Behaviour of Fungi Antagonistic to *Sclerotinia sclerotiorum* on Plant Tissue Segments

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(Received 14 October 1986; revised 2 February 1987)

To assess the ability of fungi antagonistic to *Sclerotinia sclerotiorum* to inhibit the formation of sclerotia and to grow through plant tissue from cut surfaces, a plant-tissue-based system has been developed using celery, lettuce and tomato segments. *Pythium oligandrum* consistently showed no ability to grow through plant tissue or prevent sclerotium formation whereas *Gliocladium roseum* and three isolates of *Trichoderma harzianum* showed significant ability to grow through plant tissue and significantly decreased the production of sclerotia. Treatment of the plant tissue with *G. roseum* or *T. harzianum* 24 h before, or at the same time as, application of *S. sclerotiorum* gave significantly greater inhibition of sclerotium formation than did treatment 24 h after application of *S. sclerotiorum*. Of the fungi tested, *G. roseum* had the greatest ability to grow through the tissue and *T. harzianum* WT6 the greatest ability to inhibit sclerotium formation.

INTRODUCTION

Most systems for the biological control of soil-borne plant pathogens involve incorporation of propagules of an antagonistic micro-organism into the soil or onto the surface of seeds or plant tissue transplants in an attempt to decrease populations of pathogens or inhibit their growth before contact with the plant (e.g. Suslow & Schroth, 1982; Hubbard *et al.*, 1983; Van den Boogert & Jager, 1984; Lewis & Papavizas, 1985; Loper *et al.*, 1985; Fravel *et al.*, 1985 and references therein). These systems have had varying rates of success. However, one of the most successful and routinely used systems for biocontrol consists of inoculation of stumps of pine with *Phlebia* ((*Peniophora*) *gigantea* to control heart-rot caused by *Heterobasidion annosum* (*Fomes annosus*) (Rishbeth, 1975). Here, no attempt is made to control the pathogen in the soil, but by precolonization of the dead stump with *P. gigantea* the pathogen is excluded from a potential source of food. Such a treatment could be of use in other agricultural and horticultural situations. For instance, in the glasshouse after harvest of lettuce and celery considerable quantities of crop debris are present. Under good horticultural practice this debris is removed but often the cut stumps and roots are incorporated to increase the organic matter content of the soil. If, however, some of this material is infected with *Sclerotinia sclerotiorum*, disease carryover can be encouraged by this procedure. In the field, techniques other than debris removal are sometimes used to lessen disease carryover. For example, the availability of food bases for sclerotium formation may be decreased by spraying the debris with contact herbicides immediately after harvest and cycles of soil flooding can be used to diminish survival of sclerotia (Coley-Smith & Cooke, 1971). Irrespective of these treatments, the incidence of disease caused by pathogens such as *S. sclerotiorum* builds up with time, necessitating soil sterilization by steam or chemical treatment such as methyl bromide (Lynch & Ebben, 1986). A possible novel approach to the control of such pathogens would be to inoculate the residues at harvest with a fungus which has both the capability of entering plant tissues at cut surfaces (so that the residues become occupied by the inoculated fungus) and antagonistic properties towards pathogens. Thus, an ideal inoculant would outcompete pathogens already in occupation of infected residues but would also be able to use the residues as a food base after incorporation from which to grow into the soil and decrease populations of pathogens there.

As a prerequisite to glasshouse experiments some plant-tissue-based tests have been carried out and the results of these are reported here. They were designed firstly to see if a plant-tissue-based system could be used as an indicator of the ability of different antagonistic fungi to prevent formation of sclerotia of *S. sclerotiorum*, and secondly to determine the capability of the antagonists to infect plant tissue from cut surfaces. The study included tomato as well as lettuce and celery to see if the interactions were similar in a range of different plant tissues likely to be found in the glasshouse.

METHODS

Fungal and plant material. The pathogen, *Sclerotinia sclerotiorum* Lib. (de Bary), was isolated from a sclerotium obtained from glasshouse soil under routine cropping with lettuce and celery. The antagonists used were *Pythium oligandrum* Dreschler (IMI 133857), a well-documented mycoparasite (Dreschler, 1943; Deacon, 1976; Lutchmeah & Cooke, 1984; Vesely & Hejdánek, 1984), *Gliocladium roseum* (Link) Bain (IMI 300400) obtained from the same glasshouse soil as *S. sclerotiorum*, and three isolates of *Trichoderma harzianum* Rifai – IMI 275950, isolated from straw (Harper & Lynch, 1984), WT-6 (from G. C. Papavizas, Beltsville, Md, USA) and IMI 298374, isolated from baited hyphae of *Rhizoctonia solani*. In preliminary tests on a range of agars all antagonists were shown to be capable of parasitizing mycelium of *S. sclerotiorum* directly; *G. roseum* and all strains of *T. harzianum* were also able to produce metabolites that inhibited normal growth of *S. sclerotiorum*. All fungi were maintained on potato dextrose agar (PDA) (Oxoid) at 18 °C. For experiments, agar plugs (1 cm diameter) were taken from the growing margin of all fungi.

Plants of tomato (*Lycopersicon esculentum* Mill., cv. Moneymaker), lettuce (*Lactuca sativa* L., cv. Ostinata) and celery (*Apium graveolens* L., cv. Celebrity) were grown in pots in the glasshouse at 18–25 °C. The tomatoes were grown until 40–60 cm tall and the lettuce and celery until market sized.

The tissue system. Tomato stem from the first to the seventh leaf was cut into 2 cm lengths with a sterile razor blade and the pieces were randomized in sterile 15 cm Petri dishes. All operations were then carried out under clean but not sterile conditions. Three 2 cm segments were placed on moist 7 cm filter paper in a 9 cm diameter Petri dish and arranged around a PDA plug containing either an antagonist (treatment A), a plug of antagonist on top of a plug of *S. sclerotiorum* (treatment B) or a plug of *S. sclerotiorum* (treatment C), with the cut ends of the segments touching the plugs of agar (Fig. 1). The agar plugs were always placed in the Petri dishes with the upper surface facing upwards. Each treatment was replicated three times. The plates were then placed in a controlled-environment plant growth room at 18 °C with a 14 h daylength with an irradiance of 25 W m⁻² from a bank of 65/80W white fluorescent tubes. After 24 h, treatment A received a plug of *S. sclerotiorum* and treatment C a plug of antagonist. The plates were observed every day for fungal growth and sclerotium formation and the filter papers kept moist with sterile distilled water for 14 d; the watering was then stopped. After 28 d the sclerotia that had formed on the tissue and those that had formed on the surface of the Petri plate were separated, counted and weighed. The tissue segments were sampled for the presence of antagonist at the distal end of the tissue relative to the agar plug. The distal surface (1–2 mm) of the tissue segment was removed with a sterile scalpel and tissue blocks (2 × 2 × 2 mm) were taken from within the segment and directly plated onto PDA + 2 µg aureomycin ml⁻¹ to assess general fungal colonization, V8 juice medium (Papavizas & Lumsden, 1982) for specific isolation of *Trichoderma* spp., or cornmeal agar (Oxoid) for detection of *P. oligandrum*.

Similar treatments were carried out for lettuce mid-rib, obtained from leaves 14–20 cm in length, and celery petioles, obtained from between 2 cm from the base to below the lamina expansion point. Outer or centre leaves or petioles for lettuce and celery respectively were not used.

Fig. 1. Arrangement of tissue segments in a Petri dish.

Statistical analysis. Data were analysed by generalized linear model methods. In the case of binary (presence/absence) data a binomial error function with logit link was used, while the count data were analysed using a log-linear model: Poisson error function with log link (McCullagh & Nelder, 1983).

RESULTS

Effect of antagonists on production of sclerotia

The overall pattern of antagonist effect was similar for both number and weight of sclerotia produced and so for clarity the data for number of sclerotia only are given in Table 1. A comparison was made between the numbers of sclerotia produced with the pathogen alone (control) and the numbers produced with the antagonist present for each of the five antagonists

Table 1. Mean number of sclerotia produced per Petri plate and significance of effect of antagonist and time of application

	Tissue†					Plate†				
	Mean no. of sclerotia			Significance of differences§		Mean no. of sclerotia			Significance of differences§	
	Treatment‡:			Control v. antag.	Between treatments	Treatment‡:			Control v. antag.	Between treatments
	A	B	C			A	B	C		
Celery										
Control¶	14.6					5.3				
<i>P. oligandrum</i>	12.3	15.0	11.7	NS	NS	13.0	17.0	14.7	NS	NS ^a
<i>G. roseum</i>	7.0	3.0	9.0	***	**	0	0	0	—	—
<i>T. harzianum</i>	3.0	1.0	10.7	•	* ^a	0	0	0.7	—	—
IMI 298374										
<i>T. harzianum</i>	1.0	0	5.7	***	*** ^b	0	0	1.7	**	NS ^{ab}
IMI 275950										
<i>T. harzianum</i>	0	0	1.7	***	** ^b	0	0	0	—	—
WT6										
Lettuce										
Control¶	3.7					6.7				
<i>P. oligandrum</i>	3.0	3.0	2.6	NS	NS	3.0	4.3	2.0	NS	NS
<i>G. roseum</i>	1.0	0	1.7	**	* ^b	0.7	0	0.7	***	NS ^b
<i>T. harzianum</i>	0.3	0	1.0	***	NS ^b	0	0	1.0	***	* ^b
IMI 298374										
<i>T. harzianum</i>	0	0	2.0	***	** ^b	0	0	0.7	***	NS ^b
IMI 275950										
<i>T. harzianum</i>	0	0	0	—	—	0	0	0	—	—
WT6										
Tomato										
Control¶	19.0					29.7				
<i>P. oligandrum</i>	16.0	8.0	10.7	**	•	27.0	26.0	25.7	NS	NS ^a
<i>G. roseum</i>	0	0.3	6.3	***	*** ^b	0	0	14.3	**	* ^{ab}
<i>T. harzianum</i>	2.0	2.0	5.3	**	NS ^a	0	0	2.0	***	•* ^b
IMI 298374										
<i>T. harzianum</i>	4.3	0.3	6.7	***	***	0	0	18.6	***	*** ^b
IMI 275950										
<i>T. harzianum</i>	0	0	9.3	***	** ^b	0	0	15.7	**	** ^{ab}
WT6										

† On any Petri plate sclerotia could be produced on the plant tissue alone or on the plant tissue and the surface of the Petri plate.

‡ Treatments A, B and C have antagonist applied 24 h before, simultaneously with, or 24 h after *S. sclerotiorum* respectively.

§ Effects are not significant (NS) or significant at the 5% (*) or 1% (**) or 0.1% (***) level respectively. No test (—) is possible with large numbers of zero values. Superscript letters indicate the following: *a*, variation between pieces within dishes was much greater than expectation; *b*, analyses suspect due to large number of zero values.

¶ The comparison is for control v. mean of treatments A, B and C.

¶ *S. sclerotiorum* alone.

Table 2. *Incidence of sclerotia on tissue*

(a) Effect of antagonist						Significance of differences between antagonists†
Incidence of sclerotia (out of 27)						
<i>P. oligandrum</i>	<i>G. roseum</i>	<i>T. harzianum</i>				
		IMI 298374	IMI 275950	WT6		
Celery	27	23	13	6	4	***
Lettuce	23	8	5	6	0	***
Tomato	27	10	13	17	9	***
SE range 1.85–2.60‡						
(b) Effect of time of application						Significance of differences between treatments‡
Incidence of sclerotia (out of 45)						
	Treatment§:					
	A	B	C			
Celery	21	17	35		***	
Lettuce	12	8	22		***	
Tomato	18	13	35		***	
SE range 2.6–3.4‡						

† ***, effect significant at the 0.1% level.

‡ SEs under a binomial hypothesis vary depending on the score according to the formula $r(n-r)/n$ where r is the score (out of n). Note that the SE is 0.0 for scores of 0 and n .

§ Treatments A, B and C have antagonist applied 24 h after *S. sclerotiorum* respectively.

separately as well as a comparison of the number of sclerotia produced at different times of antagonist application. Each comparison was made using a log-linear model (analysis of count data) and it should be noted that the analyses are not independent inasmuch as the control was included in all of them for each tissue. *P. oligandrum* had no effect on the number of sclerotia produced on celery or lettuce tissue but did significantly reduce the number produced on tomato tissue. It had no effect on the number of sclerotia produced on the plate. All the other antagonists significantly reduced the number of sclerotia produced on all tissues and plates, *T. harzianum* WT6 consistently being associated with the lowest number of sclerotia. There was no effect of time of application of *P. oligandrum* except on tomato tissue but *G. roseum* and *T. harzianum* (IMI 275950) consistently showed an effect of time of application on all three tissues. The greatest number of sclerotia were produced when the *T. harzianum* isolates and *G. roseum* were applied after the *S. sclerotiorum* (treatment C, Table 1). A similar pattern was shown for the sclerotia produced on the plate, but due to the large number of zero values, the plate analyses must be treated with some caution.

In order to ascertain whether the system could be streamlined to enable quicker assessment of effect of antagonists, the data for production of sclerotia were subsequently analysed on the basis of presence or absence of sclerotia on the tissue pieces (Table 2). To facilitate analysis, the control group was omitted as it was indistinguishable from the *P. oligandrum* treatment and the remaining 5×3 factorial was analysed for each tissue type using a binomial analysis with a logit link. *G. roseum* and all *T. harzianum* isolates caused a significant reduction in the incidence of sclerotia on all tissues, with the *T. harzianum* isolates causing the greatest reduction on celery and lettuce (Table 2a). On tomato, *G. roseum* decreased the incidence of sclerotia almost as much as *T. harzianum* WT6, which was consistently associated with the lowest incidence of sclerotia on all tissues. Differences between time of application were significant for all three tissue types (Table 2b). Treatment B (simultaneous application of antagonist and *S. sclerotiorum*) consistently gave the lowest incidence of sclerotia while treatment C (antagonist applied after *S. sclerotiorum*) gave the highest.

Table 3. Incidence of recovery of antagonists from distal end of plant tissue

	Incidence of recovery (out of 9)				Significance of differences§	
	Control†	Treatment‡			Control v. antag.	Between treatments
		A	B	C		
Celery						
<i>P. oligandrum</i>	0	0	0	0		
<i>G. roseum</i>	9	9	9	9		
<i>T. harzianum</i> IMI 298374	4	4	8	9	NS	***
<i>T. harzianum</i> IMI 275950	3	3	5	9	NS	***
<i>T. harzianum</i> WT6	4	4	3	6	NS	***
Lettuce						
<i>P. oligandrum</i>	0	0	0	0		
<i>G. roseum</i>	9	9	9	9		
<i>T. harzianum</i> IMI 298374	8	9	9	9		
<i>T. harzianum</i> IMI 275950	9	9	9	9		
<i>T. harzianum</i> WT6	9	9	9	9		
Tomato						
<i>P. oligandrum</i>	0	0	0	0		
<i>G. roseum</i>	8	9	9	9		
<i>T. harzianum</i> IMI 298374	1	3	6	8	*	***
<i>T. harzianum</i> IMI 275950	1	8	5	1	*	***
<i>T. harzianum</i> WT6	4	8	7	8	*	***

† Antagonist without *S. sclerotiorum*.‡ Treatments A, B and C have antagonist applied 24 h before, simultaneously with, or 24 h after *S. sclerotiorum* respectively.

§ Effects are not significant (NS) or significant at the 5% (*) or 0.1% (***) level respectively. A blank space indicates 'not tested'.

|| The comparison is for control v. mean of treatments A, B and C.

Recovery of antagonists from distal end of tissue

The incidence of recovery of antagonists from the distal ends of tissue pieces is given in Table 3. *P. oligandrum* failed to reach the distal end of all tissues whereas *G. roseum* (almost) always succeeded. This latter feature was similarly exhibited by all the *T. harzianum* isolates on lettuce. For this reason, all these data were excluded from subsequent analysis, leaving two 3 × 4 matrices for celery and tomato. These were examined by using a simple binomial analysis relating the response (out of 9) to the factors of antagonist type and time of application (treatments A, B and C). For celery there was a significant effect of time of application but no difference between the mean of the antagonist type and the respective controls nor any interaction. Early application of the antagonist (treatment A) was similar to the control, with progressively later application leading to greater recovery. In tomato, significant effects were found between the controls and the mean of the antagonist types as well as between the times of application. An interaction was found between antagonist type and time of application but *T. harzianum* WT6 was regularly recovered at the highest incidence at all treatment times.

DISCUSSION

Consistent effects of the antagonists were shown on all three tissues examined. Irrespective of time of application, *P. oligandrum* had no effect on the number of sclerotia formed except on tomato tissue. When the results were expressed as incidence of sclerotia a similar pattern was found and the tomato effect was not detected. *P. oligandrum* had no ability to grow through any tissue. In contrast, *G. roseum* and the *T. harzianum* isolates significantly reduced the number and incidence of sclerotia formed on all tissues and showed marked ability to grow through

tissue from cut surfaces. This was particularly so with *G. roseum*. For *G. roseum* and *T. harzianum* isolates, treatment C (when the antagonist was applied after *S. sclerotiorum*) gave the greatest number and incidence of sclerotia produced, indicating that pretreatment with antagonists was beneficial in preventing sclerotium formation.

On all three tissues the presence of *T. harzianum* WT6 resulted in the lowest incidence of sclerotia, and this isolate was potentially the most effective biocontrol agent tested, particularly as it has some ability to enter the plant tissue. However, if occupation of residues from cut surfaces is of greater importance than *G. roseum* could be of more value. Significantly, after treatment of intact lettuce leaves with spore sprays of all these fungi no morphological or pathological symptoms were noted (unpublished observations), implying the need for cut or damaged tissue for entry.

The pattern of behaviour of the antagonists observed on these tissue pieces agrees well with the known ecological properties of each of the fungi. *P. oligandrum* has little or no ability to infect higher plants (Deacon & Henry, 1978) and survives in soil as long-lived oospores with the capability to act as a mycoparasitic biological control agent of plant-pathogenic *Pythium* spp. (Al-Hamdani *et al.*, 1983). Interestingly, it does have the ability to parasitize mycelium of *S. sclerotiorum* on agar (unpublished observations) but appears of little use as a biocontrol agent of *S. sclerotiorum* from this system. Both *G. roseum* and *T. harzianum* can behave as mycoparasites and biocontrol agents of a range of plant pathogens and can survive in the soil as spores. They grow rapidly when substrates such as plant debris become available and exhibit little ability to infect intact plants (Papavizas, 1985). It may well be that these fungi could enter cut surfaces of plant residues and use them as a food source after harvest, particularly if normal resistance mechanisms were impaired by senescence in the residue. Of note here is the recent report that *G. roseum* has been found as a non-pathogenic systemic parasite of soybeans, particularly of roots and older parts of the stem (Mueller & Sinclair, 1986), but the method of entry into the soybean is not known.

Other workers have treated sclerotia of *S. sclerotiorum* derived from agar culture or autoclaved tissue pieces with putative antagonists as a screen for biocontrol activity (e.g. Makkonen & Pohjakallio, 1960; Mueller *et al.*, 1985) as this provides uncontaminated, uniform sclerotia. However, sclerotia produced in culture differ in physiological properties and resistance to biocontrol agents from those produced on living material (Turner & Tribe, 1976; Trutmann *et al.*, 1980; Coley-Smith, 1985). Thus, this is the first example of antagonist tests utilizing sclerotia formed from initially living systems and as such it may give more reliable results.

The system described here also has the potential to give information on the viability of the sclerotia produced. Preliminary assessments of apothecial formation and sclerotium contamination were technically easy and simply carried out on the low numbers of sclerotia produced in these systems (unpublished observations). However, for meaningful statistical analyses to be obtained, greater numbers of sclerotia would have to be used in the future.

Overall the system appears to provide a simple, rapid and reproducible method of screening antagonists for their ability to inhibit sclerotium formation in plant tissue. The use of the incidence of sclerotia (Table 2) as a guide to efficacy appears particularly valuable.

I thank Sarah Molyneux for technical assistance and John Fenlon for the statistical analyses.

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