Special Topics

Penetration of Sclerotia of *Sclerotium rolfsii* by *Trichoderma* spp.

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ABSTRACT

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Penetration of sclerotia of *Sclerotium rolfsii* by *Trichoderma* isolates was followed by a direct and an indirect approach. Sclerotia were immersed in an aqueous suspension of *Trichoderma* conidia (1.5×10^7/ml) and incubated on water agar or on soil. Periodically, sclerotial samples were surface sterilized with 1% sodium hypochlorite solution for 2 min, washed, and plated out on a selective medium for *Trichoderma* and on a medium that allowed germination of sclerotia only. When the sclerotia were preincubated on either agar or soil, *Trichoderma* isolates differed in their ability to penetrate the sclerotia. Penetrated sclerotia that showed different stages of degradation were fixed, sectioned, stained, and examined with a

light microscope. Hyphae of *Trichoderma* penetrated the rind and cortex, lysed the medullar tissue, produced chlamydospores inside and conidia outside the sclerotia, and finally underwent autolysis. Degraded sclerotia became dark in color, soft, and empty, and disintegrated under slight pressure. Sclerotia that were allowed to be penetrated on agar without being degraded and that were further incubated in soil, retained their firmness and were not degraded by the penetrating antagonist. Penetration capacity is an important but not the only property required for *Trichoderma* isolates to be efficient biocontrol agents.

Isolates of the genus *Trichoderma* have long been known for their capacity to reduce plant diseases caused by fungal pathogens (2). The properties for a successful antagonist, which have been suggested as necessary for an efficient disease control agent, include strong competitive ability, antibiotic production, direct parasitism, and lysis (1,2). The mechanism whereby antagonists control diseases caused by sclerotial fungi such as *Sclerotium rolfsii* Sacc. may involve (1) interference with sclerotial germination by the antagonist that may or may not be followed by sclerotial degradation; (2) inhibition of the growth of the pathogen in the soil; and (3) prevention of host penetration by the pathogen. Resistance of sclerotia to microbial attack in the soil (2,4) might be related, at least in part, to their resistance to penetration by segments of the soil microbiota.

The purpose of this work was to evaluate penetration of *S. rolfsii* sclerotia by isolates of *Trichoderma* and its impact on sclerotial viability. A preliminary report was published (8).

MATERIALS AND METHODS

Isolates. Isolate Sr-3 of *S. rolfsii*, provided by R. Rodriguez-Kabana, Auburn, AL, was used throughout this study. Twenty-two isolates of *Trichoderma harzianum* Rifai, five isolates of *T. viride* Pers., and one isolate of *T. hamatum* (Bon.) Bain., all from the collection of the Soilborne Diseases Laboratory at Beltsville, MD, were used.

Production of sclerotia of *S. rolfsii*. Sclerotia of *S. rolfsii* were produced on Difco potato-dextrose agar (PDA). Agar disks (5 mm in diameter) of *S. rolfsii* from PDA cultures were transferred to plates containing 15 ml of PDA, and the plates were incubated at 28 C for 3 wk in sealed polyethylene bags to prevent drying. The sclerotia were dislodged from the agar surface with a soft brush.

Sclerotia were also produced on natural soil (Sassafras loamy sand, SLS) by the following procedure. A mixture of oat seed and water (1:1, w/w) was autoclaved for 90 min, seeded with an agar plug (5 mm in diameter) of *S. rolfsii*, and incubated for 7 days at 28 C. Portions (2 kg) of SLS were passed through a sieve (0.85-mm pore size), adjusted to 80% moisture holding capacity, and spread in autoclavable polypropylene pans ($12 \times 22 \times 48$ cm). The soil surface was then smoothed with a petri dish cover. Fifty grams of oat seed colonized by *S. rolfsii* were evenly spread on the soil surface, and the seed was covered with 1 kg of moist soil to a depth of 4 mm. The containers were placed in plastic bags to prevent drying and incubated for 10 days at 28 C. Sclerotia formed on the soil surface were scraped with a metal spatula and separated from soil particles by wet-sieving on a sieve of 0.85-mm pore size. The sclerotia were then washed with sterile water and air-dried. Twenty grams of sclerotia were formed from 50 g of oats.

Production of conidia of *Trichoderma*. Conidia of *Trichoderma* were produced by transferring agar disks (5 mm in diameter) from the edge of 1-wk-old PDA cultures to plates of PDA and incubating these for 7-8 days under continuous fluorescent light ($\sim 700\ \mu\text{Ein}/\text{m}^2/\text{sec}$). Conidia were removed from the agar surface by pipetting 3-5 ml of sterile water on the surface and gently rubbing the surface with a sterile cotton-tipped applicator. Conidia were counted with a hemacytometer and aqueous suspensions were adjusted to provide the desired concentration of conidia in each test.

Infestation of sclerotia with *Trichoderma* conidia and subsequent surface sterilization. One hundred sclerotia were immersed in a suspension of *Trichoderma* conidia (1.5×10^7/ml) and incubated for 4 hr at 28 C. The sclerotia were collected on filter paper (Whatman no. 4) in a Büchner funnel, immersed for 2 min in a 1% sodium hypochlorite (NaClO) solution, and washed with sterile distilled water on filter paper. Fifty sclerotia from each treatment were plated out on each of two media (ie, the selective *Trichoderma* medium [TME] developed by Papavizas [15] and recently modified [16] for isolation of *Trichoderma* spp. from soil and on PDA-8HQ). Medium TME, containing V-8 juice, antimicrobial agents, and tergitol, was used at half-strength of the selective inhibitors (½ TME) to detect *Trichoderma* associated

with sclerotia and to allow for limited germination of sclerotia of isolate Sr-3. PDA-8HQ, consisting of PDA supplemented with 10 μg/ml of 8-hydroxyquinoline, allowed sclerotia to germinate while inhibiting germination of conidia of *Trichoderma* for 24–48 hr. The plates were incubated at 28 C for 7 days and examined for growth of *Trichoderma* and germination of sclerotia. The NaClO eradicated the surface conidia of *Trichoderma* without affecting sclerotial germinability.

Penetration of sclerotia by *Trichoderma* isolates. SLS was passed through a sieve (0.85-mm pore size) and adjusted to 80% of its moisture-holding capacity. Soil portions of 60 g were placed in 90-mm-diameter petri dishes, and the surface was smoothed with a small (30-mm-diameter) glass petri dish cover. One hundred sclerotia from agar or soil were immersed for 30 min in 5-ml suspensions containing 1.5×10^7 spores per milliliter of each of the *Trichoderma* isolates tested and placed either on water agar or on the natural sandy loam soil. The sclerotia were then pushed into the soil, allowing only their tops to be uncovered. All plates were placed in polyethylene bags to prevent drying and incubated for 1 wk at 28 C. Sclerotia were removed at intervals, surface sterilized, plated out on ½ TME and on PDA-8HQ, and incubated for 7 days at 28 C. To prevent interference between sclerotia, those that did not germinate after 3 days on the PDA-8HQ medium were removed to another plate of the same medium for further incubation. Experiments were run at least twice with five replications.

Histological studies. Test sclerotia were fixed in formalin-propionalcohol (9), dehydrated in tertiary-butyl alcohol, embedded in paraffin, sectioned, and stained with safranin-fast green (9,12). Sections were examined with a standard light microscope (Leitz) at magnifications of ×540 and ×950. Three sectioned sclerotia represented a treatment. Twenty-seven sections were examined from each sclerotium.

RESULTS

Comparison of penetration capacity of different *Trichoderma* isolates. Twenty-eight isolates of *T. harzianum, T. viride,* and *T. hamatum* were compared for their sclerotial penetration capacity. Growth of *Trichoderma* from surface-sterilized sclerotia on WA was interpreted as penetration. The tested isolates differed greatly in their capacity to penetrate Sr-3 sclerotia. *Trichoderma* isolates WT-6, WT-6-24, Th-5, Th-19, T-1-R5, T-1-R6, TR40 (ipro-3M), and Th-1 (Br-3M) penetrated 60% of the sclerotia or more. Isolates WT-6-5, WT-6-6, T-1-R1, Th-8, Th-14, Th-17, Tri(4), TMP-R2, and TR40(ben-25) penetrated 20% of the sclerotia or less; isolates

Th-1, Th-6, Th-11, Th-20, T-1, T-3, T-1-R3, TR40, TMP, and Kalo-1 were between 20 and 60%.
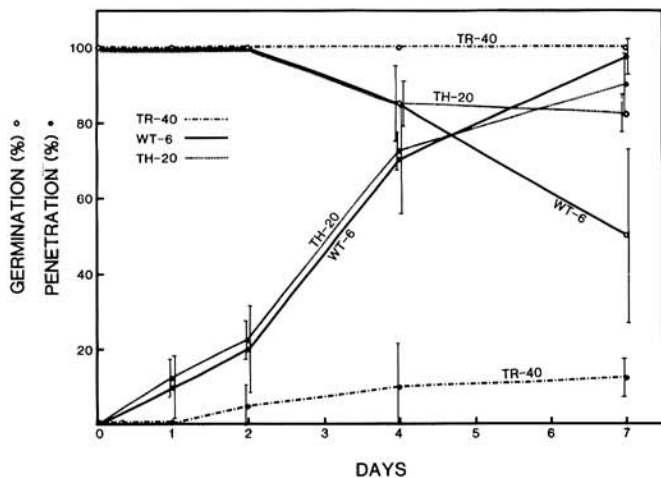
The incidence and degree of penetration of three isolates of *T. harzianum* (WT-6, Th-20, and TR-40) into sclerotia and the subsequent sclerotial germination were also determined. Sclerotia, naturally produced on soil and surface sterilized, were immersed in individual conidial suspensions of WT-6, Th-20, and TR-40 and plated on water agar. Samples were taken from the water agar at 1-day intervals, surface sterilized, and plated on ½ TME to detect *Trichoderma* penetration and on PDA-8HQ to follow sclerotial viability. Isolates Th-20 and WT-6 penetrated 90% of the sclerotia within 7 days, whereas TR-40 penetrated only 10% (Fig. 1).

Sclerotial germinability was affected to a lesser degree. Isolate WT-6 caused a 50% reduction, whereas Th-20 and TR-40 caused only a 20 and 0% reduction, respectively. Similar effects on infection and germinability were obtained with PDA-produced sclerotia. Surface sterilization appeared not to affect the infection process by *Trichoderma*, but it eliminated contaminating soil microbiota.
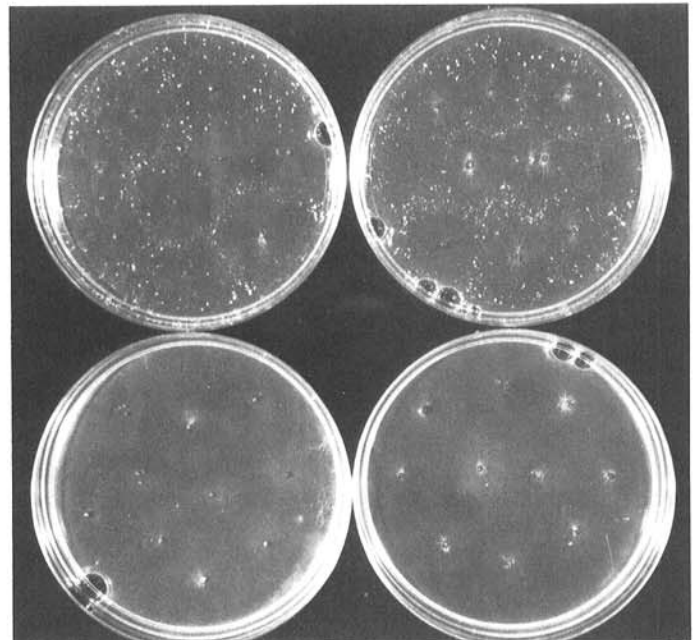
Penetration was also observed on natural soil. Naturally produced sclerotia were immersed in spore suspension of WT-6, Th-20, and TR-40, incubated on natural soil in plates for 7 days, surface sterilized, plated on ½ TME, and incubated for 7 days. Penetration percentage was 55 ± 8, 19 ± 5, and 10 ± 5 with Th-20, WT-6, and TR-40, respectively, with a statistically significant difference ($P = 0.05$) between Th-20 and either WT-6 or TR-40. No penetration by native *Trichoderma* was observed in noninoculated control soil plates.

An experiment was performed to determine the minimum *Trichoderma* spore concentration necessary to obtain the maximum sclerotia penetration. Naturally produced sclerotia were immersed in various concentrations ($1.5 \times 10^2 - 1.5 \times 10^7$) of a spore suspension of *T. harzianum* (WT-6) and incubated on WA for 4 days at 28 C. The sclerotia were surface sterilized and incubated on ½ TME for 7 days. Penetration on water agar was relatively unaffected by a spore concentration between 1.5×10^2 and 1.5×10^7 spores per milliliter. As few as 1.5×10^3 spores per milliliter resulted in as much penetration as that with 1.5×10^7.

Interaction between *Trichoderma* and sclerotia on water agar. Fresh, PDA-, and naturally produced surface-sterilized sclerotia were immersed in a conidial suspension of *T. harzianum* (WT-6)

Fig. 2. Interaction between sclerotia of *Sclerotium rolfsii* (strain Sr-3) and *Trichoderma harzianum* (strain WT-6) on water agar. Lower and upper left, sclerotia produced in PDA; lower and upper right, sclerotia produced in soil. Upper row, with *T. harzianum*; lower row, without the antagonist.

Fig. 1. Penetration of naturally produced sclerotia of *Sclerotium rolfsii* (strain Sr-3) by three strains of *Trichoderma harzianum* (Th-20, TR40, and WT-6), examined on water agar, and subsequent germinability of sclerotia examined on the *Trichoderma* medium TME and on the PDA-8HQ medium. Bars represent standard deviation of the mean. Days indicate incubation time on the water agar.

and placed on water agar (Fig. 2). No sporulation or degradation of the sclerotia was observed during the first 2 wk of incubation. In the noninoculated control in the absence of *Trichoderma*, sclerotia germinated profusely. In the plates containing *Trichoderma*-inoculated sclerotia, the antagonist grew towards the periphery and sporulated at a distance of 2–3 cm from the sclerotium. After 10–14 days, some direct sporulation on the sclerotia was also observed. After 3–4 wk, some of the sclerotia (~10%) lost their firmness, darkened, and finally degraded. The majority of sclerotia, however, appeared not to be affected by the antagonist.

Microscopic observations of the penetration process. In sclerotia incubated with *Trichoderma* (Th-20), and sectioned and stained, mycelium was observed in the cortex and the medulla. No degradation of either the rind or the cortex was observed at the initial stages of penetration. At a later stage, colonized sclerotia that were soft disintegrated upon slight pressure. The medulla cells of these sclerotia appeared lysed, and *Trichoderma* hyphae were seen forming chlamydospores. Conidia could not be seen inside the disintegrated sclerotium, but were observed on the sclerotial surface. In degraded sclerotia, the cortex tissue was replaced by *Trichoderma* hyphae, which grew out through the rind. Stained control sclerotia had a typical structure, ie, a deeply stained rind, a cortex composed of a layer of few large cells, and a medulla consisting of thick-walled cells and air spaces between them (3).

DISCUSSION

This is the first detailed report on penetration of sclerotia of *S. rolfsii* by *Trichoderma* isolates. Sclerotia produced on PDA or in soil were penetrated by isolates of *Trichoderma* on both water agar and in natural soil, but isolates differed in their capacity for penetration. Sclerotia produced in natural soil and on PDA were similarly affected. For example, *T. viride* (T-1-R6) and *T. harzianum* [TR40-(ipr-3M)] were the best penetrators, whereas *T. viride* (T-1-R3) and *T. harzianum* (Th-11) showed a poor penetration capacity. Penetration was not always followed by degradation of the sclerotia. Thus, some sclerotia that had been penetrated by *Trichoderma* and incubated in soil or on water agar remained firm and did not show any degradation. Among the strains of *Trichoderma* that showed a poor penetration capacity, only *T. hamatum* [Tri-(4)] was found to be an effective biocontrol agent against *S. rolfsii* (G. C. Papavizas, *unpublished*). Not all of the strains showing high penetration capacity proved to be efficient control agents against *S. rolfsii*. Similarly, there seems to be no clear relationship between penetration capacity and its effect on sclerotial germination. Thus, of the three *T. harzianum* isolates examined for their penetration capacity and for their effect on sclerotial germination, only WT-6 inhibited sclerotial germination on PDA-8HQ medium, whereas Th-20 affected germination only slightly in spite of its high penetration capacity (Fig. 1). Although we used a selective medium that was supposed to inhibit *Trichoderma* growth completely, some growth of the antagonist was observed after few days of incubation, indicating that the observed values of germinability could be lower than the real ones.

In spite of generally being resistant to attack on water agar, some sclerotia were also degraded by the penetrating *Trichoderma*. These sclerotia were sectioned, fixed, and stained. When observed with the light microscope, the stained sections revealed a picture similar to that described elsewhere (3), using a transmission electron microscope, showing clearly the rind, cortex, and medulla layers of the sclerotium. In the *Trichoderma*-inoculated sclerotia, penetrating mycelium seemed to run through the rind and cortex without affecting the host cells. However, upon reaching the medulla, it ramified, lysed the medullar tissue, and produced chlamydospores. During this process it also underwent autolysis. No conidia could be found inside the degraded sclerotia, but they were abundantly formed on the sclerotial surface. Coley-Smith et al (5) first reported on chlamydospore production by *T. hamatum* invading sclerotia of *Sclerotium delphinii*, whereas Henis et al (8) first reported on chlamydospore production by *T. harzianum* in sclerotia of *S. rolfsii*. It seems that different conditions are required for production of conidia and chlamydospores. Possible factors involved may include nutrients (11), carbon dioxide, and/or oxygen concentrations. The role of chlamydospores in *Trichoderma* survival and capacity to parasitize other fungi in the soil and in other environments remains to be evaluated.

T. viride has been isolated from decayed sclerotia of *S. rolfsii* (6,7), *Sclerotinia sclerotiorum* (10), and *Botrytis convoluta* (13). There is some doubt about the ability of *Trichoderma* spp. to parasitize healthy sclerotia, but more evidence exists that it attacks those that are injured in some way (4). High specificity is also reported. Some isolates of *T. viride* can cause a high level of decay in *S. sclerotiorum*, whereas others do not attack sclerotia of this fungus but will infect those of *S. trifoliorum* and *S. borealis* (10,14).

The hyphae of *Coniothyrium minitans* can apparently grow through sclerotium tissue of *S. trifoliorum* without causing much physical disturbance, and pycnidia are frequently found on the exterior. Later, secondary invasion of the tissue by other microorganisms occurs, and the sclerotium disintegrates (4). Penetration of the rind of *S. sclerotiorum*, *S. trifoliorum*, *Botrytis cinerea*, and *Claviceps purpurea* by *Verticillium roseum* (=*Acrostalagmus roseus*) has also been observed (4).

Penetration capacity may be one of the major properties required for an isolate of *Trichoderma* to be an efficient biocontrol agent. It is not known what other properties are required. Preliminary, unpublished tests indicated that all the isolates tested were capable, at least to some degree, of parasitizing mycelium of *S. rolfsii* on glass slides. Therefore, properties other than direct attack and lysis by lytic enzymes or nonenzymatic toxins, may be involved.

From the data presented in this work it appears that certain isolates of *Trichoderma* can penetrate intact sclerotia of *S. rolfsii*. However, penetration alone does not lead to sclerotial degradation, and naturally produced sclerotia will have an attached soil microbiota of which a part has penetrated the sclerotium without affecting its germinability. The environmental factors and the properties of the antagonist that lead to sclerotial attack and degradation remain to be elucidated.

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