Pertanika J. Trop. Agric. Sci. 20(1): 35-41 (1995) ISSN: 0126-6128
© Universiti Putra Malaysia Press

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

J. JOMDUANG[1] and M. SARIAH[2]

[1]*Agricultural Research and Trainning Centre*
P.O. Box 89, A. Maung
52000 Lampang, Thailand

[2]*Department of Plant Protection*
Faculty of Agriculture
Universiti Putra Malaysia
43400 UPM. Selangor, Malaysia

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

ABSTRAK

Trichoderma harzianum *dan* Gliocladium virens *yang dipencilkan daripada sklerotia yang diparasit dinilai potensi antagonis mereka terhadap* Sclerotium rolfsii. *Berdasarkan kepada mekanisma antibiosis in vitro; dwikultur dan ujian degradasi koloni, interaksi hifa, antibiosis dan parasitisma sklerotia. kedua dua antagonis didapati berkesan terhadap* S. rolfsii. *Walaubagaimanapun,* G. virens *didapati lebeh berkesan daripada* T. harzianum *dalam dwi-kultur dan ujian degradasi koloni. Di bawah SEM, keduadua antagonis mengkoloni, menembusi dan mengeluarkan spora didalam sklerotia patogen yang diuji, mencadangkan parasitisma keatas sklerotia sebagai mekanisma utama yang terlibat.* T. harzianum *and* G. virens *adalah serasi didalam aktiviti antibiosisnya. Disamping itu, mereka boleh hidup bersama dan sinergis apabila di uji didalam dwikultur.*

ABSTRACT

Trichoderma harzianum *and* Gliocladium virens *isolated from parasitized sclerotia were evaluated for their antagonistic potential against* Sclerotium rolfsii. *Based on the mechanisms of antagonism in vitro, dual culture and colony degradation tests, hyphal interaction, antibiosis and parasitism of sclerotia, both antagonists were found to be effective against* S. rolfsii. *However,* G. virens *was more effective than* T. harzianum *in dual culture and colony degradation tests. Under SEM, both antagonists colonized, penetrated and sporulated inside the sclerotia of the test pathogen, suggesting parasitism of sclerotia could be the principal mechanism involved.* T. harzianum *and* G. virens *were comparable in their antibiosis activity. In addition, they can co-exist together and are synergistic in activity when tested simultaneously in dual culture.*

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 Manibhushanrao 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 *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 *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.

Compatibility Studies between T. harzianum *and* G. virens

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

RESULTS

Antagonism in Culture

In dual culture, *G. virens* exhibited stronger antagonistic potential than *T. harzianum* against *S. rolfsii* (Table 1). At four days after incubation, *G. virens* showed 70.48% inhibition of radial growth compared with 64.44% for *T. harzianum.* Sclerotia parasitized by the antagonists 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, *G. virens* completely overgrew the colony of *S. rolfsii* while *T. harzianum* was able to colonize *S. rolfsii* after eight days of co-inoculation.

Both antagonists actively lysed the *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, *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. *G. virens* produced several short

TABLE 1
Antagonistic effects of *Trichoderma harzianum* and *Gliocladium virens* on *Sclerotium rolfsii* in dual culture and colony degradation tests

Antagonistic effects	Dual culture		Colony degradation	
	T harzianum	*G. virens*	*T. harzianum*	*G.virens*
% inhibition of radial growth	64.44[a]	70.48[b]	-	-
% colonized sclerotia	98.30[b]	100[b]	97.50[a]	100[b]
Number of days to colonize colony	8	7	11	9

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

branches which coiled compactly around the hyphae of *S. rolfsii* causing it to become granulated and malformed. However, penetration into the pathogen's hyphae was not observed.

Parasitism of Sclerotia

Sclerotia of *S. rolfsii* exposed to either *T. harzianum* or *G. virens* for 24 - 120 h failed to germinate. Colonies of *T. harzianum* and *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 *T. harzianum* and *G. virens* on the sclerotia of *S. rolfsii* examined by scanning electron microscopy showed there was no difference in the mode of parasitism of the sclerotia by either *T. harzianum* or *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).

Production of Volatile and Non-volatile Inhibitors

T. harzianum and *G. virens* were able to produce volatile inhibitors that inhibited radial growth of *S. rolfsii* on PDA although the percentage inhibition was low (Table 2). Age of antagonists

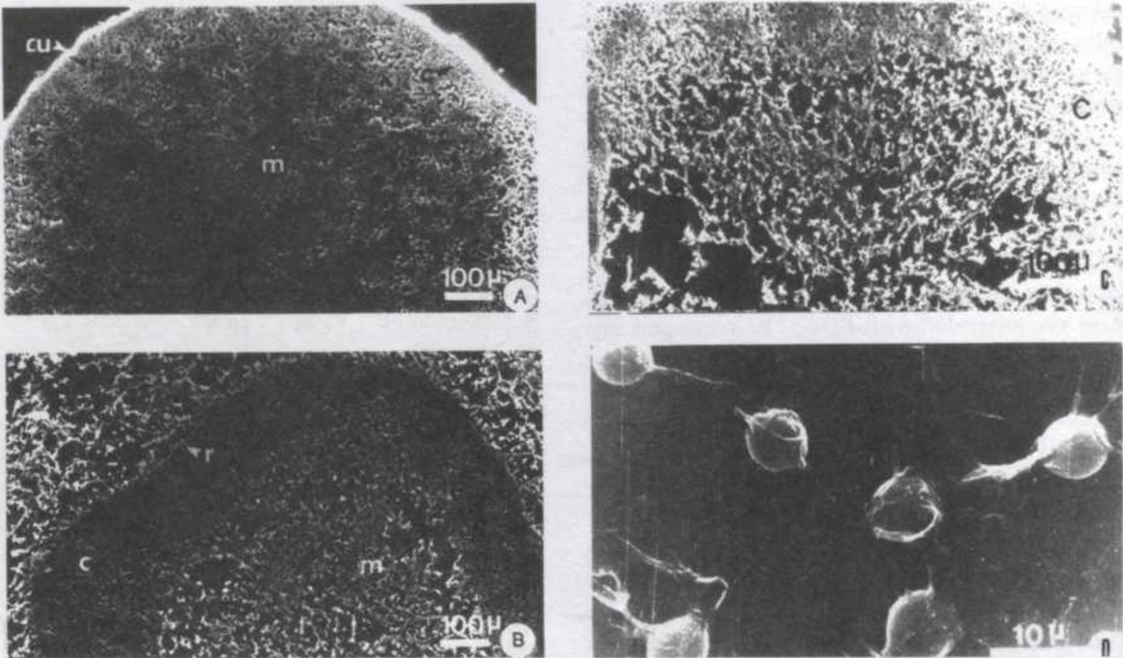

Plate 1. Scanning electron micrographs of S. rolfsii *sclerotia A. Healthy sclerotia; B. Sclerotia parasitized by* T. harzianum; *C. Sclerotia parasitized by* G. virens; D. Chlamydospores *of* G. virens *in the medulla region of the parasitized sclerotia (cu cuticle; r rind; c cortex; m medulla)*

had a significant effect ($p<.01$) 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*

Antagonists	Age of culture (days)	% inhibition of radial growth
Trichoderma harzianum	1	21.76[b]
	7	19.07[c]
	14	8.47[e]
	21	7.30[ef]
	28	6.23[f]
Gliocladium virens	1	24.12[a]
	7	7.35[d]
	14	2.86[g]
	21	2.47[g]
	28	2.56[g]

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

TABLE 3
Effect of non-volatile inhibitors produced by *Trichoderma harzianum* and *Gliocladium virens* on growth of *Sclerotium rolfsii*

Antagonists	Age of culture (days)	% inhibition of radial growth
Trichoderma harzianum	6	9.37[d]
	10	11.50[bcd]
	17	12.8[b]
Gliocladium virens	6	30.25[a]
	10	11.75[bc]
	17	10.38[cd]

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.

The principal mechanism of antagonism is antibiosis 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 endochitinase (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 pla ngs 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

BRATHWAITE, C.W. and H.G.A. CUNNINGHAM. 1982. Inhibition of *Sclerotium rolfsii* by *Pseudomonas aeruginosa*. *Canadian Journal of Botany* **60**: 237-239.

CHAMSWARING, C. 1992. Biological control of *Sclerotium* stem rot of tomato in Thailand. *Journal of Plant Protection in the Tropics* **9**: 77-83.

CHAMSWARING, C. and K. SANGKAHA. 1988. *In vitro* screening for effective antagonists of *Sclerotium rolfsii* Sacc. a causal agent of tomato stem rot. *Kasetsart Journal (Nat. Sci. Suppl.)* **22**: 713.

CHET, I. 1987. *Trichoderma* - application, mode of action, and potential as biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control ed. I Chet* p, 137-160. New York: Wiley.

CHET, I., Y. HENIS, and N. KISLEV. 1969. Ultrastructure of sclerotia and hyphae of *Sclerotium rolfsii* Sacc. *Journal General Microbiology* **57**: 143-147.

COOK, R.J. and K.F. BAKER. 1983. *The Nature and Practice of Biological Control of Plant Pathogens.* St Paul: American Phytopathology Society.

DENNIS, C. and J. WEBSTER. 1971a. Antagonistic properties of species-groups of *Trichoderma* I. Production of non-volatile antibiotics. *Transactions British Mycological Society* **57**: 25-39.

DENNIS, C and J. WEBSTER. 1971b. Antagonistic properties of species-groups of *Trichoderma* II. Production of volatile antibiotics. *Transactions British Mycological Society* **57**: 41-48.

ELAD, Y., I CHET and J. KATAN. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathology* **70**: 119-121.

GAUDET, D.A. and E.G. KOKKO. 1984. Application of scanning electron microscopy to paraffin-embedded plant tissues to study invasive process of plant-pathogenic fungi. *Phytopathology* **74**: 1078-1080.

GHISALBERTI, E.L. and K. SIVASITHAMPARAM. 1991. Antifungal antibiotics produced by *Tric hoderma* spp. *Soil Biology Biochemistry* **23**: 1011-1020.

HARMAN, G.E., C.K. HAYES, M. LORITA, R.M. BROADWAY, A. DI PIETRO, C. PETERBAUER and A. TRONSMO. 1993. Chitinolytic enzymes of *Trichoderma harzianum:* Purification of chitobiosidase and endochitinase. *Phytopathology* **83**: 313-318.

HENIS, Y. and G.C. PAPAVIZAS. 1983. Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. *Phytopathology* **73**: 1469-1474.

HENIS, Y., P. B. ADAMS, J.A. LEWIS and G.C. PAPAVIZAS, 1983. Penetration of sclerotia of *Sclerotium rolfsii* by *Trichoderma* spp. *Phytopathology* **73**: 1043-1046.

LORITA, M., G.E. HARMAN, C.K. HAYES, R.M. BROADWAY, A. TROSMO, S.L. WOO and A. DI PIETRO. 1993a. Chitolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase chitobiosidase. *Phytopathology* **83**: 302-307.

LORITA, M., A. DI PIETRO, C.K. HAYES., S.L. WOO and G.E. HARMAN. 1993b. Antifungal synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. *Phytopathology* **83**: 721-728.

LORITA, M., C.K. HAYES, A. DI PIETRO, S.L. WOO and G.E. HARMAN. 1994. Purification, characterization and synergistic activity of a glucan 1,3-B-glucosidase and an N-acetyl-B-glu-cosamine from *Trichoderma harzianum. Phytopathology* **84**: 398-405.

MAROIS, J.J. and J.C.LOCKE. 1985. Population dynamics of *Trichoderma viride* in steamed plant growth medium. *Phytopathology* **75**: 115-118.

ORDENTLICH, A., Z. WILESMAN, H.E. GOTTLIEB, M. COJOCARU and I. CHET. 1992. Inhibitory furanone produced by the biocontrol agent *Trichoderma harzianum. Phytochemistry* **31**: 485-486.

PAPAVIZAS, G.C. and D.J. COLLINS. 1990. Influence of *Gliocladium virens* on germination and infectivity of sclerotia of *Sclerotium rolfsii. Phytopathology* **80**: 627-630.

PAPAVIZAS, G.C. and J.A. LEWIS. 1989. Effect of *Gliocladium* and *Trichoderma* on damping-off and blight of snapbean caused by *Sclerotium rolfsii* in the greenhouse. *Plant Pathology* **38**: 277-286.

PUNJA Z.K. 1985. The biology, ecology and control of *Sclerotium rolfsii. Annual Review of Phytopathology* **23**: 97-127.

RISTAINO J.B., K.B. PERRY and R.D. LUMSDERM. 1991. Effect of solarization and *Gliocladium virens* on sclerotia of *Sclerotium rolfsii,* soil microbiota,

and the incidence of southern blight of tomato. *Phytopathology* **81**: 117-124.

ROYCE, D.C. and S.M. RIES. 1978. Influence of fungi isolated from peach twigs on the pathogenecity of *Cytospora cincta*. *Phytopathology* **68**: 603-607.

SARIAH, M. 1986. Structural studies of the interaction between *Trichoderma* spp. and *Sclerotium rolfsii* Sacc. In *Abstracts of the 9th Malaysian Microbiology Symposium,* 22-23 August 1986. Serdang. Selangor. UPM.

SARIAH, M. 1995. Effect of flooding on the viability and pathogenicity of sclerotia of *Sclerotium rolfsii* in Malaysian soil. *International Journal Pest Management* **41**: 97-99.

SREENIVASAPRASAD, S. and K. MANIBHUSHANRAO, 1990. Antagonistic potential of *Gliocladium virens* and *Trichoderma longibrachiatum* to phytopathogenic fungi. *Mycopathologia* **109**: 19-26.

TAYLOR, A. 1986. Some aspects of the chemistry and biology of the genus *Hypocrea* and its anamorphs, *Trichoderma* and *Gliocladium*. *Proceedings NS International Science* **36**: 27-58.

VAJNA, L. 1985. Mutual parasitism between *Trichoderma pseudokoningii*. *Phytopathology Z.* **113**: 300-303.

WELLS, H.D., D.K. BELL and C.A. JAWORSKY. 1972. Efficacy of *Trichodermaharzianum* as a biocontrol for *Sclerotium rolfsii*. *Phytopathology* **62**: 442-447.

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