Biological Control of Gray Mold Disease (*Botrytis cinerea*) on Tomato and Bean Plants by Using Local Isolates of *Trichoderma harzianum*

Radwan Mahmoud Barakat and Mohammad Ibraheem Al-Masri

ABSTRACT

Replacement of fungicides with biocontrol of foliar diseases is an alternative mean of managing plant pathogens. One of the most important biocontrol agents is *Trichoderma* spp. Forty-seven isolates of *Trichoderma* spp. recovered from one hundred sixty nine surface soil samples, were collected from different agricultural fields throughout the West Bank. The antagonistic potential of the obtained isolates against gray mold disease (*Botrytis cinerea*) was investigated in dual culture and bioassays under growth chamber conditions. The results of this study indicated that the isolates (H1, H2, Jn14, T33, T35, N40, and R43) were significantly (P<0.05) reducing the disease by 50%-55%. The highly effective *Trichoderma harzianum* Jn14 isolate was applied as spore suspension and in talc formulation. Disease was reduced significantly (P<0.05) in both methods with different spore concentrations (10⁴, 10⁵, 10⁶ and 10⁷ CFU/ml) on tomato and bean plants. The disease reduction at concentration 10⁷ was 56% and 73% applied as talc formula and 59% and 77% as spore suspension on tomato and bean plants, respectively.

KEYWORDS: Biological control, *Trichoderma harzianum*, Gray mold Disease, *Botrytis cinerea*.

INTRODUCTION

Gray mold is one of the ubiquitous and potentially serious fungal diseases of greenhouse tomato (*Lycopersicon esculentum* Mill.). The causal agent, *Botrytis cinerea* Pers.: Fr., infects a wide range of host plants under a wide range of conditions and over a widely distributed area and survives saprophytically for long periods in plant debris. Resistance to gray mold has not been recorded yet in cultivated tomato. The protectant fungicides currently registered for gray mold on greenhouse tomato inhibit germination of spores but cannot cure an infection in progress (Jana et al., 2001). Furthermore, *B. cinerea* has developed resistance to benomyl, vinclozolin, and other fungicides (Moorman and Lease, 1992). In addition, many fungicides labeled for gray mold control on greenhouse tomato are under Environmental Protection Agency (EPA) scrutiny as potential carcinogens. Future availability of fungicides for greenhouse use on vegetables may be limited because of concerns for the environment and human safety. There has been a strong research and development efforts in the area of biological control of plant pathogens (Elad et al., 1996; 2000). Bioagents are generally found in all soils including forest humus layer (Wardle et al., 1993), and in agricultural and orchard soils (Domsch et al., 1980; Chet, 1987; Roiger et al., 1991). *Trichoderma* spp. are known antagonists of other fungi (Papavizas, 1985; Samules, 1996) and have been shown to be a very potent biocontrol agents of several soil borne plant pathogenic fungi under both greenhouse and field conditions (Chet, 1987; Inbar et al., 1994). *Trichoderma harzianum*, alone or in combination with other *Trichoderma* species or chemicals and other agriculture practices has been used for control of several plant diseases ; *Rhizoctonia* damping off radish (Lifshitz and Baker, 1985), corn and soybean (Kommedahl et al., 1981); gray mold on tomato (Migheli et al., 1994; O'Neill et al., 1996; Elad, 2000); gray mold on grapes and strawberry (Elad et al., 1995; Harman et al., 1995); *Colletotrichum* storage rot of apple (Tronsmo and Hjeljord, 1995); cucumber fruit rot (*Rhizoctonia solani*) (Lewis and Papavizas, 1980); *Sclerotinia sclerotiorum* in pea (Knudsen and Eschen, 1991); lettuce drop (*Sclerotinia minor*) (Vannacci et al.,

* Dept. of Plant Production and Protection, College of Agriculture, Hebron University, Hebron, Palestine. Received on 5/7/2004 and Accepted for Publication on 9/3/2005.
1991); and a wilt-complex caused by Sclerotium rolfsii, Rhizoctonia solani and Fusarium oxysporum in lentil and chickpeas (Mukhopadhyay, 1995).

The aim of the present work was to study the distribution and presence of the bioagent Trichoderma species in Palestinian agricultural soils in the West Bank, and to evaluate their antagonistic potential against gray mold disease (Botrytis cinerea) of tomato and bean plants in an attempt to prepare a suitable formulation of the most promising Trichoderma isolates to be considered for future control application.

**MATERIALS AND METHODS**

**Bioagents Isolation**

One hundred sixty nine soil samples were collected from various agricultural fields from eight areas in the West Bank (Hebron, Jericho, Nablus, Jenin, Tulkarim, Qalqelia, Ramallah, and Bethlehem) during summer 2000. Soil samples were collected from the upper 5-10 cm of soil surface. Sampled fields were planted previously with either vegetables or wheat.

Isolates were recovered from soil samples according to the following procedure: Twenty-five grams of air-dry soil sample were suspended in 225 ml of 0.1% water agar and shaken for 20-30 minutes in a rotary shaker at 200 rpm. Serial dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were made out of each soil sample, and 0.1 ml of 10^{-4} soil suspension was pipetted into 90-mm diameter Petri dishes and spread with a glass rod on Trichoderma-Selective Media (TSM) surface. Five Petri plates considered as replicates were used for each soil sample and suspension concentration. The plates were incubated for 5-7 days at 25°C, and then Trichoderma spp. were transferred to potatoes dextrose agar medium (PDA) for purification.

Trichoderma - Selective Media (TSM) consisted of the following components (g/l distilled water): magnesium sulfate heptahydrate (MgSO_{4} \cdot 7H_{2}O), 0.2; potassium phosphate dibasic – anhydrous (KH_{2}PO_{4}), 0.9; potassium chloride (KCl), 0.15; ammonium nitrate (NH_{4}NO_{3}), 1.0; glucose, 3.0; chloramphenicol (Chloromycetin, Sigma Chemical Co., USA), 0.25; dexon (p-dimethylaminobenzene diazo sodium sulfonate 60% W.P. (Bayer A.G., Germany), 0.3; pentachloronitrobenzene (Terracolor 75% W.P, Olin Chemicals, USA) PCNB, 0.2; rose bengal (Tetrachlorotetradiodfluorescein, BDH Chemicals Ltd., England), 0.15 and agar, (Difco Laboratories, USA), 20 (Elad et al., 1981).

**Evaluation of Antagonistic Potential**

**A. Dual Culture**

The interaction between Botrytis cinerea strain (Bc.A) previously isolated from naturally infected tomato leaves growing in greenhouse and the hyphae of forty-four Trichoderma isolates was evaluated. Five mm diameter mycelial plugs collected from 6 days-old cultures of Trichoderma isolates with the phytopathogen strain (Bc.A) were placed 3 cm apart on the surface of 5 PDA-plates and incubated at 25°C under continuous light. The experimental design used was the Completely Randomized Design (CRD) with five replicates (plates). The most effective Trichoderma isolates (H7, Jn11, Jn14, Jn20, Jn21, Jn23, Q27, Q29, T35, T36, and N40) were reevaluated as mentioned above at 15, 20, 25, 30 and 35°C under continuous light. The experimental design was completely randomized (CRD) as well and included five replicates (plates). The antagonist and the plant pathogen grew towards each other, and the over growth of B. cinerea mycelium by the hyphae of Trichoderma spp. isolates occurred at 3-5 days after inoculation. The interactions were measured during 10 days and the Coiling Growth Rate (CGR) mm/day was calculated, in addition to the mycelium growth rate (MGR) (cm²/day) of the Trichoderma isolates, measured at 15, 20, 25, 30 and 35°C.

**B. Bioassay**

**Plants**

Tomato (Lycopersicon esculentum, L., cv. Facultia 1684) and bean (Phaseolus vulgaris, L., cv., Helda) plants were grown in a peat/vermiculate/perlite mixture (1:1:1, v/v) in 17-cm pots in a greenhouse at 20-25°C. Plants were fertilized weekly with commercial fertilizer (20:20:20 plus trace elements). Eight weeks-old tomato plants and six weeks-old bean plants at flowering stage were used in the experiments.

**Bioagent Application**

**Spore Suspension**

Trichoderma isolates collected from soil were prepared as spore suspension. Ten-day old cultures were...
suspended in Sterile Distilled Water (SDW) containing 2% sucrose and 1% potassium dihydrogen phosphate (KH$_2$PO$_4$) to get final concentration of *Trichoderma* spores $3\times10^6$/ml (CFU). Tomato plants were sprayed (40 ml/plant) with spore suspension 8 hours before inoculation with *B. cinerea* suspension. *B. cinerea* used in the inoculation was previously isolated from naturally infected tomato leaves and maintained and grown on potato dextrose agar (PDA, Oxiod). Conidia suspension of *B. cinerea* were prepared from 14-days old cultures by harvesting conidia from sporulating cultures in SDW and filtered through muslin. Spore concentration was determined with a hemocytometer and adjusted as (5x10$^5$/spore/ml). Additives of 2% sucrose and 1% KH$_2$PO$_4$ were added to spore suspension (ONeill et al., 1996). *Trichoderma* treated plants were then inoculated by micro sprayer with conidial suspension of *B. cinerea* (20ml/plant). The application of water containing 2% sucrose and 1% KH$_2$PO$_4$ served as the negative control treatment, where as the same solution with *B. cinerea* conidial suspension served as the positive control. Plants were covered with transparent polyethylene sacks and incubated in a growth room at 25ºC and a 12h photoperiod. Disease severity was evaluated 7, 14, 21, and 28 days after incubation as disease severity (%). The disease severity data at the end of the evaluation period (28 days) are presented.

**Talc Formulation**

The most effective isolate (Jn14) was taxonomically identified to be *Trichoderma harzianum* according to taxonomic key described by Bissett (Bissett, 1991). The *T. harzianum* strain Jn14 was grown on 90 mm PDA medium plates for 7-10 days. Autoclaved and sealed erlenmeyer flasks (500ml) containing 100 ml potato dextrose broth (PDB, Difco) were inoculated with 5mm mycelium plug of the strain Jn14. The flasks were fitted on a shaker at 200 rpm and incubated at 25ºC in a growth room for 12 days. The suspension was filtered through sterile glass funnel (max. pore size: 40-100 µm), and the mycelial mat discarded. The filtrate suspension containing spores was centrifuged at 5000 rpm for 15 mins and the supernatant was discarded. The spore pellets were washed with adding sterile distilled water, vortexed to ensure homogeneous suspension and centrifuged as discussed earlier (Repeated 2 times). After washing, the pellets were recovered in sterile water and the final volume was brought up to 100ml. The 100 ml spore suspension was added to 200ml of talc powder (Sigma, T-2015) and stirred with a sterile spatula. The mixture was placed on aluminum foil in a sterile tray (in the form of small heaps) in a disinfected aerated oven at 20ºC for 48-72 hours. Once dried, the heaps looked like “cakes” were crushed and sieved (200 µm) under sterile conditions to avoid contamination. The talc-formula can be stored dried in a tightly closed flask either at 4ºC or at room temperature. The density can be evaluated by counting the number of CFU/g of talc by using dilute plates techniques.

**Effect of the Isolate Jn14 on Gray Mold Disease Severity**

Eight weeks-old tomato plants and six weeks-old bean plants at flowering stage were sprayed with Jn14 as spore suspension and in talc formulation. Each plant was treated (30ml/plant) by both methods at concentrations of 0, 10$^3$, 10$^4$, and 10$^5$ CFU/ml. For each concentration, 5 plants (replicates) were used for both tomato and bean plants in a completely randomized design. *Trichoderma* treated plants were inoculated with conidial suspension of *B. cinerea* after 24h by micro sprayer (20ml/plant and concentration of 5*10$^5$ CFU/ml). Negative control treatment plants (without pathogen) were sprayed with water containing 2% sucrose and 1% KH$_2$PO$_4$, and positive control treatment plants (with pathogen) were inoculated with *B. cinerea* conidial suspension. Plants were covered with transparent polyethylene sacks and incubated in a growth room at 25ºC and photoperiod 12h. Disease was evaluated after 7, 14, 21, and 28 days of incubation and expressed as disease severity (%).

**Antibiosis**

The ability of the isolates, Jn14, T35, and J8 to inhibit the mycelium growth of *B. cinerea* through the production of fungitoxic metabolites at 15, 20, and 25ºC was tested according to the method of Dennis and Webster (1971). The three autoclaved, sealed erlenmeyer flasks (250ml) containing 50 ml of potato dextrose broth (pH =6) were inoculated with 5mm mycelial disks of 6-days old cultures of the isolates Jn14, T35, and J8 and incubated at 25ºC without shaking. After 10 days, the culture filtrate was sterilized through Millipore membrane filter (0.45 µm) and added to PDA at 10% (V/V). The filtrate-amended PDA was incorporated into 15 plates for each isolate. The plates were centrally inoculated with 5mm mycelial disks of *B. cinerea*. Each
five plates of the three isolates were incubated at 15, 20, and 25°C. Un-amended PDA served as control. Colony diameters were measured at 48, 82, and 130h. The growth rate (cm²/day) of the fungus at 130h was calculated.

Statistical Analysis
Data of mycelium growth rate, coiling growth rate and disease severity were analyzed statistically using one-way repeated measurement analysis of variance (ANOVA) to test for significance, and Fisher LSD test was used for mean separations (Sigma Stat program).

Results

Trichoderma Isolates
Forty-seven Trichoderma spp. isolates were recovered from 169-soil samples collected from different areas in the West Bank (Table 1 and Fig. 1). Trichoderma isolates are ubiquitous in soil found in open field and greenhouse soils. Most of the isolates were found in non-treated soils for the last three seasons; samples were taken from soils previously fumigated with methyl bromide, irrigated with fungicide Danion, or solarized. Some isolates were recovered from treated soils; the isolate Q29 was recovered from a greenhouse soil cultivated with cucumber and was irrigated with the fungicide Danion during the same season and fumigated with methyl bromide four years earlier. The isolate T32 was recovered from greenhouse soil cultivated with jews mallow and treated with the fungicide Danion during the same season through the irrigation system. The isolate B45 was recovered from intensively cultivated open field with bean and fumigated with methyl bromide three years earlier.

Dual Culture and Bioassay
The growth rate of Trichoderma spp. isolates over B. cinerea isolate (Bc.A) mycelium was significant \((P \leq 0.05)\) between isolates, with the lowest growth \((1\ \text{mm/day})\) recorded for the isolate H3 and the highest \((14.9\ \text{mm/day})\) for the isolate Jn14. The growth rate of the most effective isolates \((\text{Jn14, Q24, Q29, Q30, R42, Jn20, Jn18, Q26, T33, T36, R43, B45, R34, and Q25})\) ranged between 12-15 mm/day (Fig.1A).

Most of the Trichoderma spp. isolates significantly reduced gray mold disease severity on tomato plants and disease reduction ranged between 5.5% and 55%. Variability between isolates observed was significant \((P \leq 0.05)\). The most effective isolates \((\text{H1, H2, Jn14, T33, T36, N40, and R43})\) reduced the disease by 50%-55% (Fig.1B).

The Trichoderma harzianum isolates were repeatedly tested for growth rate and coiling growth rate over B. cinerea at 15, 20, 25, 30, and 35°C. The results showed that the CGR and MGR between isolates were significantly observed at 25 and 30°C, but not at 15°C, 20°C and 35°C. The most coiling isolate was Jn14 (31mm/day) (Fig. 2 A and B) and the lowest isolate was Q29 (7.5 mm/day).

The T. harzianum isolate Jn14 was applied as spore suspension and in talc formulation. Disease reduction was significant \((P \leq 0.05)\) in both methods at the concentrations of \(10^{4}-10^{7}\) CFU/ml on tomato and bean plants. The reduction of disease on tomato plants in both methods was similar at the concentrations of \(10^{5}\) and \(10^{7}\) CFU/ml while the reduction was significantly different on bean plants. Disease reduction at the concentrations \(10^{7}\ CFU/ml\) was 56% and 73% applied as talc formulation and 59% and 77% as spore suspension on tomato and bean plants, respectively (Fig. 3 and Table 2).

Antibiosis
The effect of fungitoxic metabolites produced by the isolates Jn14, T35, and J8 in broth was indicated by mycelium growth rate of B. cinerea in amended media (Table 3). The growth rate of B. cinerea was significantly \((P \leq 0.05)\) reduced after incubation at temperatures 15, 20, and 25°C in amended media that assumingly containing fungitoxic compounds. The variation in growth rate of B. cinerea on PDA-amended media with metabolites of Jn14, T35 and J8 was evaluated. Growth rate of B. cinerea was lowest on media amended with T35 toxic metabolites, and growth decreased with increasing incubation temperature.

Discussion
The number of Trichoderma spp. isolates (47) showed that this fungus is abundant in Palestinian soils especially those non-chemically treated for the last three seasons. Solarized soils proved to enhance the growth and proliferation of Trichoderma spp (Chet, 1987). The efficiency of Trichoderma spp in reducing mycelial growth of B. cinerea in dual culture and bioassay experiments are shown in Fig.1. This data showed significant variation between different isolates in this respect. Disease reduction in bioassay experiments
ranged between 5.5%-55%. The most effective *Trichoderma* spp. isolate (Jn14) was taxonomically identified to be *T. harzianum*. The reduction of disease using isolate Jn14 was 56% and 73% applied as talc formulation and 59% and 77% as spore suspension on tomato and bean plants, respectively, using the highest concentration $10^7$ CFU/ml. There were no significant differences between the two formulations used (talc and spore suspension) in reducing disease severity (Fig. 3 and Table 2). Similar results were observed when *T. harzianum* strain T39 was used in reducing the incidence of gray mold on tomato leaves infection (31%) and on fruit (84%) with a 57% reduction of the disease incidence (Elad et al., 1995). Similarly, gray mold disease on tomato plants was reduced by at least 77% by *T. harzianum* T39 when applied as Trichoderex™ 20 SP (Makhteshim Chemical Works) at concentration of 0.4g a.i.1⁻¹ (Elad, 2000). The mode of action of *Trichoderma harzianum* on the pathogen includes mycoparasitism, competition and antibiosis. Mycoparasites produce fungal cell-wall- degrading enzymes such as chitinases and glucanases to dissolve their fungal hosts cell walls and penetrate the cells (Lorito et al., 1993). Competition is effective when the pathogen conidia need exogenous nutrients for germination and germ-tube elongation. Similar results were observed by several workers (Elad, 1995; 1996 and 2000; Lorito et al., 1993). The growth rate of *B. cinerea* was significantly ($P \leq 0.05$) reduced due to the production of fungitoxic substance by the isolates Jn14, T35, and J8 in liquid culture at incubation temperatures of 15, 20, and 25°C (Table 3). Antibiotic substances have been suggested earlier to be involved in biocontrol by *Trichoderma* spp. (Sivasithamparam and Ghisalberti, 1998), who listed 43 substances produced by *Trichoderma* spp. that have antibiotic activity. Of these, alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines and steroids have frequently been associated with biocontrol activity.

**CONCLUSION**

Results of the present study support the hypothesis that replacement of fungicides with biocontrol of foliar diseases can be a potential alternative management of plant pathogens. In this context, the isolates of *T. harzianum* used in this study were effective in reducing gray mold disease on tomato and bean plants. Applying biological control in the field by using *T. harzianum* is influenced by many environmental, biological, and physical factors. In light of this, further investigations are needed to improve the ability of the promising isolates in controlling the disease under field conditions, in addition to further studies that need to take place in respect to the mode of action.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>District</th>
<th>Site</th>
<th>Cultivation</th>
<th>Soil treatment in previous two seasons</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7</td>
<td>Hebron</td>
<td>Wad Fraides</td>
<td>Cucumber (Greenhouse)</td>
<td>Non-treated</td>
</tr>
<tr>
<td>J8</td>
<td>Jericho</td>
<td>Ketif Al-Wad</td>
<td>Grape</td>
<td>Non-treated</td>
</tr>
<tr>
<td>J10</td>
<td>Jericho</td>
<td>Ayne Al-Sultan</td>
<td>Banana</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn11</td>
<td>Jenin</td>
<td>Al Jalameh</td>
<td>Cucumber (O.field)</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn14</td>
<td>Jenin</td>
<td>Qabatyia</td>
<td>Cucumber (O.field)</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn17</td>
<td>Jenin</td>
<td>Qabatyia</td>
<td>Eggplant</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn20</td>
<td>Jenin</td>
<td>Al-Jarbaah</td>
<td>Tomato</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn21</td>
<td>Jenin</td>
<td>Al-Jarbaah</td>
<td>Pea</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Jn23</td>
<td>Jenin</td>
<td>Al-Jarbaah</td>
<td>Pepper</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Q25</td>
<td>Qalqilya</td>
<td>Hableh</td>
<td>Tomato (O.field)</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Q29</td>
<td>Qalqilya</td>
<td>Azon</td>
<td>Cucumber (Greenhouse)</td>
<td>Irrigated by fungicid; Dainon in present season and fumigated with methyl bromide before four years.</td>
</tr>
</tbody>
</table>
Table 2. Effect of different concentrations (CFU/ml) of *Trichoderma harzianum* isolate Jn14 applied in talc-formulation and spore suspension on gray mold disease severity (%) caused by *B. cinerea* on tomato and bean plants incubated under growth chamber conditions at 25°C.

<table>
<thead>
<tr>
<th>CFU/ml concentration</th>
<th>Tomato</th>
<th>Bean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Talc</td>
<td>Spore suspension</td>
</tr>
<tr>
<td>0</td>
<td>71*±5.47 a</td>
<td>71±5.47 a</td>
</tr>
<tr>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>46±4.18 b</td>
<td>46.8±4.18 b</td>
</tr>
<tr>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>46.5±2.23 b</td>
<td>44.3±5.3 b</td>
</tr>
<tr>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>17±2.73 c</td>
<td>18±4.47 c</td>
</tr>
<tr>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>15±5 c</td>
<td>12±3.43</td>
</tr>
</tbody>
</table>

* Means of five replicates ± standard deviation followed by the same letter within a column or row for each plants is not statistically significant according to Fisher LSD method multiple comparison test.

Table 3. Mycelium growth rate (mm/day) of *B. cinerea* growing on PDA medium amended with 10% PDB containing compounds produced by *Trichoderma harzianum* isolates Jn14, T35, and J8 and incubated at 15°C, 20°C and 25°C.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>7*±1.6 a</td>
<td>7.2±1.66 a</td>
<td>3.25±0.25 c</td>
</tr>
<tr>
<td>Jn14</td>
<td>3.1±0.25 c</td>
<td>2.75±0.34 c</td>
<td>2±0.49 d</td>
</tr>
<tr>
<td>T35</td>
<td>2.77±0.40 c</td>
<td>2±0.53 d</td>
<td>0.92±0.32 e</td>
</tr>
<tr>
<td>J8</td>
<td>4.85±0.71 b</td>
<td>6.02±0.47 b</td>
<td>2.95±0.37 c</td>
</tr>
</tbody>
</table>

* Means of five replicates ± standard deviation followed by the same letter within a column or row is not statistically significant according to Fisher LSD method multiple comparison test.
REFERENCES


