ISOLATION AND *IN VITRO* SCREENING OF SOIL FUNGI FOR BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM*

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ABSTRACT

A study was carried out to identify fungi with biocontrol activity against *Sclerotinia sclerotiorum* on cabbage. Sixty three fungal isolates from 10 vegetable sites in Canterbury, and four isolates with known biocontrol activity from culture collections, were evaluated using a cabbage petiole bioassay for their ability to inhibit colonisation of host tissue by *S. sclerotiorum.* Twenty seven isolates reduced infection of cabbage petioles and subsequent sclerotial production by *S. sclerotiorum* compared to the pathogen control. Eight isolates (five *Trichoderma* sp., two *Gliocladium virens* and one *G. roseum*) reduced petiole infection by more than 75% and completely prevented sclerotial production.
Keywords: *Sclerotinia sclerotiorum*, cabbage, biological control, *Trichoderma* sp., *Gliocladium* sp.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a widespread plant pathogen affecting many economically important vegetable crops in New Zealand including brassicas, lettuce, peas and beans. Control of this pathogen relies on the use of fungicides but these have become less effective due to increased disease pressure, development of pathogen resistance and problems with enhanced microbial degradation of fungicides in soil (Slade *et al*. 1995). As a result, there is increasing interest in the use of biological control agents for the control of *S. sclerotiorum.* Biological control can be targeted towards reducing numbers of sclerotia in the soil with microbial parasites or inhibiting infection of host tissue by propagules of the pathogen. Isolates of *Coniothyrium minitans* (Budge *et al.* 1995) and *Trichoderma* spp. (Dos Santos and Dhingra 1980) have been identified as promising sclerotial parasites in glasshouse and field evaluations whilst *Trichoderma* spp. (Jones and Stewart 1997*)* and *Gliocladium* spp. (Budge *et al.* 1995) have been shown to prevent or reduce host infection by *Sclerotinia* species.

This paper reports experiments carried out to isolate and identify naturally occurring fungi with the ability to inhibit mycelial growth and sclerotial production of *S. sclerotiorum* as demonstrated with a cabbage petiole bioassay.

METHODS

Soil sampling and isolations

Soil samples were taken from 10 vegetable growing areas near Christchurch (eg. Marshlands, Russley, Lincoln, Ashburton) during September 1996. Soil was taken at 10 cm depth. Samples were sieved and air dried for 3-5 d at 18°C. After drying, samples were kept at 10°C until used. Two methods were used to isolate microorganisms from the soil samples. In the pre-colonised agar plate method (modified from Mulligan and Deacon 1992), a culture of *S. sclerotiorum* (G18*),* isolated from field carrots in 1991, was used for selective baiting. A mycelial plug (10 mm diam.) was taken from a stock culture. Petri plates containing quarter strength potato dextrose agar (PDA, 9.75 g/litre) were inoculated with this isolate and incubated at 18°C in the dark until colony growth reached the periphery of the plates. Soil samples (approx.

0.1 g) were ground to a fine dust and placed onto the culture surfaces of the pre-colonised plates in a circular pattern (nine samples in an outer circle and three in an inner circle). There were five replicate plates for each soil type. Plates were then incubated at 18°C in the dark and fungi growing from soil particles were observed after 2 weeks. Selected fungal colonies were removed and purified on PDA plates amended with aureomycin (20 mg/litre). Isolated fungi were stored on PDA slopes at 4°C until further use.

The second method used to isolate microorganisms was a soil dilution technique. Seived soil samples (10 g) from four sites (Marshlands, Lincoln University, Lincoln organic block and Ashburton) were shaken in 90 ml of 0.01% agar in sterile water for 10 min then left standing for a further 20 min. A dilution series was made up to 10^{-6}. Aliquots (0.5 ml) were spread onto three Czapek-dox agar (CZA, 45.4 g/litre) plates amended with Triton X-100 (2 ml/litre) and aureomycin (20 mg/litre) and incubated at 20°C for 2 weeks (modified method of Jones and Stewart 1997). Resulting colonies were purified on PDA plates amended with aureomycin (20 mg/litre) and identified using standard mycological keys.

In addition to those isolates obtained from the field, four isolates with known biocontrol activity against other sclerotial pathogens (held in the Lincoln University Microbial Culture Collection) were selected. These were *Coniothyrium minitans* A69 obtained from a parasitised sclerotium of *Sclerotium cepivorum* in 1994 at Pukekohe, South Auckland; two isolates of *Gliocladium virens* (GV4 and B19) isolated from Canterbury soils in 1995 and *Trichoderma harzianum* C52 isolated from soil in 1991 at Pukekohe, South Auckland.

Cabbage petiole bioassay

Petioles and/or leaf midribs (4-7 mm thick) were excised from 65-75 d old cabbage plants (*Brassica oleraceae* cv. Leprechaun mini) grown in potting mix in a glasshouse (mean temperature 18.8°C, range 7 - 45°C). Tissue segments were surface sterilised in 1% NaOCl for 3 min and then rinsed in sterile water three times. The ends of the tissue pieces were removed using a sterile scalpel and the remaining tissue was cut into 20 mm length segments.

Test isolates were grown on PDA for 4-14 d depending on the growth rate of individual isolates (minimum of 8 cm diam. colony). Mycelial discs (10 mm diam.) of test isolates were placed on top (mycelial side down) of a similarly sized mycelial disc taken from a 4 d old PDA culture of *S. sclerotiorum* (mycelial side upwards) in the centre of 90 mm Petri dishes each containing 30 g sterilised (48 h at 80-85°C) silica sand. In each dish, three cabbage tissue segments were placed against the agar plug such that they radiated outwards and were equidistant from each other. In addition, mycelial discs of test isolates were tested in the absence of pathogen inoculum to determine any phytotoxicity towards cabbage tissue. Controls consisted of a pathogen only inoculation and an uninoculated treatment (PDA disc). The plates were watered with sterile water (4.5 ml/plate) to 50% saturation. Petri dishes were incubated at 20°C under fluorescent light (irradiance of 1.05 W/m^2), 14 h day length for 21 d (modified from Gerlagh *et al.* 1996). All treatments were replicated three times and watered weekly to original saturation. Each replicate was placed at one of three levels in the incubator viz. top, middle and bottom. A total of 67 isolates were tested in six screens (9-13 isolates per screen).

After 4 and 7 d, cabbage petioles were examined using sterile forceps, from the distal end next to the agar plug to the proximal end, for evidence of softening of tissue. Length of area softened was measured and percentage of infected tissue calculated. After 21 d, the number and mean weight of sclerotia produced per Petri dish were recorded. The consistency of the tissue was assessed on a 1-3 scale where 1= rigid and green tissue; 2 = semi-soft tissue with discolouration; 3 = disintegrated tissue.

Statistical analysis of data for mean percentage of infection, number of sclerotia and sclerotial weight was conducted using the SAS package (SAS Institute, 1989). For each treatment, median values of tissue consistency were analysed by the Kruskal-Wallis test in the SYSTAT package.

RESULTS

Soil isolations

Fifty one fungal isolates (34 *Trichoderma* or *Gliocladium* sp.; 17 unidentified organisms) were obtained from the pre-colonised plate assay and 12 isolates (3 *Trichoderma* or *Gliocladium* sp; 9 unidentified organisms) were recovered by soil dilution.

TABLE 1: Mean proportion (%) of cabbage petioles infected by *Sclerotinia sclerotiorum* after 7 d, mean number and weight of sclerotia of the pathogen produced and consistency of host tissue after 21 d in a leaf petiole bioassay.

Test isolate infection	Mean %[1]	Mean no.[1] sclerotia	Mean wt.[1] sclerotia (mg)	Tissue[2] consistency
Screen 1				
Control	100 ± 0 a	9 ± 1 a	32 ± 16	3 a
Trichoderma 7Sr2	44 ± 22 de	2 ± 0 c	4	2 a
G. virens GV4	22 ± 11 de	0	0	1 b
G. virens B19	11 ± 11 de	0	0	1 b
Screen 3				
Control	100 ± 0 a	10 ± 1 a	49 ± 8	3 a
Unid. 9Sbe	55 ± 22 bc	8 a	36 ± 3	3 a
Trichoderma 5Sr5-2	45 ± 5 c	4 bc	52	
Trichoderma 3Sr3	11 ± 11 d	0	0	1 b
Trichoderma 6Sr4	5 ± 5 d	0	0	1 b
Trichoderma 5Sr2-2	0 ± 0 d	0	0	1 b
Unid. 7Sr4-1	0 ± 0 d	1	43	2 a
Unid. 8S-Y	7 ± 3 d	3 ± 2 b	16 ± 14	2 a
Trichoderma 4Sr4-3	5 ± 5 d	0	0	1 b
Screen 4				
Control	100 ± 0 a	9 ± 2	34 ± 1	3 a
Unid. 6Sr1	55 ± 11 bc	1	30	3 a
Trichoderma 6Sr5	55 ± 29 bc	1	20	3 a
Trichoderma 1Sr1	33 ± 19 bcd	0	0	3 a
Unid. 2Sr1	34 ± 22 bcd	2	25	3 a
Trichoderma 4Sr5-2	11 ± 11 d	1	13	3 a
Trichoderma 3Sr4-2	11 ± 11 d	0	0	1 b
Trichoderma 5Sr3	22 ± 22 cd	2	10	3 a
Trichoderma 2Sr2	33 ± 19 bcd	5	33	3 a
Unid. 2Sr4	11 ± 11 d	0	0	3 a
Trichoderma 8Sr4-2	0 ± 0 d	0	0	1 b
Screen 5				
Control	100 ± 0 a	9 ± 2	34 ± 1	3 a
Trichoderma 4Sr5-1	55 ± 29 bc	2 ± 1	35	3 a
Trichoderma 3Sr3-1	61 ± 14 bc	0	0	1 b
Unid. 4Sr1	2 ± 23 c	0	0	3 a
Screen 6				
Control	100 ± 0 a	7 ± 1	40 ± 4	3 a
Unid. S1DYgr4	35 ± 2 de	0	0	3 a
Trichoderma S1BYG	10 ± 10 ef	0	0	1 b
G. roseum 9Sr4	0 ± 0 f	0	0	1 b

[1]Means ± SEM. Means within columns followed by the same letter are not significantly different (P≤0.05, ANOVA, LSD test). [2] Tissue consistency scored from 1 to 3; 1= rigid and green; 2= semisoft and discoloured; 3= disintegrated.

Cabbage petiole bioassay

All isolates tested in the second screen 2 (of the six screens conducted) caused rotting of cabbage petiole tissue when inoculated alone and these isolates were excluded from the study. From the remaining screens, 27 isolates reduced ($P<0.05$) the extent of infection of cabbage petiole segments and subsequent production of sclerotia by *S. sclerotiorum* compared to the pathogen control (Table 1). Eleven isolates reduced petiole infection by more than 75% compared to the controls and completely prevented sclerotial production. The most inhibitory fungi included five *Trichoderma* sp. (6Sr4, 5Sr2-2, 3Sr4-2, 8Sr4-2, and S1BYG), two *Gliocladium virens* isolates (GV4 and B19) and one isolate of *Gliocladium roseum* (9Sr4). In these treatments, tissue segments were more rigid ($P<0.05$) than the pathogen control after 21 d. Three isolates (3Sr3, 4Sr4-3 and 3Sr3-1), whilst providing good inhibition of *Sclerotinia* infection of cabbage petioles, caused some softening of the tissue when applied alone.

DISCUSSION

The majority (59%) of isolates recovered from the soil isolations were *Trichoderma* and *Gliocladium* spp. Both isolation techniques used favoured the recovery of fast growing isolates so it was not unexpected that these genera would predominate. In addition, since *Trichoderma* and *Gliocladium* spp. have been widely reported in the literature to show biocontrol activity against a range of plant pathogens including *Sclerotinia sclerotiorum* (Huang 1992), isolates from these two genera were preferentially selected. Infection of host plants by *S. sclerotiorum* can occur either from myceliogenic germination of sclerotia in the soil or as a result of aerial infection by ascospores. In both instances, saprophytic colonisation of senescent leaves, stamens and anthers by *S. sclerotiorum* often occurs prior to infection of healthy tissue. During this early colonisation stage, the pathogen is susceptible to the antagonistic activities of other microorganisms and, therefore, this phase of the disease cycle is considered suitable to target for biological control.

The cabbage petiole assay was designed to identify fungi able to inhibit growth and development of *S. sclerotiorum* within host tissue. Low light and suitable temperatures (1.05 W/m^2, 20°C respectively) were used to favour pathogen growth, providing a rigorous assay of the antagonistic ability of test isolates.

Eight isolates (five of *Trichoderma* sp., two of *G. virens* and one of *G. roseum*), inhibited infection of cabbage petiole tissue by *S. sclerotiorum* and prevented sclerotial production. Similar observations of antagonism by *Trichoderma* and *Gliocladium* isolates have been reported previously. For example, *T. harzianum* and *G. roseum* inhibited growth and sclerotial production of *S. sclerotiorum* in celery, tomato and lettuce tissue (Whipps 1987) and *T. harzianum* inhibited infection by *S. sclerotiorum* in cucumber and lettuce seedlings (Inbar *et al.* 1996). Although *Trichoderma* spp. are well known as biological control agents of plant diseases, there have been reports of problems with phytotoxicity by *Trichoderma* isolates. In this study, several isolates of *Trichoderma* were pathogenic on cabbage tissue. Similar observations have been made on cucumber, pepper and tomato seedlings (Menzies 1993). Clearly this criterion must be considered during initial biocontrol screens to prevent the selection of unsuitable isolates.

This study has demonstrated that a number of naturally occurring fungal isolates can inhibit growth of *S. sclerotiorum.* We intend to test several of the most inhibitory of these for their ability to parasitise sclerotia of the pathogen and/or inhibit apothecial production.

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