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Efficiency of isolates of *Coniothyrium minitans* as mycoparasites of *Sclerotinia sclerotiorum*, *Sclerotium cepivorum* and *Botrytis cinerea* on tomato stem pieces

M. Gerlagh[1], J.M. Whipps[2], S.P. Budge[2] and H.M. Goossen-van de Geijn[1]
[1]*DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands (Fax: 31 317 410113);* [2]*Horticulture Research International (HRI), Wellesbourne, Warwickshire, CV37 9EF, UK (Fax: 44 1789 470552)*

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Summary

Twenty five isolates of *Coniothyrium minitans* were screened for antagonism to *Sclerotinia sclerotiorum* in a Petri dish bioassay using tomato stem segments placed on sterile sand. The antagonistic activity of 23 isolates was quite uniform and only two less antagonistic isolates were identified. Antagonism, expressed as a reduction in the rate of tissue colonization by *S. sclerotiorum*, occurred, whether *C. minitans* was co-inoculated at the same time, one day before or one day after *S. sclerotiorum*, but was slightly restricted when *S. sclerotiorum* was given a lead of one day. On average, 50–80% of sclerotia of *S. sclerotiorum* formed on the stem pieces were infected by *C. minitans* two weeks after inoculation. Excluding the less antagonistic isolates, *Coniothyrium minitans* was recovered from over 80% of *S. sclerotiorum*-infected stem segments when co-inoculated but from a maximum of only 7% of stem pieces when exposed to *C. minitans* alone. When the experiments were carried out on non-sterile soil instead of sterile sand, infection of stem pieces by *S. sclerotiorum* was reduced and recovery of *S. sclerotiorum* and *C. minitans* from stem segments was decreased. Seven *C. minitans* isolates were also screened against *Sclerotium cepivorum* and *Botrytis cinerea* and, whereas the effect of *C. minitans* on *S. cepivorum*-infected tissue and sclerotia was essentially similar to that observed with *S. sclerotiorum*, *B. cinerea* infected tissue and sclerotia were not invaded by the antagonist.

Introduction

Coniothyrium minitans Campbell is a mycoparasite of a number of sclerotia forming plant pathogens, notably of *Sclerotinia* spp. (Adams, 1989; Ahmed and Tribe, 1977; Whipps and Gerlagh, 1992). Most isolates of *C. minitans* have been obtained from sclerotia of *S. sclerotiorum* (Lib.) de Bary or *S. trifoliorum* Erikss. (Whipps and Gerlagh, 1992). Isolates of *C. minitans* from *Sclerotium cepivorum* Berk. are rare, and sclerotia of *Botrytis cinerea* Pers. have only been described as hosts of *C. minitans* in laboratory conditions (Turner and Tribe, 1976). Relatively little is known of the ecology of *C. minitans*. It does not appear to be a plant pathogen (Turner and Tribe, 1975, 1976; Huang, 1977). Although *C. minitans* is generally found only in sclerotia (Sandys-Winsch et al., 1993; Whipps et al., 1993), it may also follow *S. sclerotiorum* through infected plant tissue and produce pycnidia on the plant surface (Trutmann et al., 1982; Huang, 1977). Consequently, application of *C. minitans* onto crop debris infected with *S. sclerotiorum* may reduce disease carry-over. This would require colonization of mycelium and sclerotia of *S. sclerotiorum* both on and in diseased tissue. Colonization of senescent plant tissue by *C. minitans* in the absence of *S. sclerotiorum*, thereby occupying the potential foodbase of the pathogen, would also be an advantage.

In this paper, the tomato stem segment assay of Whipps (1987) was used to screen 25 isolates from a worldwide collection of *C. minitans* for their capacity to colonize infected stem tissue and sclerotia of *S.*

sclerotiorum. The purpose was to obtain an impression of variation in efficiency of isolates and effects of mycoparasitism on *S. sclerotiorum*. A limited number were also screened for antagonism to *S. cepivorum* and *B. cinerea*.

Materials and methods

Fungal and plant material

Isolates of *C. minitans* were obtained from the culture collection of Horticulture Research International and maintained on potato dextrose agar (PDA, Oxoid) at 18 °C. Sources of these isolates can be found in Sandys-Winsch et al. (1993). *Sclerotinia sclerotiorum* and *Botrytis cinerea* were originally isolated from lettuce (*Lactuca sativa* L.) and *Sclerotium cepivorum* from onion (*Allium cepa* L.) and maintained as above.

Stem segments of tomato (*Lycopersicon esculentum* Mill.) cv Moneymaker, 2 cm long and 5–12 mm diameter, were obtained from two to three month-old plants grown in potting compost in 15 cm diameter pots in a glasshouse at 18–25 °C.

Experimental procedure

Two experiments using 21 isolates in total, were carried out to screen for antagonism against *S. sclerotiorum*. The experimental set-up was essentially as described by Whipps (1987). A 9 cm-diameter plastic Petri dish was filled with 30 g sterilized acid-washed silver-sand and 4.6 ml water added to obtain 75% of field capacity. Tomato stem segments were placed, three per Petri dish, in a radiating arrangement, cut ends just touching a centrally positioned inoculum of 9 mm-diameter agar discs. There were five treatments: I. *S. sclerotiorum* only; II. *C. minitans* only; III. *C. minitans* applied simultaneously on top of *S. sclerotiorum*; IV. *C. minitans* applied one day later on top of *S. sclerotiorum*; V. *S. sclerotiorum* applied one day later on top of *C. minitans*. All agar discs were placed with the fungal growth upwards and were taken from the margin of colonies of *S. sclerotiorum* and *C. minitans* grown on PDA at 20 °C for 3 days and 2 weeks, respectively.

In the first experiment, 10 isolates of *C. minitans*, CH3, G5, G10, A3 (as an internal standard), A4, A6, SI1, ST1, ST3 and D1 were screened. All *C. minitans* treatments consisted of 3 replicate Petri dishes, with the 'control' treatment I replicated 30 times. In the second experiment 12 isolates, CH2, CH8, G8, G9, A3, CM/MG/C9, MAR2, MAR3, C10, C18, C100B and C106 were screened, with 5 replicate Petri dishes for each *C. minitans* treatment and 10 for treatment I. Due to contamination of the stem pieces by saprophytes in an experiment not further reported here, stem pieces were surface sterilized before the second experiment. Briefly, tomato stems, approximately 25 cm long, were immersed for 3 min in 1% NaOCl, then rinsed three times in sterile water. Segments (2 cm) were then cut from these, discarding cut ends which had been exposed to the sterilizing agent.

In addition, to estimate the effect of microbial competition, the experiment was repeated using soil rather than sterile sand. The soil from the Rhine valley (loamy mixed calcareous mesic family of Typic Eutrochrepts, 25% < 2μm; pH/KCl 7.0; 2.8% organic matter) was air-dried, sieved (< 2mm) and brought to 75% of field capacity before use.

In a third experiment, 7 isolates (Conio, A1, A3, C10, C10A, C101A and ST3) were screened against *B. cinerea* and *S. cepivorum* in comparison to *S. sclerotiorum*. Five tomato stem pieces were used in each of 5 replicate plates and the pathogen and antagonist were inoculated simultaneously. A repeat of the third experiment, with only three replicates, yielded similar results and is not discussed further.

All Petri dishes in each experiment were incubated at 18 °C under fluorescent light (14 h day; 25 Wm^{-2} irradiance). Each experiment lasted for two weeks, with water added after one week to bring all Petri dishes back to their original weight to maintain the water content. The infection of stem segments by *S. sclerotiorum*, *S. cepivorum* and *B. cinerea* was assessed daily by measuring the length of browning along the stem, and then expressed as a percentage of total stem length. These values were averaged per Petri dish. Occurrence of pycnidia of *C. minitans* on the stem segments was also recorded.

Sclerotial viability and infection by C. minitans

At the end of the experiment, sclerotia of *S. sclerotiorum* from replicates of each treatment were counted, combined and weighed. They were surface sterilized by immersing for three minutes in Chloros (sodium hypochlorite, containing approximately 11% available chlorine) and absolute alcohol (50/50, v/v), followed by four rinses with sterile water. Subsequently, they were bisected and placed on 13 mm diameter plugs of PDA plus Aureomycin (0.32g l^{-1} of a powder containing 5.5% chlortetracyclin hydrochloride; Cyanamid, U.K.) (Whipps and Budge, 1990). In the third exper-

iment sclerotia were collected from each Petri dish separately. Sclerotia of *B. cinerea* were collected, surface sterilized for 3 min in Chloros only, rinsed four times in sterile water, bisected and placed onto plugs of PDA plus Aureomycin. Collections of sclerotia of *S. cepivorum* from the proximal or distal halves of the stem pieces were surface sterilized for 3 min. with chloros only, washed four times in sterile water and crushed. The crushed material was subdivided in ten lots and plated on plugs of PDA plus Aureomycin.

Small pieces of stem tissue from the distal end, including surface tissue, were plated on 13 mm diameter plugs of PDA plus Aureomycin and on Petri dishes containing PDA plus Aureomycin plus Triton X-100 (2 ml l^{-1}). Attempts were also made to take a 'sterile' sample from the interior (pith) of stem pieces which still had a rigid structure, as was generally the case for treatment II and for pieces which escaped infection by the pathogen.

After 1–2 weeks, colonies of *S. sclerotiorum, C. minitans* or other fungi developing from sclerotia or stem pieces were identified. When both *S. sclerotiorum* and *C. minitans* grew from a tissue piece, the result was scored as *C. minitans*, as *C. minitans* would eventually outcompete *S. sclerotiorum.*

Statistics

Percentage recovery of *C. minitans* from stem pieces was calculated as %C = 100 × C/T, where C is the number of stem pieces yielding *C. minitans*, T is the total number of stem pieces exhibiting browning and tested. For sclerotia, %C and %S represent repectively the percentage of sclerotia yielding *C. minitans* and either *S. sclerotiorum* or *S. cepivorum.*

All statistical analyses were performed using Genstat 5 (Genstat 5 Committee, 1987). Percentage rot (after angular transformation) and numbers and weights of sclerotia were subjected to analysis of variance (ANOVA). In experiments 1 and 2, sclerotia numbers and weights could not be assessed for individual replicates, therefore the interaction mean square was used to estimate the residual variance. All other data are binomial and were analysed using Generalized Linear Models (GLM). Pairwise comparisons were carried out using Student's t-test where the F-test in ANOVA yielded overall significance ($P \leq 0.05$); analogous pairwise comparisons were done for GLM analyses using the Genstat 5 Procedure RPAIR.

Results

Generally, inoculation of the stem segments with *S. sclerotiorum, S. cepivorum* or *B. cinerea* alone, resulted in browning of the tissue closest to the inoculated end within two days and led to complete rotting of the plant tissue usually within four days. In the *S. sclerotiorum* only control, all stem segments were infected by the end of the experiments. Sclerotia of *S. sclerotiorum* developed mainly at the distal end of the stem pieces while those of *S. cepivorum* and *B. cinerea* were present at any position along the stem segments. There was no extensive tissue browning or collapse following inoculation with *C. minitans* only, but the stem ends immediately in contact with the inoculum often turned dark brown to black. Rarely, this discoloration progressed into the pith of the segment for up to 5 mm and here *C. minitans* was isolated. This occurred in 7% of the stem segments (6 out of 90) in the first experiment, and in 4% of stem segments (8 out of 180) in the second experiment.

The effect of isolates of *C. minitans* on the rate of browning was significant, but the ranking of isolates was irreproducible when tested in replicates. Therefore, isolates can be considered as equivalent in this respect. No isolate × treatment interaction was found, and therefore data are presented as treatment or isolate averages.

Co-inoculation of *C. minitans* with *S. sclerotiorum, S. cepivorum* or *B. cinerea* resulted in slower progress of the pathogens through the stem segments (Table 1) and prevention of infection in about 8% of the stem segments. When *S. sclerotiorum* was given a lead of one day, the rate of browning of the stem segments (measured 3 or 4 days after inoculation) was consistently higher than with simultaneous inoculation or with *C. minitans* inoculated one day in advance. In addition, production of pycnidia of *C. minitans* occurred on numerous rotted stem pieces. Co-inoculation also resulted in a lower number of sclerotia of *S. sclerotiorum* and a smaller weight per sclerotium, particularly if *C. minitans* was inoculated first (Table 2). It proved impossible to collect, count and weigh all sclerotia of *B. cinerea* and *S. cepivorum* that were formed and so corresponding sclerotial data for experiment 3 are omitted.

Differences in antagonistic effects between isolates of *C. minitans* were limited. Only two isolates of low antagonistic capacity, ST3 and C100B, could be clearly distinguished from 23 effective antagonists (Tables 3 and 4). The different co-inoculation treatments did

Table 1. Effect of *Coniothyrium minitans* isolates on infection of tomato stem pieces by *Sclerotinia sclerotiorum*, *Sclerotium cepivorum* and *Botrytis cinerea*

Treatment		Percentage browning[1]				
		Exp. 1	Exp. 2	Exp. 3		
		S. sclerotiorum		*S. sclerotiorum*	*S. cepivorum*	*B. cinerea*
I	Pathogen only	91a	99a	93a	61a	67a
II	*C. minitans* only	0	0	0	0	0
III	Pathogen and *C. minitans* simultaneously	35d	69b	69b	39a	40b
IV	Pathogen first	79b	99a	–[2]	–	–
V	*C. minitans* first	61c	53c	–	–	–

Figures in the same column followed by the same letter are not significantly different ($P \leq 0.05$; ANOVA after angular transformation, pairwise comparison by t-test).
[1] Browning measured on day 3 (exp. 3) and day 4 (exp. 1 and 2) as progress of rot was faster in exp. 3 compared with 1 and 2.
[2] Not tested.

Table 2. Effect of *Coniothyrium minitans* isolates on the number, weight and infection of sclerotia of *Sclerotinia sclerotiorum* formed on tomato stem pieces

Treatment		Mean no. sclerotia per stem piece		Mean weight per sclerotium (mg)		Percentage infection of sclerotia by *C. minitans*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
I	Pathogen only	1.39a	1.47a	40a	48a	11a	0a
III	Pathogen and *C. minitans* simultaneously	1.12bc	1.06c	25c	36b	69b	81b
IV	Pathogen first	1.21ab	1.22b	33b	45a	70b	78b
V	*C. minitans* first	0.93c	0.98c	23c	36b	63b	52c

Figures in the same column followed by the same letter are not significantly different ($P \leq 0.05$; ANOVA (columns 2–5) and GLM (columns 6 and 7), pairwise comparison by t-test).

not lead to consistent differences in % recovery of *C. minitans* from stem pieces (data not shown). However, treatment V (antagonist first) led to a significantly lower percentage recovery of *C. minitans* from sclerotia in experiment 2 (Table 2). On average, the isolates of *C. minitans* were recovered from 83% of the *S. sclerotiorum* infected stem pieces and 69% of the sclerotia in experiments 1 and 2.

Stem segments placed on soil instead of sand resulted in very infrequent isolation of *S. sclerotiorum* and *C. minitans* with numerous colonies of saprophytes, such as *Fusarium* spp. present (data not shown). Consequently, fewer sclerotia were formed. However, those sclerotia that were produced were equally infected by *C. minitans* in soil as in sand.

In experiment 3, the isolates of *C. minitans* were not recovered from stem pieces infected by *B. cinerea*, nor from sclerotia of this pathogen. Stem segments infected with *S. cepivorum* yielded as many colonies of the effective *C. minitans* isolates as with *S. sclerotiorum* (Table 4). However, the sclerotia of *S. cepivorum* were much less infected by *C. minitans* than those of *S. sclerotiorum*. Isolate ST3 was an equally poor antagonist of both *S. cepivorum* and *S. sclerotiorum*. Spontaneous infection of control stem pieces and sclerotia by *C. minitans* frequently occurred in this experiment.

Discussion

Most isolates of *C. minitans*, whatever their origin and cultural characteristics (see Sandys-Winsch et al., 1993), were effective antagonists of *S. sclerotiorum*

Table 3. Recovery of specific isolates of *Coniothyrium minitans* from tomato stem pieces and sclerotia of *Sclerotinia sclerotiorum*

Experiment 1			Experiment 2		
Isolate	%C[1]		Isolate	%C	
	Stem pieces	Sclerotia		Stem pieces	Sclerotia
Control	0a	11	Control	0a	0a
A3	80b	77	A3	98b	77bc
A4	92b	75	CH2	98b	78bc
A6	92b	70	CH8	100b	96b
CH3	92b	84	MAR2	98b	72c
D1	96b	53	C10	93bc	72bc
G10	91b	72	C106	91bcd	68c
SI1	93b	87	MAR3	88bcd	61c
ST1	88b	78	C18	85bcd	80bc
G5	87b	65	CM/MG/C9	83bcd	75bc
ST3	12c	10	G8	73cd	72c
			G9	68d	65bc
			C100B	38e	27d

Figures in the same column followed by the same letter are not significantly different ($P \leq 0.05$; GLM, pairwise comparison by RPAIR).
[1] %C = percentage recovery of *C. minitans*.

Table 4. Recovery of *Sclerotinia sclerotiorum, Sclerotium cepivorum* and *Coniothyrium minitans* from stem pieces and sclerotia, following simultaneous inoculation of stem pieces with individual isolates of the mycoparasite and each pathogen

Isolate	Stem pieces				Sclerotia			
	S. sclerotiorum		*S. cepivorum*		*S. sclerotiorum*		*S. cepivorum*	
	%S	%C	%S	%C	%S	%C	%S	%C
Control	44	48	66a	6a	55a	45a	100	0
ST3	17	25	47a	0a	52a	48a	100	0
Average of 6 isolates: Conio, A1, A3, C10, C10A and C101A	3	81	5b	78b	5b	95b	80	20

Figures in the same column followed by the same letter are not significantly different ($P \leq 0.05$; GLM, pairwise comparison by RPAIR).
%S = % of stem pieces or sclerotia yielding *S. sclerotiorum* only.
%C = % of stem pieces or sclerotia yielding *C. minitans*.

under the experimental conditions used. The two isolates with less antagonistic activity, ST3 and C100B, had a rapid mycelial growth and poor pycnidial production (colony types 6 and 5 of Sandys-Winsch et al., 1993). In the third experiment, the antagonistic capacity of the culturally unique ST3 was compared with isolates of *C. minitans* from *S. sclerotiorum* on its original host, *S. cepivorum*. Clearly, ST3 was not especially adapted to *S. cepivorum* as all isolates from *S. sclerotiorum* were more effective against *S. cepivorum* than ST3. The factors controlling antagonistic capability and cultural characteristics in *C. minitans* deserve further study.

In general, the recovery of *C. minitans* was greater in stem pieces than in sclerotia (Table 3). Mixed recovery of *S. sclerotiorum* together with *C. minitans* was rare from stem pieces, but common from sclerotia. Presumably this was due to the more delicate structure of mycelium of *S. sclerotiorum* in tissue pieces compared with that in sclerotia. Nevertheless, *S. sclerotiorum* sclerotia were often non-viable in these assays of short duration. This consideration justified

the scoring of those sclerotia yielding both *S. sclerotiorum* and *C. minitans* as *C. minitans* only, since infected sclerotia would have been completely killed within a few weeks.

Recovery of *S. sclerotiorum* and *C. minitans* from stem pieces incubated on unsterilized soil instead of sand, was negligible. This relates to the relatively low competitive saprophytic ability of both fungi in plant residue in soil (Gerlagh et al., 1994) which is further expressed during the isolation procedure on the rich agar media. The lower production of sclerotia of *S. sclerotiorum* on plant tissue incubated on soil further illustrates the influence of the natural soil microbiota on inhibiting the development of *S. sclerotiorum*. However, recovery of *C. minitans* from surface sterilized sclerotia was similar under both sterile conditions and on soil and demonstrates that the mycoparasite has been fully competitive with the soil microflora in infecting sclerotia of *S. sclerotiorum*. Also, the soil microbiota did not prevent a high percentage of spontaneous infection of sclerotia by *C. minitans*. Thus, *C. minitans* is highly adapted to infect sclerotia of *S. sclerotiorum* despite a low competitive saprophytic ability in general.

None of the seven isolates of *C. minitans* tested against *B. cinerea* could be recovered from sclerotia or stem pieces, although they did reduce the speed of rotting. This fact, combined with the absence of any reports on isolation of *C. minitans* from sclerotia of *B. cinerea* from the field, makes it improbable that the former can be considered an effective antagonist of *B. cinerea*. The laboratory data of Turner and Tribe (1976), describing infection of *B. cinerea* by *C. minitans*, may not be representative of most isolates in nature.

S. sclerotiorum and *S. cepivorum* are both good hosts for *C. minitans*. The recovery of both fungi from infected stem pieces is about equal but sclerotia of *S. cepivorum* yield less *C. minitans* than those of *S. sclerotiorum*. The much smaller sclerotia may disintegrate quickly after attack, so that sclerotia of *S. cepivorum* infected by *C. minitans* can rarely be collected. However, it is also possible that *S. cepivorum* is a less preferred host than *S. sclerotiorum*. This is corroborated by the rarity of spontaneous infection by *C. minitans* of the control stem pieces with *S. cepivorum*, whereas in the same experiment it was frequent with *S. sclerotiorum*. Spontaneous infection was difficult to prevent, since the Petri dishes had to be opened during the daily measurements of the progress of rotting.

Apart from a few pith infections of stem segments by *C. minitans* in the absence of *S. sclerotiorum*, the mycoparasite did not colonize healthy tomato tissue. This is a desirable characteristic but it will prevent multiplication of the antagonist in field situations in the absence of *S. sclerotiorum*. As soon as tissue was infected by *S. sclerotiorum* or *S. cepivorum*, abundant pycnidia of *C. minitans* developed, though less with *S. cepivorum* (data not shown). This is another argument for considering the latter as a less preferred host of *C. minitans*.

Considering the ability of most strains of *C. minitans* to invade plant tissue infected by *S. sclerotiorum* and subsequently decrease formation and survival of sclerotia of the pathogen, it would appear that *C. minitans* has considerable potential to prevent disease carryover of *Sclerotinia* on residues in the field.

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