An In Vitro Test for Evaluating Efficacy of Mycoparasites on Sclerotia of *Sclerotinia sclerotiorum*

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ABSTRACT

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A modification of Kohn's technique for apothecial induction in the Sclerotiniaceae was developed to test the efficacy of mycoparasites on sclerotia of *Sclerotinia sclerotiorum*. The modification is an incubation period at 25 C prior to Kohn's initial cold treatment. The new method was compared with the previously used method of pairing the fungi on an agar medium (such as potato-dextrose agar) and determining the effects on growth of *S. sclerotiorum* mycelium. This method tested the effects of *Gliocladium roseum, G. virens, Trichoderma harzianum*, and *T. viride* on carpogenic germination of *S. sclerotiorum*. Numbers of carpogenically germinating sclerotia and apothecia produced per sclerotium were used to measure the effects of the mycoparasites. Mycoparasites were also evaluated on the basis of their percentage of reisolation from sclerotia. A good separation of the fungi was obtained using this technique. *G. virens* was the mycoparasite most frequently recovered from *S. sclerotiorum* sclerotia and significantly reduced all measurements of carpogenic germination.

Sclerotinia sclerotiorum (Lib.) de Bary is a ubiquitous plant pathogen with a host range of more than 360 species distributed in 64 families (9). Crop rotation, sanitation, reduced irrigation, resistant varieties, and the use of protectant chemicals often cannot economically and consistently control diseases caused by *S. sclerotiorum* (13,14).

Since 1979, more than 30 species of fungi and bacteria as well as insects and other organisms have been reported to be antagonists of *Sclerotinia* spp. (13). *Coniothyrium minitans* Campbell (4), *Gliocladium roseum* (Link.: Fr.) Bain. (8), *G. virens* Miller & Foster (15), and *Trichoderma viride* Pers.: Fr. (3,5,8) parasitize sclerotia of *S. sclerotiorum* in soil. *G. virens* prevented the formation of *S. sclerotiorum* sclerotia and parasitized preformed sclerotia in an in vitro study (15). *C. minitans* and *T. viride* destroyed *S. sclerotiorum* sclerotia after 100 days of incubation in soil in vitro at an ambient air temperature of 20–22 C (4). Neither of these studies reported the frequency of myceliogenic or carpogenic germination of surviving sclerotia (7).

In this study, we examined the effect of the length of incubation period with a mycoparasite prior to a cold treatment on the ability of *S. sclerotiorum* sclerotia to germinate carpogenically. The technique used was adapted from Kohn (6) and differs from other in vitro methods in that the mycoparasite contacts mature sclerotia instead of mycelia.

MATERIALS AND METHODS

We evaluated Kohn's (6) technique using *G. roseum, G. virens,* and *T. viride*, all reported as antagonists of *S. sclerotiorum*, and *T. harzianum* Rifai, an antagonist of other sclerotium-forming fungi (8). The isolate of *S. sclerotiorum* used was obtained from naturally infected soybean (2). The designations for the mycoparasites (and their sources) are as follows: *G. roseum*-12 (Illinois soybean), *G. roseum*-40 (West Virginia University culture collection), *G. virens* (J. C. Tu, Agriculture Canada, Harrow, Ontario), *T. harzianum* (Kalo Laboratories, Kansas City, MO), and *T. viride* (West Virginia University culture collection). Stock cultures of *S. sclerotiorum* and the mycoparasites were maintained on potato-dextrose agar (PDA).

A spore suspension ($1–4 \times 10^6$ conidia per milliliter of each mycoparasite) was prepared in sterile water from 7- to 10-day-old PDA cultures incubated in

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darkness at 21 C. Forty milliliters of the spore suspension was added to 14 g of sterile vermiculite previously moistened with 65 ml of sterile distilled water in preparation dishes. Twenty 1-mo-old sclerotia 4–6 mm in diameter selected from *S. sclerotiorum* cultured at 21 C in darkness on autoclaved carrot disks were added to the preparation dishes containing the mycoparasite conidial suspensions. The mycoparasite and sclerotial treatment combinations were 1) *S. sclerotiorum* alone (control), 2) *S. sclerotiorum* and *G. roseum*-12, 3) *S. sclerotiorum* and *G. roseum*-40, 4) *S. sclerotiorum* and *G. virens*, 5) *S. sclerotiorum* and *T. harzianum*, and 6) *S. sclerotiorum* and *T. viride*. Each combination was replicated five times (each replicate consisting of 20 sclerotia), and the experiment was repeated once.

The preparation dishes were sealed with Parafilm and incubated for either 0, 7, or 14 days at 25 C in darkness, then for 2 wk at 4 C in darkness, and finally for 2–3 wk at 15 C with a 14-hr photoperiod to induce apothecial production. The number of mature apothecia and stipes were recorded for each replicate. Calculation of an FLSD value allowed comparison of fungal combinations both within and between incubation periods.

Percentage recovery of the respective mycoparasites from sclerotia that had not produced apothecia, sclerotia that had produced apothecia, and from apothecia was determined. For each treatment, 25 sclerotia that produced apothecia and all apothecia were surface-disinfested in a 0.5% sodium hypochlorite solution for 4 min, plated on PDA, incubated in darkness at 21 C, and evaluated for percent recovery of the mycoparasites after 7 days. This was conducted for all three incubation periods from the second experiment.

Number and size of apothecia produced per sclerotium varied greatly despite initial screening of sclerotia for size. Therefore, we examined the relationship between weight of a sclerotium and the apothecia and stipes it produced. Ten apothecia-producing sclerotia in each replicate of the control after 14 days of incubation in the second experiment were collected. Fresh weight of the apothecia plus stipes and the sclerotia were recorded. The apothecia plus stipes and the sclerotia were oven-dried for 72 hr and the dry weights recorded. Two regression analyses were conducted. In the first, fresh weight of apothecia and stipes was regressed on fresh weight of sclerotia. In the second, dry weight of apothecia and stipes was regressed on the dry weight of sclerotia. Linear and curvilinear models were tested for goodness of fit.

Growth of *S. sclerotiorum* in the presence of *S. sclerotiorum*, *G. roseum*-12, *G. roseum*-40, *G. virens*, *T. harzianum*, or *T. viride* was evaluated. Plugs (3 mm in diameter) were taken from the advancing margins of 4-day-old cultures of both *S. sclerotiorum* and the respective fungus and transferred to 9-cm culture plates containing PDA. The two plugs were placed 1.5 cm from the edge of the plate at opposite sides. Plates were evaluated 4 days later to determine if the fungi either formed zones of inhibition, overgrew each other, or met but did not overgrow. In addition, 3-mm plugs of either *G. roseum*-12 or *G. roseum*-40 were placed on four plates, and plugs of *S. sclerotiorum* were added 4 days later. Growth of the two fungi was evaluated 4 days after inoculation with *S. sclerotiorum*.

RESULTS

Fresh weight of apothecia and the number of stipes produced per sclerotium increased with increasing fresh weight of the sclerotium. Total fresh weight of the apothecia produced by a sclerotium was predicted by the linear model $Y = 0.0284 + 1.1339X$, where Y = total predicted fresh weight in grams and X = fresh weight of the sclerotium in grams. The coefficient of determination (r^2) was 0.62 and was significant at $P = 0.01$. A curvilinear model did not significantly improve the r^2 value.

On PDA plates, *G. virens* and *T. viride* overgrew the colonies of *S. sclerotiorum*. *T. harzianum*, *S. sclerotiorum*, *G. roseum*-12, and *G. roseum*-40 grew up to colonies of *S. sclerotiorum* but did not form zones of inhibition or overgrow *S. sclerotiorum*. When either *G. roseum*-12 or *G. roseum*-40 was placed on PDA 4 days before subsequent inoculation with *S. sclerotiorum*, a distinct zone of inhibition formed between the two colonies.

The mean number of sclerotia failing to produce either apothecia or stipes in the control was not affected by length of incubation period prior to apothecial induction (Tables 1 and 2). The mean number of apothecia and stipes per sclerotium in the control increased between 0 and 1 wk of incubation in the second experiment but not in the first (Tables 1 and 2). The number of sclerotia failing to produce apothecia increased with an increased incubation period prior to apothecial induction for *G. virens* but not for the other four mycoparasites (Tables 1 and 2). Increased length of incubation with *G. roseum*-12, *G. roseum*-40, *T. harzianum*, and *T. viride* increased the mean number of apothecia and stipes observed per sclerotium in the second experiment (Table 2).

G. virens was the only mycoparasite that suppressed stipe production within an incubation period. This was observed at 14 days in the first experiment and at 0, 7, and 14 days in the second (Tables 1 and 2). *G. virens* was also the only mycoparasite that significantly affected the number of apothecia plus stipes per sclerotium within an incubation period (Tables 1 and 2).

Recovery of *S. sclerotiorum* in the control was greater from sclerotia than from either apothecia or stipes (Table 3). Recovery was greater from sclerotia that produced apothecia than those that did not produce apothecia. Recovery of *S. sclerotiorum* from either *G. roseum* treatment was similar to that of the control. Recovery was lowest from the *G. virens* treatment, and recovery from *T. viride* and *T. harzianum* was lower than from the control.

Recovery of the respective mycoparasites from their treatments was higher from *S. sclerotiorum* sclerotia

Table 1. Mean apothecial production of *Sclerotinia sclerotiorum* after incubation with *Gliocladium roseum*, *G. virens*, or *Trichoderma viride* for either 0, 1, or 2 wk prior to apothecial induction in the first experiment

Fungal combination	No. of sclerotia with no apothecia[a] (incubation period [wk])			No. of apothecia plus stipes per sclerotium[a] (incubation period [wk])		
	0	1	2	0	1	2
S. sclerotiorum[b]	2.4[c]	2.8	3.8	2.2[d]	2.5	3.0
S. sclerotiorum + *T. viride*[b]	2.2	1.8	5.4	2.6	2.5	3.8
S. sclerotiorum + *G. virens*[b]	5.2	4.6	15.4	2.0	1.0	1.4
S. sclerotiorum[e]	3.0[f]	1.0	2.2	4.6[g]	2.7	4.2
S. sclerotiorum + *G. roseum*-12[e]	0.8	1.4	0.6	3.1	4.2	4.6
S. sclerotiorum + *G. roseum*-40[e]	0.8	0.2	1.6	2.4	3.0	3.8

[a] Each number is the mean of five replicates, 20 sclerotia per replicate.
[b] These three treatments were run simultaneously.
[c] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods run simultaneously is 4.1.
[d] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods run simultaneously is 3.6.
[e] These three treatments were run simultaneously.
[f] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods run simultaneously is 3.0.
[g] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods run simultaneously is 1.8.

than from apothecia (Table 3). Recovery was greatest from the *G. virens, T. viride*, and *T. harzianum* treatments. *G. roseum*-40 was recovered only from sclerotia not producing apothecia at the zero incubation period.

DISCUSSION

We have demonstrated that the modification of Kohn's technique is better than pairing the fungi on artificial media. A similar technique has been used by Radke and Grau (10) to evaluate the effects of herbicides on carpogenic germination. Environmental conditions greatly affect the interactions of a mycoparasite and its host (1). The interaction between temperature and length of incubation of sclerotia with mycoparasites prior to cold conditioning could be studied further using the technique described in this paper.

Comparison of the results of pairing *G. virens* and *T. viride* with *S. sclerotiorum* illustrates a shortcoming of screening techniques using artificial media to evaluate possible antagonists. *G. virens* and *T. viride* both overgrew colonies of *S. sclerotiorum* on PDA. This could be interpreted as an indication of the ability of these organisms to be good biological control agents. When the modification of Kohn's technique was used, *G. virens* reduced the viability of preformed sclerotia and the number of apothecia produced per sclerotium, whereas *T. viride* did neither. Another shortcoming of in vitro media studies can be illustrated by our study where zones of inhibition developed between colonies of *S. sclerotiorum* and different-aged colonies of *G. roseum*. Production of antimicrobial compounds and staling products can be dependent on the age of the fungi and the media's physical characteristics (micronutrient composition, osmotic pressure, and pH [11]), rendering media studies such as these inadequate.

In this study, equal levels of mycoparasitism measured by rate of recovery of the respective antagonist from sclerotia of *S. sclerotiorum* did not result in equivalent levels of reduction in apothecial production. In some instances, the presence of the mycoparasite appeared to increase apothecial production as in the case of *G. roseum*. The extent of parasitism of the sclerotium should be measured not only by incidence of recovery of the antagonist but also by the failure of the sclerotium to germinate either carpogenically or myceliogenically.

Screening sclerotia for uniformity of size is an important factor that should be considered in future studies. Our results indicate that the potential number of apothecia increases with the increasing fresh weight of the sclerotium. This concurs with Saito's (12) observation that production of apothecia uses constituents of the sclerotium. *S. sclerotiorum* sclerotia are more uniform in size when produced on PDA (M. N. Cline and J. D. Mueller, *unpublished*) than on autoclaved carrot pieces. To optimize experimental conditions, therefore, sclerotia should be grown on PDA and/or selected on a weight basis.

Table 2. Mean apothecial production of *Sclerotinia sclerotiorum* after incubation with *Gliocladium roseum*, *G. virens*, *Trichoderma harzianum*, or *T. viride* for either 0, 1, or 2 wk prior to apothecial induction in the second experiment

Fungal combination	No. of sclerotia with no apothecia[a] (incubation period [wk])			No. of apothecia plus stipes per sclerotium[a] (incubation period [wk])		
	0	1	2	0	1	2
S. sclerotiorum	1.4[b]	0.2	0.6	3.5[c]	6.7	6.0
S. sclerotiorum + *G. roseum*-12	4.8	2.8	3.4	3.6	4.7	5.0
S. sclerotiorum + *G. roseum*-40	3.2	4.8	0.2	2.5	5.4	6.4
S. sclerotiorum + *T. harzianum*	0.6	0.6	0.8	3.0	6.3	6.0
S. sclerotiorum + *T. viride*	1.0	1.4	0.6	3.4	4.6	6.5
S. sclerotiorum + *G. virens*	7.2	19.0	12.2	2.2	0.8	2.3

[a] Each number is the mean of five replicates, 20 sclerotia per replicate.
[b] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods is 4.0.
[c] FLSD ($P = 0.05$) for all comparisons between treatments and incubation periods is 1.8.

Table 3. Recovery of *Sclerotinia sclerotiorum* and the mycoparasites *Gliocladium roseum, G. virens, Trichoderma harzianum,* and *T. viride* on potato-dextrose agar from *S. sclerotiorum* apothecia and sclerotia after incubation at 25 C prior to apothecial induction

Fungal combination	Sample size[b]	Percentage recovery[a]	
		S. sclerotiorum	Mycoparasite
S. sclerotiorum			
Apothecia	139	64	...
Sclerotia-a[b,c]	73	81	...
Sclerotia-na[d]	12	75	...
S. sclerotiorum + *G. virens*			
Apothecia	60	28	33
Sclerotia-a	42	5	62
Sclerotia-na	96	1	77
S. sclerotiorum + *T. viride*			
Apothecia	162	17	15
Sclerotia-a	72	0	81
Sclerotia-na	17	18	77
S. sclerotiorum + *T. harzianum*			
Apothecia	157	22	10
Sclerotia-a	75	25	44
Sclerotia-na	11	9	82
S. sclerotiorum + *G. roseum*-12			
Apothecia	115	57	18
Sclerotia-a	75	72	27
Sclerotia-na	66	52	50
S. sclerotiorum + *G. roseum*-40			
Apothecia	140	51	0
Sclerotia-a	67	67	0
Sclerotia-na	41	49	15

[a] Each number is the mean of three incubation times prior to apothecial induction.
[b] Sample size dependent on availability of apothecia and sclerotia in each category.
[c] Sclerotia that produced apothecia.
[d] Sclerotia that did not produce apothecia.

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