

Interaction between *Sclerotinia sclerotiorum* and *Coniothyrium minitans* Strains with Different Aggressiveness

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The effects of *Sclerotinia sclerotiorum* live and autoclaved sclerotia, and sclerotial exudates, and commercial oxalic acid were tested *in vitro* on seven *Coniothyrium minitans* strains differing in aggressiveness towards *S. sclerotiorum*. Only sclerotial exudates and autoclaved sclerotia affected the mycelial growth rate of almost all the strains tested, whereas a change in the *C. minitans* mycelial growth pattern was observed in the presence of autoclaved sclerotia and live sclerotia germinating by the myceliogenic eruptive germination. In addition, sclerotial exudates had a stimulatory effect on spore germination. These findings indicate that the various treatments could influence the *C. minitans* strains regardless of their aggressiveness.

KEY WORDS: *Sclerotinia sclerotiorum*; *Coniothyrium minitans*; interaction.

INTRODUCTION

Coniothyrium minitans Campbell is a worldwide mycoparasite of *Sclerotinia sclerotiorum* (Lib.) de Bary (3,15). In one *C. minitans* strain it was shown that host sclerotia have a stimulatory effect on conidial germination and germ tube growth, and that water and ethanol extracts of sclerotia affect the mycelial growth rate (19).

During the formation of sclerotia, water droplets containing relatively large amounts of nutrients are exuded (2). Moreover, *S. sclerotiorum* mycelium is known to secrete oxalic acid (OA), a common metabolite of many fungi (7).

To extend previous studies of the *C. minitans*-*S. sclerotiorum* interaction (10,11,19), we addressed the influence of OA, sclerotial exudates, and live and autoclaved host sclerotia on the growth rate and pattern of seven *C. minitans* strains differing in aggressiveness (11). We also examined the effect of sclerotial exudates on spore germination.

MATERIALS AND METHODS

Coniothyrium minitans strains

Seven *C. minitans* strains were tested: strain 286.81 was obtained from C.M.I. (Baarn, the Netherlands); strains IVT5, IVT1 and C102 from Dr. M. Gerlach (Wageningen, the Netherlands); and strain Z1 from Dr. G. Zizzerini (Perugia, Italy); strains M3 and M4 were isolated in our laboratory from two sclerotia found in a single lucerne plant naturally infected by *S. sclerotiorum*. The strains were classified according to colony type (11),

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and maintained on potato dextrose agar (PDA), at 20°C, in the dark. As done in our previous study (11), the aggressiveness of the different strains was determined based on the percentage of sclerotia attacked, the rate of symptoms appearance on inoculated sclerotia, and the number of dead sclerotia 2 weeks after inoculation with a conidial suspension (10^3 conidia ml⁻¹, 20 ml for 10 sclerotia); 20 sclerotia were inoculated in a typical experiment. After 24 h of incubation at 20°C, in the dark, sclerotia were removed from the suspension and placed on the surface of water agar (WA, Difco Bactoagar) plates under the same conditions. After 2 weeks, sclerotium mortality was assessed as follows: each sclerotium was cut in halves, which were transferred onto carrot slices, previously sterilized in H₂O₂ (28%) for 30 min. After 3–5 days of incubation at 18°C, mycelial growth of *S. sclerotiorum* was used to identify and count surviving sclerotia. Mortality was calculated from the difference between the number of inoculated and germinated sclerotia.

TABLE 1. Mycelial growth rate and aggressiveness of *Coniothyrium minitans* strains

Strains	WA ⁺	Mycelial growth rate§			OA ⁺	Aggressiveness§§
		Exudates	Sclerotia			
			Live	Autoclaved		
286.81	1.0a z ⁺⁺	3.8b z	0.7a z	3.2b y	0.7a y	9.6x
IVT1	0.7a z	3.4b y	0.6a z	2.0b z	0.5a z	9.3x
IVT5	1.3a z	4.4b y	0.9a z	3.7b y	1.0a z	6.6y
C102	2.3a y	4.5b y	1.1a yz	2.3a y	1.9a y	3.0z
M3	1.7a yz	3.9b y	1.8a y	2.2a y	2.0a y	2.6z
M4	1.1a z	4.5c y	0.9a z	2.5b y	1.6a y	0.6w
Z1	5.1a x	5.9a x	7.0a x	6.6a x	5.7a x	3.3z

§Means expressed as mm 48 h⁻¹.

§§Figures represent the number of *S. sclerotiorum* sclerotia dead (out of 20 initially), 15 days after inoculation with a *C. minitans* conidial suspension. Data refer to a typical experiment, as described in Materials and Methods.

+ WA= water agar; OA= oxalic acid.

++ Values followed by a common letter (a,b,c within lines; w,x,y,z within columns) do not differ significantly ($P=0.05$) by the Student-Newman-Keuls test.

Effects of sclerotia, sclerotial exudates and oxalic acid on C. minitans mycelial growth

(a) Preparation of *C. minitans* inoculum and determination of mycelial growth: A strip (25.0 mm long × 1.5 × 2.0 mm) of *C. minitans* was cut from the growing margin of 9-day-old PDA cultures, and placed in the center of a petri dish (9 cm in diameter) containing WA; the sector of WA 2.0 cm away from the inoculum was removed. Live or autoclaved sclerotia, or strips (25.0 × 1.5 × 2.0 mm) of WA enriched with either OA or sclerotial exudates, were then placed on the naked petri dish surface in front of the inoculum strip but in contact with the remaining WA. Controls consisted of WA strips that replaced sclerotia, OA and exudates.

The mycelial growth of *C. minitans* was observed after 4, 6 and 8 days of incubation at 20°C, in the dark, and measured along three lines that were drawn on the bottom of each plate before incubating.

These experiments were repeated three times, with at least three replicates for each treatment. Data were evaluated by analysis of variance (ANOVA) and correlation

coefficients ($P=0.05$) and significant differences (Student-Newman-Keuls test; $P=0.05$) were determined.

(b) Production and preparation of sclerotia: Sclerotia were obtained from 30-day-old cultures of *S. sclerotiorum* (strain B 24) grown on PDA. The sclerotia were collected when firm and dry, placed in sterile petri dishes at room temperature, and used after 2 weeks. Before use, groups of sclerotia of similar size (total weight: 80 mg) were either autoclaved (1 atm, 20 min), or surface sterilized with a Na hypochlorite (5%)–ethanol (95%) mixture (1:1; v/v) (17), and then rinsed twice in 25 ml of sterile water for 15 min.

(c) Sclerotial exudates and oxalic acid: Using a sterile Pasteur pipette, sclerotial exudates were collected from a sufficient number of 7-day-old sclerotia to obtain a pool of approximately 50 ml and stored at -20°C until use. Before use, sclerotial exudates were passed through a sterile $0.2\ \mu\text{m}$ cellulose acetate filter and mixed (1:1; v/v) through melted WA; test strips were then taken and placed in the petri dishes containing WA with a plug of *C. minitans*. Test strips of oxalic acid (Prolabo [Paris, France]; final concentration $1\ \text{mg}\ \text{ml}^{-1}$) were prepared as above.

Analysis of sclerotial exudates

Sclerotia were analyzed for reducing sugars (14) and protein (1) content. Preliminary experiments with exudates from 5–7- and 10-day-old sclerotia disclosed that exudates from 7-day-old sclerotia contained the maximum amount of sugars and proteins.

Qualitative determination of carbohydrates was performed by thin-layer chromatography (TLC). The exudates (10, 25 and $50\ \mu\text{l}$) were analyzed at 20°C on (a) silica gel 60 plates (Merck, ref. 5721) in acetonitrile–ethylacetate–1-propanol–water (85:20:20:15; v/v/v/v) (12) to identify glucose, mannose and mannitol; and (b) cellulose plates (Merck, ref. 5716) in 1-propanol–ethylacetate–water (140:20:40; v/v/v) (16) to identify glucose, inositol, trehalose and fructose. The carbohydrates were visualized on the plates using an alkaline silver nitrate dipping procedure (12); the pale brown background was decolorized by immersing the plates in sodium thiosulfate solution (12) diluted 1:1. The TLC profiles of the exudates were compared with appropriate standards that were run simultaneously. R_G values were: arabitol, $R_G = 131$; fructose, $R_G = 114$; mannose, $R_G = 114$; mannitol, $R_G = 82$; trehalose, $R_G = 52$; inositol, $R_G = 29$.

Electrical conductivity was determined using a conductivity meter with KCl as the standard.

Spore germination test

In view of the finding that sclerotial exudates influenced mycelial growth (see Results), we tested the effect of such exudates on spore germination. A spore suspension of each *C. minitans* strain, obtained from cultures grown on oat agar for 3 weeks, was centrifuged ($1000 \times g$, 15 min), rinsed three times in sterile water containing aureomycin ($0.05\ \text{mg}\ \text{ml}^{-1}$), and collected in water to a concentration of 2×10^6 conidia ml^{-1} ; $150\ \mu\text{l}$ aliquots were then added to $150\ \mu\text{l}$ of exudates. After 48 h of incubation at 20°C , three replicate counts of 100 spores were performed; a spore was determined to have germinated according to the criteria of Whipps *et al.* (19). In controls, $150\ \mu\text{l}$ of sterile water replaced exudates; the percentage of germination was log transformed; however, as ANOVA showed similar statistical differences between means for both transformed and non-transformed data, the original data are reported.

RESULTS AND DISCUSSION

It was reported recently that sclerotial exudates from *S. sclerotiorum* accelerated the germination of conidia of the mycoparasite *Trichoderma hamatum* (9). The present study is the first to address the effect of sclerotial exudates on *C. minitans* spore germination and the findings confirm their stimulatory effect. Indeed, we observed a 40–50% increase in *C. minitans* spore germination, with no significant differences among the strains; no spore germination occurred in the controls.

The length of time needed for sclerotia penetration did not differentiate the strains (11). Table 1 shows that regardless of the treatment, the mycelial growth rate of strain Z1 was unaffected, whereas that of the other strains tested was significantly increased in the presence of sclerotial exudates. Only the four strains showing the lowest hyphae extensions in controls were stimulated by autoclaved sclerotia; however, all the strains developed particularly dense colonies toward autoclaved sclerotia. These results may be explained by quantitative and qualitative differences in the nutrients that were diffused into the growth medium, and by the different ability of each strain to use them as an energy source; indeed, large quantities of material may be released by the autoclaved, weakened and/or collapsed sclerotia, and diffused into the medium.

Sclerotial exudates of *S. sclerotiorum* contain amino acids, lipids, ions and proteins and carbohydrates are present in sclerotial exudates as well as in sclerotia (4,5,6,9,13). We found that exudates contained $339.2 \pm 40.4 \mu\text{g ml}^{-1}$ and $271.3 \pm 34.4 \mu\text{g ml}^{-1}$ of reducing sugars and proteins, respectively; the electrolyte concentration was $120.2 \text{ micromhos cm}^{-1} \text{ ml}^{-1}$ of exudates, and pH was 5.7.

The chromatograms of the sclerotial exudates showed spots corresponding to small amounts of glucose, and traces of trehalose, mannitol and inositol, which are known components of sclerotial exudates (6). Traces of mannitol and mannose were found in exudates, although mannitol was reported to be absent in exudates of sclerotia obtained on PDA (6) and mannose has been found to date only in sclerotia (13). Fructose and arabinol, which are present in *S. sclerotiorum* sclerotia (13), were not found in exudates in which an unknown compound(s) with $R_G = 81$ on cellulose and $R_G = 196$ on silica gel plates was also detected.

Like OA, germinating live sclerotia did not influence *C. minitans* mycelial growth rate (Table 1), in contrast to the findings of others (8,19). It was reported that OA secreted into growth medium by *Sclerotium delphinii* could inhibit growth and spore germination of fungi (8). Since *C. minitans* follows the *S. sclerotiorum* infection within plant tissues (18) in which the pathogen produces OA (7), the lack of inhibitory effects of OA on this mycoparasite's mycelial growth rate could constitute a useful tool for its antagonistic activity. The difference in the results obtained using live sclerotia might be due (at least partially) to the experimental conditions. In a previous work (19), only those sclerotia that were removed from agar plates and tested within 2 weeks without any treatment, germinated. In the present study, all the sclerotia germinated and produced actively growing mycelium. Furthermore, the sclerotia were disinfected and rinsed in sterile water before use, and it is known that dried sclerotia placed in water rapidly leak substances (4).

It is noteworthy that in plates where live sclerotia occasionally germinated by myceliogenic eruptive germination (previously observed in dried and washed sclerotia [2]), *C. minitans* developed particularly dense colonies towards the host without any changes in

its growth rate. As *C. minitans* is able to parasitize the host mycelium, the presence of a dense mass of *S. sclerotiorum* mycelium associated with this type of sclerotial germination might promote the observed *C. minitans* growth pattern by offering the nutritional stimulus.

No positive correlations emerged between the aggressiveness of *C. minitans* strains and their mycelial growth rate *in vitro* in the presence of *S. sclerotiorum* sclerotia, sclerotial exudates and OA. Indeed, the correlation coefficients (r) for all pairs of variables were: aggressiveness and mycelial growth rate in controls, $r = -0.302$; aggressiveness and mycelial growth rate towards live sclerotia, exudates and OA, $r = -0.21$, $r = -0.241$ and $r = -0.37$, respectively. No significant correlation emerged between aggressiveness and mycelial growth rate towards autoclaved sclerotia. Moreover, the observation that the growth rate of strain Z1 was not stimulated by the host or its metabolites might have no bearing on its efficacy as a biocontrol agent, because this strain shows the highest hyphal extension rate among those measured in the absence of any nutritional stimulus (Table 1) (19).

The use of aggressive strains (such as 286.81, IVT1 and IVT5) that are not inhibited by OA, but are stimulated by other host metabolites, can improve the biocontrol of *S. sclerotiorum*. Indeed, host metabolites can be used as an energy source by the mycoparasite, and thus provide it with sufficient colonization potential to breach the defense mechanisms of the host and parasitize it.

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