusarium oxysporum* and *Rhizoctonia solani*.

In vitro, all fungi tested induced *P. ultimum* growth reduction by more than 18%, excepting *Aspergillus* sp., seeming to be inactive via dual culture experiments. *T. viride* (strain 2) was the most effective in reducing the mycelial growth of *P. ultimum* where the inhibition ratio was of about 61%. Previous work conducted in vitro by Kerkeni et al. (2007), showed that the whole compost extract
used for fungi isolation, inhibited the growth of this same isolate of *P. ultimum* by only 14.5%. This suggests that composts and compost extracts contain biocontrol agents that are more efficient when used alone and other agents with lesser inhibitory effect when used individually, probably this is the case with *Aspergillus* sp.

Competition for nutrients present in the media, traduced by an overlapping of the pathogen colonies by that of the tested fungi, was the main mechanism, employed by the compost fungi during antagonism of *P. ultimum* in dual culture. According to Zhang et al. (1996), competition for carbon can partially explain the suppressive effect noted against *Pythium* sp. Whereas, for Mandelbaum and Hadar (1990), competition for nutrients was considered an important mechanism of suppression of the seedling damping off caused by *Pythium aphanidermatum*.

The variable efficiency of the tested fungi may be attributed to a variable mode of action and/or a variable type of antifungal metabolite produced by the antagonists (Williams and Asher, 1996).

The overlapping of the *P. ultimum* colonies by antagonists colonies is probably due to a physical contact between pathogen and the compost fungi and parasitism thus, mycelium destruction may occur. In fact, multiple mechanisms of action including mycoparasitism, lysis, induction of mycelial cords and early chlamydospores formation were observed in dual cultures of some compost fungi with four species (Ayed et al., 2006; Daami-Remadi et al., 2006).

It is known that the *in vitro* bioassays results may be different with those obtained under *in vivo* conditions (Imam-ul-Haq et al., 2003). In fact, in the present study, the selected potential biocontrol agents are also tested, on the basis of their high inhibition ratio, for their ability to suppress the cucumber *Pythium* damping-off under greenhouse conditions.

Contrarily to the *in vitro* assay, bioassay conducted *in vivo*, showed that *Aspergillus* sp. significantly suppressed the disease severity on cucumber seeds and showed higher antifungal activity comparatively to *T. viride* (strain 1). Tests based on *in vitro* mycelial inhibition do not always correlate with biocontrol efficacy under natural conditions. This finding proved that *in vitro* and *in vivo* results may be divergent as may be due to the variable physical and chemical properties within niches occupied by the biocontrol agents who may affect both root colonization and expression of biocontrol mechanisms.

Howell (2003) reported that biocontrol agents produce enzymes such as chitinase, protease and cellulase. These enzymes have been proved to be involved in the antagonistic activity, they act by breaking down and dissolving the polysaccharides, responsible for the rigidity of fungal cell wall. Cellulase enzyme having the ability to degrade and to hydrolyze the fungal cell wall, was detected in the culture substrates used in this current study. A higher enzyme activity of cellulase was noted in the culture substrate supplemented with *T. viride* (strain 2) and with *Aspergillus* sp. (136 and 131.3 μg Glucose/g/24 h, respectively). Whereas, lesser activity was noted with *T. viride* (strain 1) (94.58 μg Glucose/g/24 h) which was also correlated to a lesser suppressive effect *in vivo* against *P. ultimum*. However, *in vivo* suppression of *P. ultimum* could not be only attributed to the action of this enzyme, since an important cellulase activity was also noted in the substrate inoculated only with the pathogen. Probably this enzyme was produced by all the fungi, including the pathogen, for the decomposition of organic matter contained in the substrates.

*Aspergillus* sp. was able to suppress *P. ultimum* only *in vivo*, probably by the induction of systemic resistance on cucumber plants, which seems to be not frequently detected *in vitro* (Schäfer et al., 1997).

These results showed that compost fungi are suitable products to suppress plant pathogenic fungi. All tested fungi showed an *in vivo* antagonistic activity against *P. ultimum*. Thus, they could be a promising way for the biological control of plant diseases and could reduce the need of fungicides use and they are safe for the environment preservation.
REFERENCES


