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Biotrophic mycoparasitism by *Verticillium biguttatum* on *Rhizoctonia solani*

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Abstract. *Verticillium biguttatum* cannot utilise cellulose or nitrate-nitrogen and it requires biotin for growth, yet it grew and sporulated abundantly on *Rhizoctonia solani* on cellulose, obtaining at least organic carbon, nitrogen and biotin from *R. solani*. Videomicroscopy of inter-hyphal interactions on films of water agar showed that *V. biguttatum* behaved as a biotrophic mycoparasite. From germinating spores, it penetrated the hyphae of *R. solani* and formed haustorium-like branches without killing the host cells, and the haustoria supported an external mycelial network of the mycoparasite. Later the mycoparasite sporulated, and the infected host cells died. On cellulosic substrata *V. biguttatum* did not reduce the growth of *R. solani*, and often enhanced the rate of cellulose degradation. However, *V. biguttatum* drastically reduced the production of sclerotia by *R. solani*, often completely suppressing sclerotium production when the mycoparasite infected only a localized region of the host colony. This is ascribed to the creation of a nutrient sink by the parasite, consistent with biotrophy. On plates of cellulose agar the suppression of sclerotia was not confined to parasitized colonies but extended to adjacent colonies of *R. solani* that had successfully anastomosed with the parasitized colony. There was no effect on adjacent vegetatively incompatible colonies, where attempted anastomoses caused cytoplasmic death. In comparable experiments the necrotrophic mycoparasite *Gliocladium roseum* had no long-distance effect on sclerotium production by *R. solani*.

Suppression of sclerotium production may explain the reported success of *V. biguttatum* in biocontrol of black scurf of potato in experimental field conditions.

Introduction

Mycoparasites are fungi that parasitize other fungi, growing at the expense of nutrients from a fungal host [Deacon, 1976]. However, this strict nutritional relationship has seldom been demonstrated, so most reported mycoparasites such as *Trichoderma* spp. and *Gliocladium* spp. have been termed presumptive mycoparasites [Deacon and Berry, 1992; Mulligan and Deacon, 1992]. They might obtain nutrients from fungi that they antagonize by antibiosis or contact-mediated interference, and they might penetrate and degrade the older, senescing zones of fungal colonies; but in any case the main role of most presumptive mycoparasites could be to exploit the sub-

strates that other fungi grow on, in which case their antagonistic actions serve to eliminate competitors that have previously occupied these substrates. Mycoparasites in the more restricted (nutritional) sense have been categorised as either necrotrophic or biotrophic [Barnett and Binder, 1973]. The necrotrophs kill functional host cells rapidly and then derive nutrients from the deal cells; biotrophs feed on cells that remain alive for quite extended times while being parasitized.

Most practical interest in mycoparasites and presumptive mycoparasites centres on their potential roles in regulating the populations of other fungi, especially of plant pathogens. This can occur both in soil [e.g. Adams, 1990] and on aerial plant surfaces [e.g. Sundheim, 1986]. It can be exploited purposefully by using inocula of mycoparasites in biocontrol programmes [e.g. Chet, 1987]. Necrotrophic mycoparasites are often preferred for this because inoculum can be produced readily *in vitro* and because necrotrophs typically are aggressive antagonists. Often, however, they require exogenous nutrients in order to be effective biocontrol agents. For example, the aggressive necrotrophs *Pythium oligandrum* Drechs. and *Pythium nunn* Lifshitz et al. are most effective in antagonising pathogens in crop residues in soil, when competition for the residue nutrients may be an integral part of their mode of action [Martin and Hancock, 1986; Paulitz and Baker, 1988]. *Trichoderma* spp. need to be added to soil together with nutrients for effective biocontrol [Lewis et al., 1991], presumably because the nutrients support antibiotic-production. Alternatively, such fungi proliferate when soil is partially sterilized, thereby releasing nutrients, or when pathogens are weakened by other factors, facilitating their displacement from crop residues [Katan et al., 1992].

Biotrophic mycoparasites need not be subject to these limitations because they always feed directly on fungal hosts in nature. Moreover, some biotrophic mycoparasites can be grown satisfactorily in nutrient media, enabling production of inocula for biocontrol. The available evidence suggested that *Verticillium biguttatum* Gams and van Zaayen could be one such fungus. It was found to be an ecologically obligate parasite of *Rhizoctonia solani* Kuhn in soil [van den Boogert, 1989a] and had been shown to reduce black scurf of potatoes, caused by *R. solani*, in experimental field conditions [van den Boogert et al., 1990, 1994; Jager et al., 1991; van den Boogert and Velvis, 1992]. Our aim was to investigate this mycoparasitic interaction and thereby to explain the efficiency of *V. biguttatum* in biocontrol. In dual-membered culture with other fungi we have confirmed that *V. biguttatum* is a biotrophic mycoparasite. We also show that it markedly suppresses the production of sclerotia by *R. solani* even in regions remote from the infection sites on host hyphae; this helps to explain the reduction of black scurf symptoms which result from sclerotial crusts on tuber surfaces.

Materials and methods

Media. Water agar was prepared from either 15 g Oxoid No. 3 agar or 20 g Difco Bacto agar in 1 litre distilled water. Mineral nutrient solution (MNS) for cellulolysis experiments comprised (g l^{-1} distilled water): KH_2PO_4 (1.23), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), $FeCl_3 \cdot 6H_2O$ (0.001), $ZnSO_4 \cdot 7H_2O$ (0.0009), $MnSO_4 \cdot 4H_2O$ (0.0004). It was supplemented with nitrogen and vitamin sources as explained in the text. Mineral nutrient agar (MNA) consisted of 1 litre MNS and 20g Difco Bacto agar. Cellulose agar contained 5 g cellulose powder (Whatman CC31) and 2 g $NaNO_3$ in 1 litre of MNA. Malt extract – peptone agar (MPA) comprised 7.5 g malt extract, 0.5 g Mycological Peptone and 15 g agar No. 3 per litre distilled water. Potato-dextrose agar (PDA) comprised 20 g Oxoid potato-dextrose agar and 7.5 g Oxoid No. 3 agar in 1 litre distilled water. All media were sterilized by autoclaving at 121 °C for 15 min.

Fungi. The fungi and their origins are shown in Table 1. The strain of *V. biguttatum* (IPO-M92) was different from that (M73) used in most previous work but had behaved identically to this in comparative tests on radial growth, germination, temperature range and efficacy as a biocontrol agent. Cultures were maintained on MPA (*V. biguttatum*, *Gliocladium roseum* Bainier) or PDA (*R. solani*, *Fusarium culmorum* (W.G. Sm.) Sacc.) in darkness at 25 °C. Conidial suspensions used as inoculum of *V. biguttatum* and *G. roseum* were obtained by rinsing the surfaces of 7–10 day-old colonies with sterile distilled water. The crude suspensions were passed through 3 layers of sterile cheese-cloth then washed in three changes of sterile distilled water, being centrifuged each time for 10 min at 3000 G. The spore concentrations were assessed with a Neubaeur counting chamber and adjusted to desired levels with sterile distilled water. Inocula of *R. solani* were always discs taken from the margins of growing colonies on PDA.

Table 1. Details of fungal strains

Species	Culture	Origin/comments
Verticillium biguttatum	IPO-M92	From soil with crop of Irises, Netherlands
Gliocladium roseum	G12	From garden soil, U.K.
Rhizoctonia solani		
AG3	IPO–3R41	From potato tuber, Netherlands
	IPO–3R09	From potato tuber, Netherlands
AG4	I13	Supplied by Y. Koltin, Israel
	I13 cured	Cured of ds-RNA (hyphal tip transfer)
	Tester	Tester strain of AG4 (ex G. C. Papavizas)
	Tester cured	Cured of ds-RNA (hyphal tip transfer)
Fusarium culmorum	CD9	From wheat, UK

Interactions on filter paper. Five filter paper circles (Whatman No. 3, 7 cm diam) were accurately weighed into each 250 ml conical flask and saturated with 12 ml MNS supplemented with $NaNO_3$ (5 g l^{-1}) and sometimes also with biotin (10 μg ml^{-1}) and thiamine (100 μg ml^{-1}). The flasks were plugged, and autoclaved for 15 min at 121 °C. Each stack of filter papers was inoculated at the edge with two adjacent agar discs (5 mm diam) of *R. solani* or *F. culmorum*. The strains of *R. solani* (see Table 2) were inoculated alone (with two agar discs) or in combination (one disc of each strain). Six replicate flasks were used in each case. Three of these replicates received no further treatment. The other three replicates received a conidial suspension of *V. biguttatum* (0.1 ml, containing 1×10^6 spores) which was pipetted onto the filter paper 2 days later, so that it was confined to a circle about one-third of the way across the filter paper surface from the inoculum of *R. solani* or *F. culmorum*. Flasks were incubated in darkness at 25 °C. At sampling (4–6 wk) the contents of each flask were removed and dried for 24 h in a forced-air oven at 80 °C, then weighed. Uninoculated control flasks were treated similarly, to calculate the weight loss caused by over-drying of the originally air-dry filter papers. After correction for this, the weight loss caused by fungi in the inoculated flasks was calculated.

Table 2. Weight loss (mg) of flasks containing filter paper stacks when inoculated with *Rhizoctonia solani* or *Fusarium culmorum* in the presence or absence of *Verticillium biguttatum* (means ± SEM, for 3 replicates)

	Cellulolytic fungus alone	Cellulolytic fungus + *V. biguttatum*
Expt. 1[a]		
R. solani 3R41 (AG3)	125 ± 5	185 ± 15*
R. solani 3R09 (AG3)	149 ± 4	255 ± 5*
R. solani 3R09 + 3R41	127 ± 8	206 ± 9*
R. solani I13 (AG4)	421 ± 37	441 ± 53
R. solani I13 cured (AG4)	327 ± 6	318 ± 2
Expt. 2[b]		
R. solani 3R41 (AG3)	229 ± 55	386 ± 56
R. solani Tester (AG4)	557 ± 87	658 ± 36
R. solani Tester cured (AG4)	426 ± 6	525 ± 13*
F. culmorum CD9	424 ± 22	388 ± 49

[a, b] Experiment 1 was sampled after 4 wk at 25°; biotin and thiamine were supplied in the flasks. Experiment 2 was sampled after 6 wk at 25°; no vitamins were supplied.

* Significant enhancement of weight loss in presence of *V. biguttatum* (*t* test).

Interactions on cellulose film. Transparent cellulose film (Rayophane PU525, British Sidac Ltd., Merseyside, UK) was cut into strips 20 × 50 mm and autoclaved in distilled water. The strips were dipped into conidial suspension of *V. biguttatum*, or distilled water for controls, then placed

either singly or in parallel pairs on plates of MNA supplemented with $NaNO_3$ (2 g l^{-1}). The conidial suspension had been adjusted to provide 60 conidia mm^{-2} on the surface of the cellulose film after excess conidial suspension had been drained. It was used at this concentration and also diluted 5- and 10-fold with distilled water to provide, respectively, 12 and 6 conidia mm^{-2} of film surface. Some plates received no further treatment. For others, an inoculum disc of *R. solani* (strain 3R41) was placed in the corner at one end of each cellulose strip; it was placed either directly on the strip or on top of a glass cover-slip (see Fig. 2) to prevent nutrients in the inoculum disc from diffusing onto the cellulose film. The plates were incubated at 25 °C in darkness and the positions of the colony margins of *R. solani* were marked daily on the base of each plate. At sampling, after 4 days, the strength of the cellulose film was assessed by applying successive weights (5 g intervals) to a needle penetrometer [Deacon and Henry, 1978] which was raised and lowered each time that the weight was increased, until the needle punctured the film. Uninoculated cellulose film was punctured by a consistent weight (depending on the sharpness of the needle that was used). Any reduction in the weight supported by inoculated film, relative to uninoculated controls, was used as a quantitative assessment of cellulolysis. Each strip of cellulose film was assessed at 4 separate points within each daily zone of growth of *R. solani*, marked on the base of the dish. There were three replicate plates for each treatment.

Interactions on cellulose agar. Cellulose agar plates were inoculated with *R. solani* (strains 3R41 and 3R09) and mycoparasites in three different ways, shown in Fig. 1. For consistency, the inoculation positions were

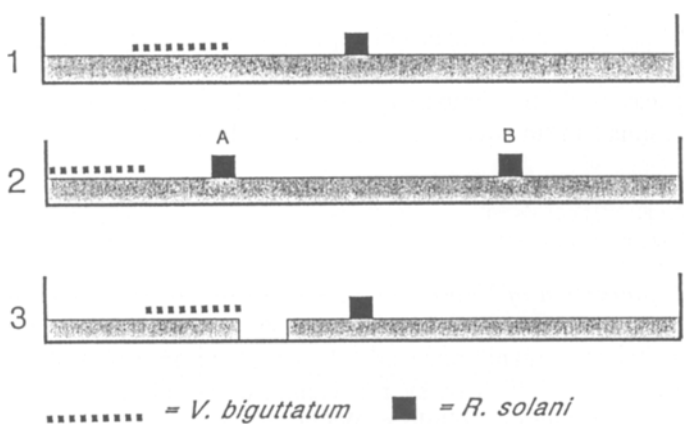

Fig. 1. Patterns of inoculation of cellulose agar plates with spores of *Verticillium biguttatum* and agar discs of *Rhizoctonia solani*. In methods 1 and 3 the spores of *Verticillium* were streaked as a band; in method 2 they were streaked as an arc. Method 3 involves an agar-free trench between the inocula.

marked on the bases of the agar plates with a template. Inoculum discs of *R. solani* were placed on pieces of cover-slip on the agar surface. *V. biguttatum* was added as conidial suspension (0.1 ml, containing 1×10^7 spores), which was carefully spread either as a band 15 mm wide or as an arc over the marked inoculation site. *G. roseum* was used similarly. The plates were incubated for 14 days at 25 °C and examined daily to record fungal growth, sporulation and production of sclerotia, which were counted for each plate. There were three replicate plates for each treatment.

Videomicroscopy of inter-hyphal interactions. The method was based on that of Laing and Deacon [1991] and is only briefly described here. Sterile glass cover-slips (64 × 35 mm) were dipped in molten water agar (Difco Bacto agar) and drained to leave a thin agar film, then placed on the surface of water agar plates to cool. The plates were slanted and conidial suspensions of *V. biguttatum* were poured over them and allowed to drain. An inoculum disc of *R. solani* (strain 3R41) was placed near one end of the cover-slip and the plates were incubated at 25 °C. When the *Rhizoctonia* colony had achieved a radius of *c.* 2 cm the film-coated cover-slip was removed from the agar plate, inverted onto a glass observation chamber, sealed to this with vaseline and the upper surface of the cover-slip was wiped clear. Microscopical observations were made through the cover-slip and agar film, using a ×70 oil-immersion objective. Time-lapse video recordings were made, and photographs were taken with a video copy processor, using equipment described in Berry et al. [1993].

Anastomoses between strains 3R41 and 3R09 of *R. solani* were studied, in the absence of *V. biguttatum*, by inoculating agar-coated cover-slips with opposing agar discs (2 cm apart) of *R. solani* and observing them as above.

Statistical analysis. Where appropriate, results are presented as means with standard errors of the means, and pairs of treatments were compared by Student's *t*-test. Multiple comparisons were made by analysis of variance, using the residual mean square to calculate 5% LSD.

Results

Nutritional interaction of V. biguttatum *with* R. solani. *V. biguttatum* grows readily in axenic culture if supplied with an appropriate carbon source (glucose, mannitol), organic nitrogen (glutamine) or ammonium, and biotin [van den Boogert, 1989b]. However, it cannot utilize nitrate as sole N source and cannot degrade cellulose. We confirmed these points in a series of experiments (data not shown). In contrast, *R. solani* (AG3 and AG4 strains) grew readily on cellulose film and filter paper when supplied with only nitrate as N-source and in the absence of biotin. The nutritional differences between these fungi enabled us to demonstrate that *V. biguttatum* can obtain all its major nutrients from *R. solani*. For example, *V. biguttatum*

grew and sporulated abundantly on cellulose film co-inoculated with *R. solani* (Fig. 2) when nitrate was the only N-source and biotin was not supplied, but it did not grow when inoculated alone. *V. biguttatum* also grew on filter paper in the presence of *R. solani* (Fig. 3) but not alone.

In two separate experiments with flasks containing filter papers (Table 2) the loss in dry weight of flask contents (i.e. weight of cellulose degraded and then respired to CO_2 and water) was greater when *R. solani* was coinoculated with *V. biguttatum* than when *R. solani* was inoculated alone. This effect was significant for some individual strains of *R. solani* – the AG4 tester strain cured of its double-stranded RNA, and AG3 strains 3R41 and 3R09 in one experiment. These strains were also seen to support sporulation of *V. biguttatum* on the cellulose. But the effect was not significant for AG4 strain I13 (wild type or cured of ds-RNA) and this strain was not seen to support sporulation of *V. biguttatum*. Nevertheless, a paired-sample *t*-test on the results for all 8 treatments involving *R. solani* in Table 2 showed a highly significant increase in cellulolysis (mean 77 ± 18.5 mg; $P < 0.01$) when *V. biguttatum* was co-inoculated with *R. solani*

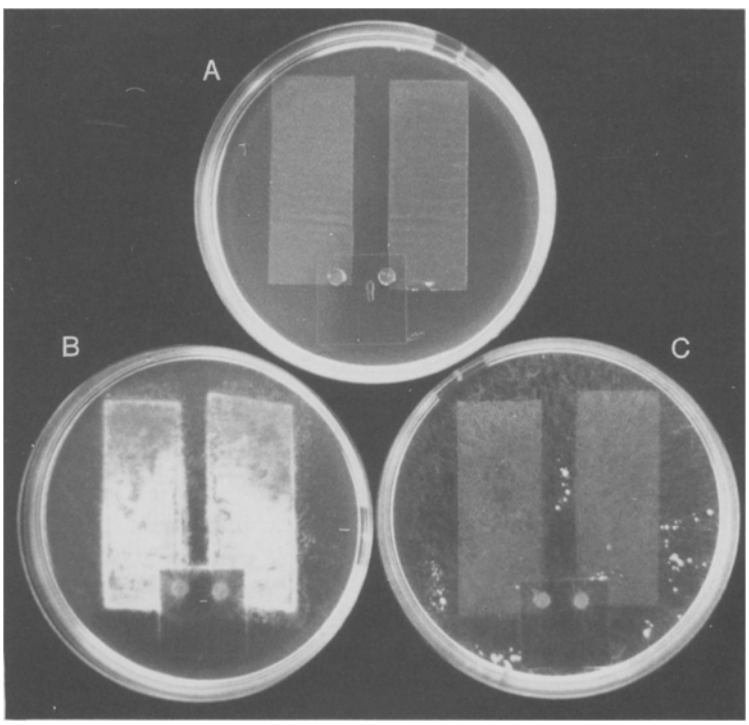

Fig. 2. Plates of mineral nutrient agar bearing strips of cellulose film. A. Inoculated with agar discs of *Verticillium biguttatum* alone (no growth). B. Inoculated with agar discs of *Rhizoctonia solani* strain 3R41 (on cover-slip) and seeded with spores of *V. biguttatum*, which has grown abundantly. C. Inoculated with agar discs of *R. solani* strain 3R41 alone; sclerotia of the fungus are seen on the surrounding agar.

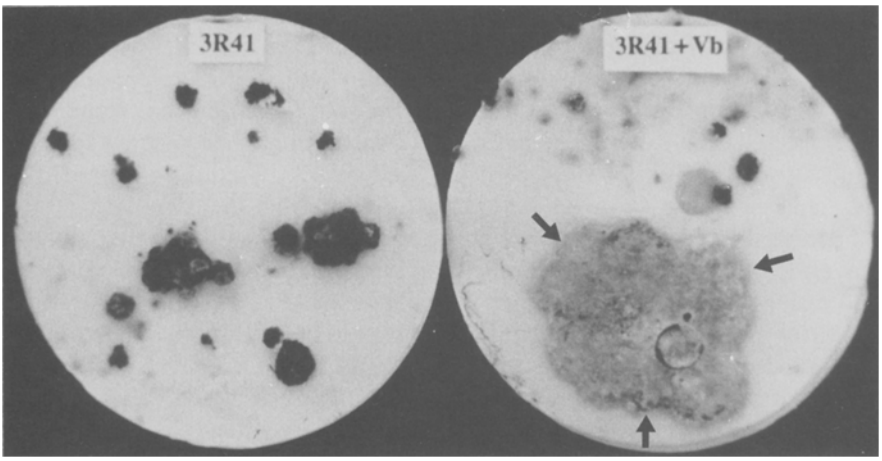

Fig. 3. Filter paper stacks removed from flasks after 6 weeks. *R. solani* strain 3R41 alone has formed many sclerotia, but few sclerotia in the presence of *Verticillium biguttatum* (Vb). The restricted extent of the colony of *V. biguttatum* (arrowed) can be seen.

than when *R. solani* was used alone. *Fusarium culmorum* was used for comparison with *R. solani* in one experiment because it is a non-host of *V. biguttatum* [van den Boogert et al., 1989]. It did not support sporulation of *V. biguttatum* in the flasks and its cellulolytic activity was not enhanced by the mycoparasite (Table 2).

In experiments with cellulose film the effect of *V. biguttatum* on *R. solani* was both density-dependent and nutrient-dependent. When the inoculum disc of *R. solani* was placed on a cover-slip on the cellulose film so that nutrients could not diffuse from it, low spore concentrations of *V. biguttatum* did not reduce the growth rate of *R. solani* across the film and did not affect cellulolysis by *R. solani*; but progressively higher spore densities seeded onto the cellulose reduced both the growth rate and cellulolysis (Figs. 4, 5). In this respect, the effect on cellulolysis was assessed independently of effect on linear growth, by measuring cellulolysis (after 4 days) in the different zones where *R. solani* had reached after 1, 2, 3 and 4 days, marked on the bases of the plates. In contrast to such experiments, when the inoculum disc of *R. solani* was placed directly on the cellulose film (no cover-slip) even a low spore density of *V. biguttatum* could reduced the amount of cellulolysis by *R. solani* (Table 3), presumably because nutrients diffusing from the inoculum disc onto the cellulose film supported growth from *V. biguttatum* spores.

Effect of V. biguttatum *on production of sclerotia by* R. solani. In all cellulolysis experiments described above the presence of *V. biguttatum* markedly reduced the production of sclerotia by *R. solani* even when there was no inhibitory effect on growth or cellulolysis. Fig. 3 shows partial suppression

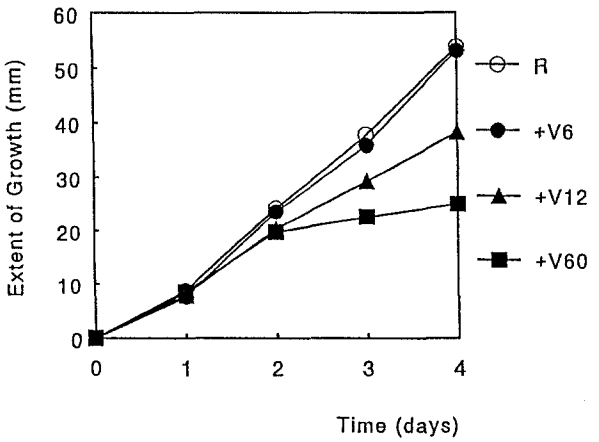

Fig. 4. Extent of growth of *Rhizoctonia solani* strain 3R41 along strips of cellulose film when inoculated alone (open circles) or on strips seeded with spores of *Verticillium biguttatum* at concentrations 6 mm^{-2} (closed circles), 12 mm^{-2} (triangles) or 60 mm^{-2} (squares). Means of 3 replicate strips of film; LSD (0.05) = 4.1 mm.

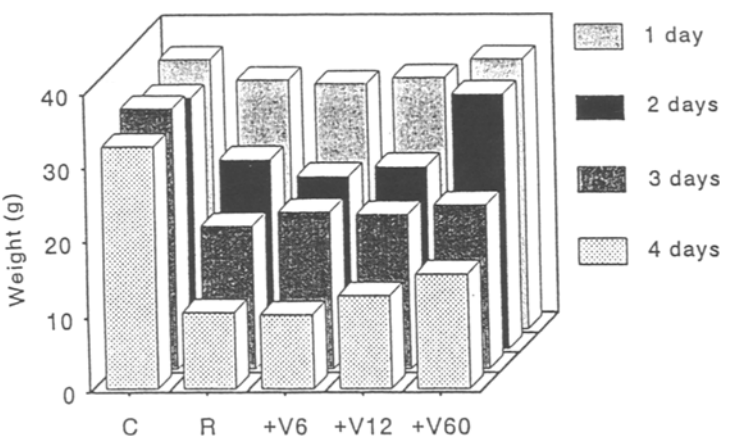

Fig. 5. Weight (g) that needed to be applied to a needle penetrometer to puncture cellulose film in uninoculated controls (C), inoculated with *Rhizoctonia solani* strain 3R41 alone (R) or inoculated with *R. solani* strain 3R41 and seeded with spores of *Verticillium biguttatum* at 6 mm^{-2} (+V6), 12 mm^{-2} (+V12) or 60 mm^{-2} (+V60). Means of 3 replicate strips of film, assessed in zones where *R. solani* had grown after 1, 2, 3 and 4 days; LSD (0.05) = 3.6.

of sclerotium production on filter paper wads and is typical of many such experiments. On cellulose film (e.g. Fig. 2) the production of sclerotia was often wholly suppressed.

To investigate this effect further, *R. solani* was grown on mineral nutrient agar with nitrate as sole N-source and cellulose powder as sole carbon source. The three types of experimental design are shown in Fig. 1.

Table 3. Effect of *Verticillium biguttatum* on breakdown of cellulose film by *Rhizoctonia solani* strain 3R41; data are weights (g) applied to a penetrometer that punctured the film (means ± SEM, for 4 replicates, each tested at 4 points on the film) after 4 days at 25°. Spores of *V. biguttatum* were seeded on the film; the inoculum disc of *R. solani* was in contact with the film (not on a glass cover-slip – see text)

Inoculation treatment	Spore concn of *Verticillium* on film	
	6 spores mm^{-2}	60 spores mm^{-2}
None (control)	30.8 a*	33.8 a
V. biguttatum (Vb)	31.3 a	34.5 a
R. solani (Rs)	11.3 b	6.7 b
Rs + Vb	25.0 c	17.6 c

* Within columns, data followed by different letters differ significantly ($P < 0.001$) by analysis of variance.

In method 1 (central placement of *R. solani*, lateral placement of spores of *V. biguttatum)* the presence of *Verticillium* spores did not impede the extension growth of *R. solani*, which reached the Petri dish edge at the same time as on the *Verticillium*–free side. However, sclerotium production was completely suppressed over the whole agar plate, even though *Verticillium* remained localized in the inoculated area. Control plates with *R. solani* alone developed numerous sclerotia.

In method 2 (Fig. 1) two inoculation positions (A and B) for *R. solani* were spaced apart and a streak of *V. biguttatum* spores was placed beyond position A. Inoculum discs of two AG3 strains of *R. solani* were used in various combinations. As shown in Table 4, when strain 3R41 was used as a single inoculum its production of sclerotia was completely suppressed, whereas it produced many sclerotia in the absence of *V. biguttatum*. Similarly, when strain 3R41 was opposed to itself the production of sclerotia in both colonies was suppressed by *V. biguttatum*, even though the mycoparasite never spread far from inoculum position A (Fig. 6). *V. biguttatum* markedly reduced sclerotium production by strain 3R09 in similar treatments. But when strains 3R41 and 3R09 were opposed to one another *V. biguttatum* only suppressed sclerotium formation on the colony that it parasitized, not on the opposing colony. In videotaped recordings when colonies of the two *R. solani* strains were opposed to one another on water agar films, the attempted inter-strain anastomoses always led to incompatibility reactions so that the fusion compartments showed cytoplasmic degeneration. Opposing colonies of a single strain always anastomosed freely and established cytoplasmic continuity.

Method 3 was like method 1 except that a strip of agar had been removed to leave an agar-free trench between the spores of *V. biguttatum* and the inoculum of *R. solani*. The hyphae of *R. solani* grew across this

Table 4. Effect of *Verticillium biguttatum* or *Gliocladium roseum* on sclerotium production by *Rhizoctonia solani* (AG3) on plates of cellulose plus nitrate agar. Strains of *R. solani* were inoculated in positions A and B (see Fig. 1) and spores of *V. biguttatum* or *G. roseum* were streaked beyond position A. Data are counts of sclerotia (means ± SEM) on three replicate plates or, where appropriate, separate *Rhizoctonia* colonies on each plate.

R. solani inocula		Number of sclerotia per colony					
		Rhizoctonia alone		With *V. biguttaum*.		With *G. roseum*	
A	B	Strain 3R41	Strain 3R09	Strain 3R41	Strain 3R09	Strain 3R41	Strain 3R09
3R41	–	158 ± 5	–	0	–	91 ± 6	–
3R41	3R41	170 ± 22	–	2 ± 1	–		
3R09	–	–	120 ± 15	–	24 ± 5	–	94 ± 12
3R09	3R09	–	134 ± 6	–	20 ± 4		
3R41	3R09	87 ± 17	62 ± 6	0	59 ± 8		
3R09	3R41	86 ± 15	58 ± 7	81 ± 22	0		

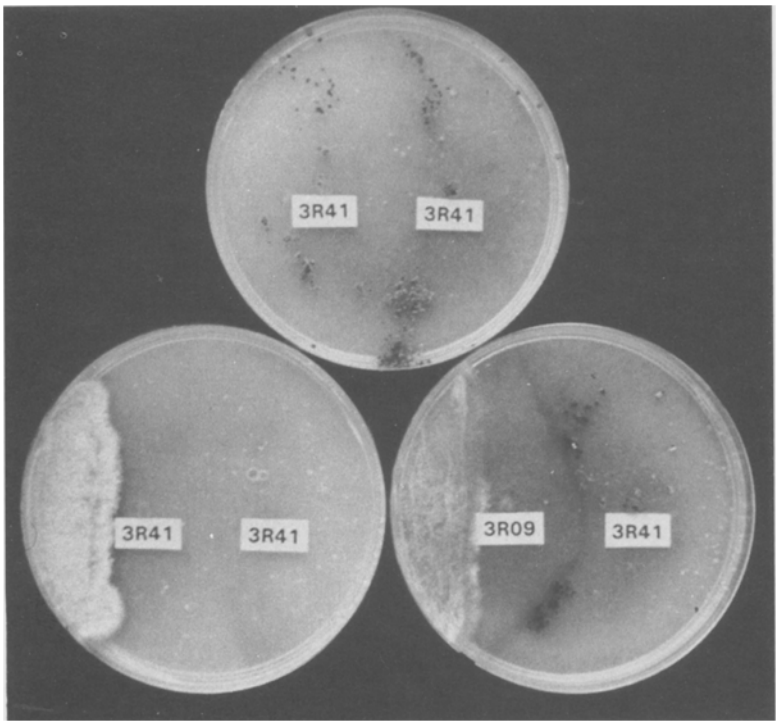

Fig. 6. Plates of cellulose agar inoculated as in method 2 of Fig. 1. A. Two opposing colonies of *R. solani* strain 3R41, producing sclerotia. B. As in (A) but inoculated with spores of *V. biguttatum* on left-hand side, causing complete suppression of sclerotium formation. C. Opposing colonies of *R. solani* strains 3R09 and 3R41, which do not anastomose successfully; sclerotium production by strain 3R09 was suppressed by *V. biguttatum* (left-hand side) but sclerotium production by strain 3R41 was unaffected.

trench, and *V. biguttatum* again suppressed sclerotium production over the whole agar plate.

These experiments thus showed the following. 1. Localized infection by *V. biguttatum* suppressed sclerotium production within a mycelial network of *R. solani*. 2. This suppression was mediated through the hyphae of *R. solani* (e.g. when these bridged a trench in the agar) and not by volatile or water-diffusible inhibitors. 3. The suppression extended only as far as there was functional cytoplasmic continuity; it did not extend past unsuccessful anastomoses between incompatible strains.

Effect of Gliocladium roseum *on* R. solani. Method 2 (Fig. 1) was used with *G. roseum* in place of *V. biguttatum* because *G. roseum* grows relatively slowly and damages other fungal hyphae by either water-diffusible inhibitors of by causing post-contact collapse of individual fungal cells [Deacon and Berry, 1992]. As shown in Table 4, it had only a small effect

on sclerotium production by strains 3R41 and 3R09 of *R. solani.* This effect was seen to be confined to the part of the *Rhizoctonia* colony that it grew on.

Videotaped interactions. Interactions on water agar films were videotaped for strain 3R41 of *R. solani* in the presence of seeded spores of *V. biguttatum.* It was not possible to videotape the mycoparasitic sequence from beginning to end, even with time-lapse, because the cycle through to sporulation of the mycoparasite took 1–2 days, during which the thin agar films dried out on a microscope stage. Instead, a composite sequence was constructed from detailed analysis of individual stages.

Spores of *V. biguttatum* germinated within 24 h. Germination was poor (< 30%) in the absence of the host and the germ-tubes then remained short. But germination increased to 100% when colonies of *R. solani* were present, and the germ-tubes grew either straight towards host hyphae or in an apparently spiral or zig-zag mode that caused them to contact the host hyphae (Fig. 7, 8). Almost all such contacts led to penetration of the host at the point where contact first occurred. There was no evidence of pre-penetration growth along the surface of the host hypha, and also no evidence of the formation of an appressorium before invasion.

The time of penetration could not be determined accurately because it was not associated with any visible change in host or parasite behaviour. For example, there was no surge of host cytoplasm to the penetration point as occurs during parasitism by *Pythium oligandrum* [Laing and Deacon, 1991] and there was no evident change in host protoplasmic content during penetration. The first sign that penetration had occurred was the appearance of a short, thin mycoparasite hypha within a host compartment. This internal hypha usually changed direction of growth and became orientated along the axis of the host hypha. During this it enlarged to normal hyphal dimensions but grew slowly and often had a club-like appearance. During all these events the penetrated host compartment showed normal cyto-plasmic streaming and retained its turgor; the septa were not seen to bulge into the parasitized compartment from adjacent ones. The optical properties of the active host cytoplasm made it impossible to photograph the internal hyphae, although they could be recorded on videotape and then traced from the video monitor, using the frame-freeze facility of the video recorder to trace from successive video frames where the microscope focus had been changed to provide a composite image. Representative tracings are shown in Fig. 8.

After penetration had occurred the mycoparasite formed external hyphae from just outside the penetration point or from further germ-tubes that arose from the spores that originally gave rise to infection. These external hyphae often grew close to the host hyphae, but without coiling, and produced clasping side branches (Fig. 9) from which successive host compartments were invaded. Thus, each successive host compartment was

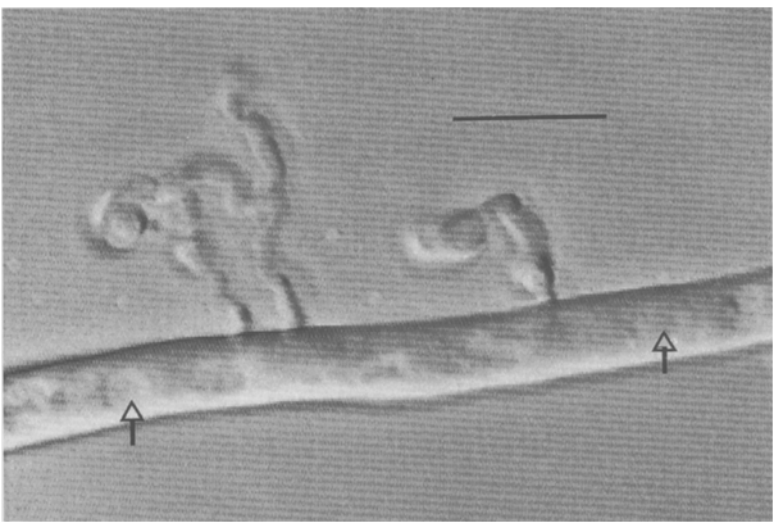

Fig. 7. Hypha of *Rhizoctonia solani* strain 3R41 parasitized from three germinated spores of *Verticillium biguttatum* on water agar, at an equivalent stage to Fig. 8. The host hypha contains internal hyphae of the mycoparasite but is still viable; host vacuoles are arrowed. Print from a video copy processor, with phase contrast illumination. Bar = 20 μm.

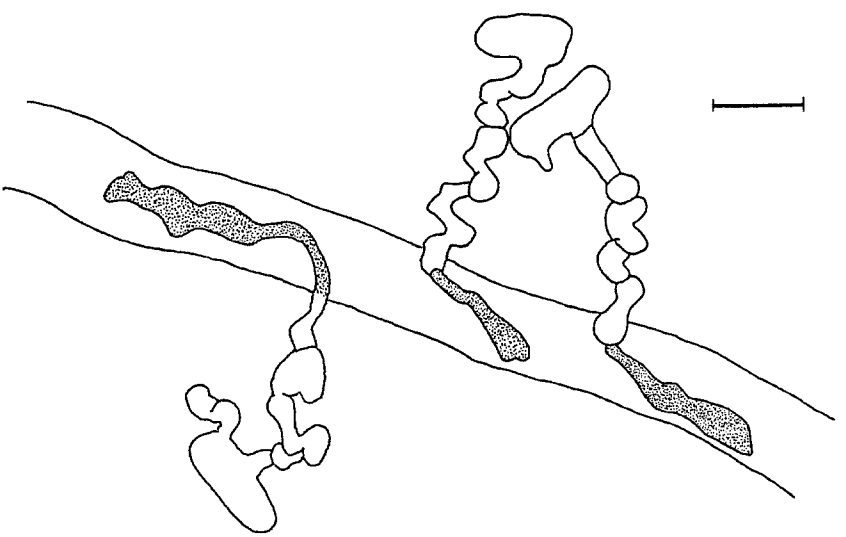

Fig. 8. Biotrophic phase of parasitism of a hyphal compartment of *Rhizoctonia solani* strain 3R41 from three germinating spores of *Verticillium biguttatum* on water agar. The germ-tubes are distorted and coiled; internal hyphae of the mycoparasite are shaded. There was no evidence of appressoria. Traced from a video monitor. Bar = 10 μm.

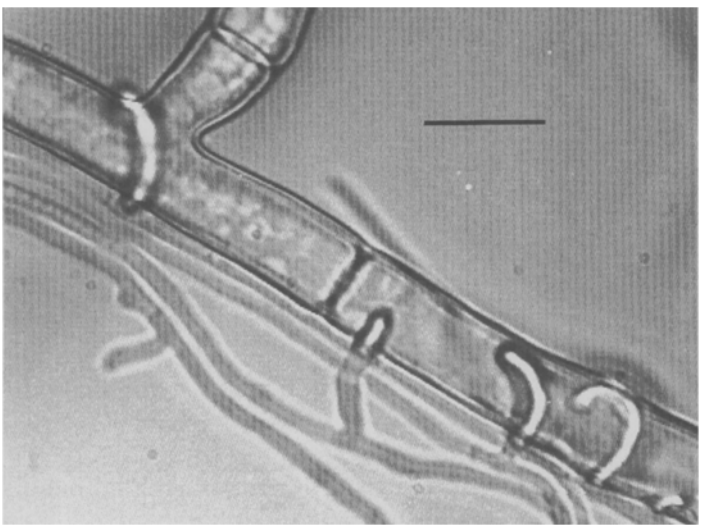

Fig. 9. Hypha of *Rhizoctonia solani* strain 3R41 (R) with living cytoplasmic contents, being parasitized from clasping side branches of *Verticillium biguttatum* on water agar. Bar = 10 μm.

parasitised by one of more short internal hyphae, and in all cases the host cytoplasm remained functional, with normal cytoplasmic streaming.

After 1–2 days *V. biguttatum* produced conidiophores and conidia from the old parasitized regions, but always from external hyphae that developed after host-penetration. At this time, also, many of the parasitized host cells appeared moribund, with only residual hyphal contents or none at all. The internal mycoparasite hyphae were then seen to have branched and subsequently grew to fill the dead host compartments (e.g. Fig. 10) and sometimes emerged through their walls.

Our interpretation of this sequence is that the mycoparasite initially established a biotrophic relationship with the host, such that the short internal hyphae functioned as haustoria. This enabled the mycoparasite to grow externally and initiate infections of adjacent host compartments. At a later stage the mycoparasite sporulated externally, and by this stage the mycoparasite was growing as a necrotroph in host compartments that had become moribund.

Two further observations were made. First, there was no evidence of conspicuous cytoplasmic streaming through the septa in parasitized regions of the host; instead, the streaming within host compartments was typical of cyclosis and the septa were evidently intact. Nevertheless, when mycelia of *R. solani* were grown on agar films in the absence of the mycoparasite to study anastomosis between strains, we sometimes saw very rapid streaming, measured at between 100 and 150 μm sec^{-1}, as the colonies aged. This

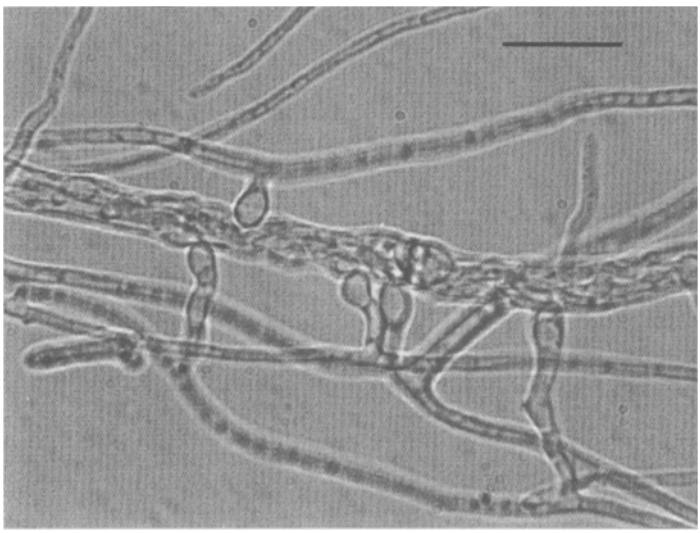

Fig. 10. Area of cellulose film showing hypha of *Rhizoctonia solani* strain 3R41 (centre) filled with hyphae of *Verticillium biguttatum*. Parasitizing side branches of the mycoparasite bear appressoria at their tips. Bar = 20 μm.

streaming was continuous along the hyphae, and at the septa the cytoplasmic flow occupied most of the hyphal diameter indicating that the dolipore septa had at least partly broken down. Only the cytosol and small particulate components of the hyphae streamed; the vacuoles remained static, as if anchored in the hyphal compartments. The second observation was for pieces of cellulose film removed from agar plates and examined on microscope slides. The later stages of interactions typical of those on water agar films were seen, except that parasitizing branches that arose from external hyphae usually bore a conspicuous appressorial swelling at the point where they are thought to have penetrated the host (Fig. 10).

Discussion

This study provides several new lines of evidence on mycoparasitism of *R. solani* by *V. biguttatum*. In flasks with filter paper as sole carbon source, nitrate as sole N source and in the absence of biotin, *V. biguttatum* grew and sporulated at the expense of *R. solani*, obtaining carbon, nitrogen and biotin requirements from the host. Similarly, *V. biguttatum* grew and sporulated at the expense of *R. solani* on films of purified water agar, and in this case it must have derived nutrients that *R. solani* had translocated from a nutrient-rich agar disc; *R. solani* is well known to do this when growing across nutrient-deficient substrata [e.g. Henis and Ben-Yephet, 1970]. The

capture of nutrients from host hyphae is evidence of mycoparasitism. But it does not distinguish between biotrophic and necrotrophic mycoparasitism because the aggressive necrotroph *Pythium oligandrum* also can grow at the expense of fungal hosts in conditions similar to those used here [Deacon, 1976].

Several details of the interaction, however, clearly demonstrate that *V. biguttatum* is a biotrophic parasite. For example, it had no adverse effect on growth or cellulolysis by its host on filter paper cellulose; on the contrary, it usually enhanced the rate of cellulolysis. This could most simply be explained if the mycoparasite utilized sugars which would otherwise repress the synthesis of cellulase enzymes by the host fungus; such sugar-repression is well documented for cellulolytic fungi [Mandels, 1981]. In contrast to the enhancement of cellulolysis by *V. biguttatum*, the mycoparasitic *Pythium* spp. such as *P. oligandrum*, which are necrotrophs, markedly reduce the cellulolytic activity of their hosts on filter paper [Deacon, 1976; Laing and Deacon, 1990]. Similarly, in videotaped interactions on films of water agar the mycoparasitic *Pythium* spp. cause rapid disruption of host hyphae, lysing or penetrating and killing the host cells within a few minutes [Laing and Deacon, 1991] whereas *V. biguttatum* did not immediately kill the host hyphae which it penetrated. This is compatible with electron microscopical studies [van den Boogert et al., 1989] which showed hyphae of *V. biguttatum* inside the hyphae of *R. solani* that had normal cytoplasmic structure; the internal mycoparasite hyphae were surrounded by a membrane that seemed to be continuous with the host plasma membrane.

The short internal hyphae of *V. biguttatum* probably functioned as haustoria, because after they had formed the mycoparasite proliferated as external hyphae. New infections were initiated from these external hyphae but did not kill the host cells during several hours of videotaped observations. Nevertheless, parasitism ultimately led to the death of host cells and then the mycoparasite apparently grew as a necrotroph, colonizing the host cells and sporulating from surface hyphae. The precise relationship between the onset of this necrotrophic phase and the onset of sporulation merits further study. We have no definitive evidence that they are related, but a transition from a presumed biotrophic phase to a necrotrophic phase has been reported for other mycoparasites, such as *Sporidesmium sclerotivorum* on sclerotia of *Sclerotinia minor* [Bullock et al., 1986]. Other details of the mycoparasitic mechanisms of *V. biguttatum* also require further study. The germination triggers and tropic factors from host hyphae seem unlikely to be general fungal metabolites because only some fungi elicit germination and some even suppress it [van den Boogert et al., 1989]. The very narrow host range of *V. biguttatum*, consistent with biotrophy, might be related to those external triggers. Current evidence suggests that *V. biguttatum* parasitizes only *R. solani* and a few other sclerotial fungi in laboratory conditions; and in nature it seems even more restricted, being most common in potato fields where it parasitizes AG3 of *R. solani* [van den

Boogert and Velvis, 1992]. In a different context, it might be useful to investigate why *V. biguttatum* formed appressorium-like structures on host hyphae on cellulose film but not on water agar (compare Figs. 9 and 10); this might relate to the availability of external nutrients.

The most remarkable feature of our study was that *V. biguttatum* markedly reduced or suppressed the production of sclerotia by *R. solani*, even when the mycoparasite grew on only a relatively small, localized region of the host colony. In some tests on large (15 cm diam) plates of cellulose agar (not detailed here) it exerted this effect at least 75 mm from the site of parasitism. The production of sclerotia typically occurs in response to nutrient-stress conditions and involves substantial remobilisation of mycelial nutrients into the developing sclerotia [Christias and Lockwood, 1973]. The levels of mycelial nutrients that Christias and Lockwood reported to be conserved in this way almost certainly come from breakdown of mycelial wall components. This was reported during the production of fruitbodies (basidiocarps) by *Schizophyllum commune*; it involved the derepression of glucanase synthesis in hyphae in response to nutrient stress, so that the hyphal and septal walls were partly degraded [Wessels and Sietsma, 1979]. We observed at least partial breakdown of septal walls of *R. solani* on water agar films, presumably when the mycelia became nutrient-stressed. We did not observe it in parasitized mycelia on water agar films, perhaps because the spores of *V. biguttatum* had been seeded across the agar so the mycoparasite soon colonized most or all of the *Rhizoctonia* hyphae (few if any were viable after 3–4 days). However, we hypothesize that a localized, slowly spreading zone of infection by *V. biguttatum* might represent a continuing nutrient sink within the mycelium of *R. solani*, perhaps causing the host to degrade its septa but, in any case, sufficient to divert nutrients from sclerotium production. This is a testable hypothesis. The alternative explanation for suppression of sclerotium formation would be that *V biguttatum* produces a suppressive factor. But, by the design of our experiments, any such factor would need to have been distributed internally throughout the mycelial network of the host, which seems implausible. The ability of biotrophic plant pathogens to create a nutrient sink in their hosts is well established [Farrar and Lewis, 1987]. Adams and Ayers [1983] suggested that the biotrophic mycoparasite *Sporidesmium sclerotivorum* destroys the sclerotia on which it grows by sequestering nutrients, thereby derepressing the synthesis of wall-lytic enzymes by the host so that the weakened sclerotia are more prone to invasion by weak parasites and saprophytes. Our work indicates that *V. biguttatum* might act more like the biotrophic plant pathogens, generating a nutrient-sink that has a long-distance effect, suppressing sclerotium formation.

These observations and suggested mechanisms have direct bearing on the role of *V. biguttatum* as a biocontrol agent in potato crops. The mycoparasite can significantly reduce the incidence of sclerotia (black scurf

symptoms) of *R. solani* on potato tubers in field conditions [van den Boogert et al., 1994]. If it does so at a distance from its sites of parasitism of the mycelia in soils, as in culture, then effective biocontrol would not be dependent on parasitism of the whole mycelium and the attendant problem of achieving uniform distribution of the mycoparasite in soil [Adams, 1990] or on tuber surfaces. This should now be investigated in soil, taking account of the population structure of *R. solani* because the effect of parasitism by *V. biguttatum* did not extend past the sites of incompatible anastomoses between genetically dissimilar host isolates. An *in vitro* comparison of strains of *V. biguttatum* is in progress, to determine whether they differ in efficiency of suppressing sclerotium formation by different strains of *R. solani*.

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