Antagonistic Effect of Malaysian Isolates of Trichoderma harzianum and Gliocladium virens on Sclerotium rolfsii

J-JOMDUANG¹ and M. SARIOH²
¹Agricultural Research and Training Centre
P.O. Box 89, A. Maung
52000 Lampang, Thailand
²Department of Plant Protection
Faculty of Agriculture
Universiti Putra Malaysia
43400 UPM. Selangor, Malaysia

Keywords: antagonism, chilli, Trichoderma harzianum, Gliocladium virens, Sclerotium rolfsii

ABSTRACT

INTRODUCTION
Foot rot of chilli (Capsicum annuum L. local var. Langkap) caused by a soilborne fungus Sclerotium rolfsii Sacc. is prevalent in almost all chilli growing areas in Malaysia. Practical and usable methods of control have yet to be developed. The use of fungicides and crop rotation is limited due to the high cost, the wide host range and long persistence of sclerotia in the soil. Flooding of soils has been reported to be effective in reducing sclerotial viability (Sariah 1995). However, difficulties can arise from climatic and regional restrictions, land use and management.

Fungi, bacteria and actinomycetes have been shown to exhibit antagonistic effects on S. rolfsii (Brathwaite and Cunningham 1982; Punja 1985; Chamswarng and Sangkaha 1988). The most studied and effective are Trichoderma harzianum Rifai (Wells et al. 1972; Elad et al. 1980; Henis and Papavizas 1983; Chamswarng 1992) and Gliocladium virens Miller, Giddens and Foster (Papavizas and Lewis 1989; Ristaino et al. 1991).
These two antagonistic fungi attack sclerotia of *S. rolfsii* causing failure in germination (Henis et al. 1983; Henis and Papavizas 1983; Papavizas and Collins 1990), hence reducing disease incidence. However, different isolates or strains of *T. harzianum* and *G. virens* were found to parasitize sclerotia of *S. rolfsii* with varying levels of efficiency (Henis et al. 1983; Sreenivasaprasad and Manibhushan Rao 1990) and they tend to be more crop- and soil-specific.

The most important factor governing the activities of a fungal biocontrol agent in the soil is the qualitative and quantitative make-up of the soil microflora and the ability of the antagonists to maintain themselves in the soil and plant environment. Interspecific and intraspecific competition between isolates of *Trichoderma* have been reported (Marois and Locke 1985; Vajna 1985). Hence the objective of this research was to isolate and evaluate the effectiveness of indigenous isolates of *Trichoderma* and *Gliocladium* from the chilli rhizosphere for controlling *S. rolfsii.*

**MATERIALS AND METHODS**

**Fungal Isolates**

*Trichoderma harzianum* and *Gliocladium virens* were isolated from parasitized sclerotia of *S. rolfsii* collected from Malaysian soils cultivated with chilli. Isolation was carried out by directly plating the sclerotia on potato dextrose agar (PDA) plates without surface sterilization. The plates were incubated at 28°C for 3-4 days for the antagonists to recover. *T. harzianum* and *G. virens* recovered on PDA were reisolated to fresh PDA for pure culture. *S. rolfsii* was isolated from naturally infected chilli plants. Cultures were multiplied and maintained on potato dextrose agar at 28°C.

**Antagonism in Culture**

Antagonism between the antagonists and the test pathogen was evaluated by the dual culture technique and the colony degradation tests. All tests were carried out at 28°C.

Pairings were carried out on PDA in five replicated petri plates. Antagonistic activity was assessed four days after incubation by measuring the radius of *S. rolfsii* colony in the direction towards the antagonist colony and transforming the data into percentage of inhibition of radial growth in relation to radius of the uninhibited *S. rolfsii* colony in the control plate (Royce and Ries 1978). The number of days for the antagonist to overgrow and degrade the whole colony of *S. rolfsii* was also recorded. The number of sclerotia of *S. rolfsii* colonized by the antagonists which failed to germinate when transferred to fresh PDA, was counted twelve days after incubation.

From the zone of interaction or overgrowth in dual culture, mycelial fragments were taken periodically, stained with cotton blue in lactophenol and observed under the light and scanning microscope for hyphal interaction.

The colony degradation tests was assessed by inoculating 5-mm diameter agar discs taken from the edge of a 4-day-old PDA culture of the antagonists onto a fully grown culture of *S. rolfsii.* Each treatment was carried out on five replicated plates. The antagonistic activity was expressed as index of the lytic activity (zone of clearing) of the antagonists on *S. rolfsii.* The number of sclerotia colonized by the antagonist was counted at twelve days after incubation.

**Parasitism of Sclerotia**

Sclerotia were placed on the edge of the colony of a 2-day-old PDA culture of each of the antagonists. Periodic observations on the colonization of the sclerotia by the antagonists were recorded. Sclerotia were examined for their viability 24, 48, 72, 96 and 120 h after incubation by plating them on PDA after surface sterilization with 1% sodium hypochlorite for 5 min.

The parasitized sclerotia were also fixed in 3% glutaraldehyde prepared in 0.1M phosphate buffer, pH 7.2. Samples were dehydrated in a graded ethanol series followed by methyl benzoate and methylbenzoate + celloidin, embedded in paraplast and sectioned to 5-10μm thickness. Paraffin sections of the sclerotia were then processed for scanning electron microscopy following the technique of Gaudet and Kokko (1984) and examined under scanning electron microscope (JEOL JSM-35C).

**Antibiosis**

The ability of the antagonists to produce volatile and non-volatile inhibitors was studied according to the method of Dennis and Webster (1971a,b). Each plate of a 48-hr-old culture of *S. rolfsii* was inverted over the plate of the antagonist, sealed and incubated at 28°C. The diameter of *S. rolfsii* colony was measured 7 days after inoculation and the antibiosis activity was
calculated as the percentage of inhibition of radial growth in relation to the average diameter of the \textit{S. rolfsii} colony in the control plates which were inverted over the PDA plates.

The effect of non-volatile inhibitors of the antagonists on the aerial growth of \textit{S. rolfsii} was determined using the culture filtrates of the antagonists. Filter-sterilized (0.45 µm cellulose nitrate membrane) culture filtrate of the antagonists grown in Richard’s solution for 10 days was amended with PDA. The pathogen was centrally inoculated on 20% filtrate amended PDA plates. Radial growth of the pathogen was observed and recorded 72 h after incubation at 28°C. The experiments were conducted in a completely randomized design with five replicates and were repeated twice.

\textit{Compatibility Studies between T. harzianum and G. virens}

Dual culture of \textit{T. harzianum} and \textit{G. virens} was carried out on five replicated plates which were incubated at room temperature. Simultaneous antagonistic effects of both the antagonists against \textit{S. rolfsii} were assessed by pairing the cultures on the same petri plate. Observations were also made seven days after incubation.

\section*{RESULTS}

\subsection*{Antagonism in Culture}

In dual culture, \textit{G. virens} exhibited stronger antagonistic potential than \textit{T. harzianum} against \textit{S. rolfsii} (Table 1). At four days after incubation, \textit{G. virens} showed 70.48% inhibition of radial growth compared with 64.44% for \textit{T. harzianum}. Sclerotia parasitized by the antagonists against \textit{S. rolfsii} were covered with conidiophores and conidia which were white when young and green when mature. However, the percentage of colonized sclerotia was not significantly different between the two antagonists tested. Within seven days, \textit{G. virens} completely overgrew the colony of \textit{S. rolfsii} while \textit{T. harzianum} was able to colonize \textit{S. rolfsii} after eight days of co-inoculation.

Both antagonists actively lysed the \textit{S. rolfsii} colony, causing disintegration of the mycelia, which resulted in clear zones. Similar effects were observed in the colony degradation test. On microscopic observation, \textit{T. harzianum} was found to have parasitized the pathogen’s hyphae by coiling and producing hooks and short hyphal branches, which penetrated into the pathogen’s hyphae. \textit{G. virens} produced several short branches which coiled compactly around the hyphae of \textit{S. rolfsii} causing it to become granulated and malformed. However, penetration into the pathogen’s hyphae was not observed.

\subsection*{Parasitism of Sclerotia}

Sclerotia of \textit{S. rolfsii} exposed to either \textit{T. harzianum} or \textit{G. virens} for 24 - 120 h failed to germinate. Colonies of \textit{T. harzianum} and \textit{G. virens} were recovered from the colonized sclerotia when plated onto fresh PDA and incubated for 48 h at room temperature. Infected sclerotia were soft and collapsed easily.

The mode of parasitism of \textit{T. harzianum} and \textit{G. virens} on the sclerotia of \textit{S. rolfsii} examined by scanning electron microscopy showed there was no difference in the mode of parasitism of the sclerotia by either \textit{T. harzianum} or \textit{G. virens}, both of which produced profuse growth and sporulation on the sclerotia. Sections of the parasitized sclerotia showed antagonist hyphae penetrating the sclerotia, parasitizing the loosely arranged hyphae of the medullary region resulting in the maceration and disintegration of the medullary hyphae and the eventual disintegration and destruction of the resting structures. Extensive mycelial growth and chlamydospores production of the antagonists in the damaged medulla region were detected (Plate 1).

\subsection*{Production of Volatile and Non-volatile Inhibitors}

\textit{T. harzianum} and \textit{G. virens} were able to produce volatile inhibitors that inhibited radial growth of \textit{S. rolfsii} on PDA although the percentage inhibition was low (Table 2). Age of antagonists...
Plate 1. Scanning electron micrographs of S. rolfsii sclerotia A. Healthy sclerotia; B. Sclerotia parasitized by T. harzianum; C. Sclerotia parasitized by G. virens; D. Chlamydospores of G. virens in the medulla region of the parasitized sclerotia (cu cuticle; r rind; c cortex; m medulla)

had a significant effect (p<0.01) on their ability to produce volatile inhibitors: the younger the cultures, the greater the ability to produce volatile inhibitors.

_T. harzianum_ and _G. virens_ also produced non-volatile inhibitors that inhibited the radial growth of _S. rolfsii_ (Table 3). The ability to produce non-volatile inhibitors by _T. harzianum_ increased with the age of the culture; in contrast that of _G. virens_ decreased.

Compatibility Studies between _T. harzianum_ and _G. virens_
The two antagonists were found to have no inhibitory effect on each other in plate culture. There was no inhibition zone in the intermingled area of the two colonies seven days after co-inoculation. Although _G. virens_ grew faster than _T. harzianum_, thus occupying more surface area of PDA in the petri plates, there was no overgrowing of one antagonist over the other. The appearance of colonies of the antagonists was the same as when grown separately.

Results from pairing cultures showed that the antagonistic effect of the two antagonists against _S. rolfsii_ could occur simultaneously, causing degradation and lysis similar to those previously described. Observation four days after incubation showed that radial growth of _S. rolfsii_ was totally restricted within the intermingled area and eventually the two antagonists grew over _S. rolfsii_ colonies.

**DISCUSSION**

Destruction of sclerotia of _S. rolfsii_ will result in the reduction of disease incidence and therefore antagonists which actively parasitize sclerotia have greater potential to control diseases caused by _S. rolfsii_. Results from dual culture and colony degradation tests showed that both _G. virens_ and _T. harzianum_ exhibited parasitic activity on the sclerotia. Sclerotia were colonized, demonstrating mycoparasitism of the antagonistic isolates on _S. rolfsii_. Mycelia of _S. rolfsii_ were lysed and disintegrated by _T. harzianum_, as seen by the clear zone that developed in the colony degradation test and the penetration of its hyphae under SEM. _G. virens_ overgrew the colony of _S. rolfsii_ and compactly coiled around the hyphae, causing them to become granulated and malformed. Parasitized sclerotia have conidia and conidiophores of the effective antagonists growing on them; hence, when plated on fresh PDA the sclerotia failed to germinate. This observation has not been demonstrated before in the screening for
TABLE 2

Effect of volatile inhibitors produced by *Trichoderma harzianum* and *Gliocladium virens* on radial growth of *Sclerotium rolfsii*

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Age of culture (days)</th>
<th>% inhibition of radial growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>1</td>
<td>21.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>19.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>8.47&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>7.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>6.23&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Gliocladium virens</em></td>
<td>1</td>
<td>24.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>19.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>2.47&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>2.56&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column with different letters are significantly different (p<.01) using DMRT.

Effective antagonists. Furthermore, the colony degradation test was found to be simple, quick and reliable as the lysis activity could be seen and the percentage of colonized sclerotia helped to distinguish the ability of mycoparasitism among the test antagonists. It appears that there could be a correlation between ability of the antagonist to parasitize sclerotia, and its ability to suppress disease. However, this needs further testing under glasshouse and field conditions.

Suggested mechanisms of antagonism of *T. harzianum* and *G. virens* are antibiosis, lysis, competition and mycoparasitism (Cook and Baker 1983; Chet 1987). The two antagonists parasitize both the hyphae and sclerotia of *S. rolfsii*. Their mechanisms of mycoparasitism were in accordance with the previous findings by Henis *et al.* (1983), Sariah (1986) and Papavizas and Collins (1990). Penetration was by the production of short-branched hyphae in the case of *T. harzianum*, while *G. virens* tightly coiled around the pathogen’s hyphae. The medulla region of sclerotia exposed to either *T. harzianum* or *G. virens* showed severe damage, while the cortical region appeared to be intact, suggesting that the preferred site of infection was the medulla tissues. Chet *et al.* (1969) found that the resistance of sclerotia to biological degradation was dependent upon the melanin-rich rind of the wall structure and the organization of cells comprising the inner layers of the sclerotium.

TABLE 3

Effect of non-volatile inhibitors produced by *Trichoderma harzianum* and *Gliocladium virens* on growth of *Sclerotium rolfsii*

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Age of culture (days)</th>
<th>% inhibition of radial growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>6</td>
<td>9.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.50&lt;sup&gt;bc,d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>12.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Gliocladium virens</em></td>
<td>6</td>
<td>30.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.75&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>10.38&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column with different letters are significantly different (p<.01) using DMRT.

The principal mechanism of antagonism is antibiotic through the production of cell-wall degrading enzymes or inhibitors by the antagonists, which results in the mycelia and sclerotia of *S. rolfsii* becoming lysed, macerated and disintegrating. Enzymes secreted by *Trichoderma* most commonly associated with biocontrol are chitinolytic enzymes, B-glucanases and cellulases (Chet 1987). Recent studies found that *T. harzianum* produced a variety of chitinolytic enzymes including N-acetl-B-D-glucosaminidases, chitin 1,4-B-chitobiosidases and endo[chitinase (Harman *et al.* 1993; Lorita *et al.* 1993a,b; 1994). The ability of *T. harzianum* and *G. virens* to produce volatile and non-volatile inhibitors which could inhibit growth of micro-organisms has been described by Dennis and Webster (1971a,b) and, recently, by Ghisalberti and Sivasithamparam (1991). The antibiotics produced by *T. harzianum* are 6-N-pentyl-2H-pyran-2-one, 6-N-pentenyl-2H-pyran-2-one, pyridone, anthraquinones, butenolides, isonitrin D and F, trichorzianines (Ghisalberti and Svasithamparam 1991) and furanone (Ordentlich *et al.* 1992). *G. virens* was also shown to produce several antibiotics such as gliotoxin, gliovirin, gliocladic acid, heptelidic acid, viridin, viridiol and valinotricin (Taylor 1986).

In addition, dual culture plates showed that *T. harzianum* and *G. virens* are compatible and complementary to each other and therefore, it is possible to mix these two antagonists for use in biological control of plant diseases caused by *S. rolfsii*.
REFERENCES


Ristaino J.B., K.B. Perry and R.D. Lumsderr. 1991. Effect of solarization and Gliocladium viriens on sclerotia of Sclerotium rolfsii, soil microbiota,


*(Received 28 October 1996) (Accepted 27 June 1997)*