Colonization of sclerotia of *Sclerotinia minor* by a potential biocontrol agent, *Penicillium citrinum*[1]

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ABSTRACT

Sclerotia of *Sclerotinia minor* were soaked in a conidial suspension (1.3×10^7 conidia/mL) of *Penicillium citrinum* at 25 ± 2 C for 1 h. This resulted in coating each sclerotium with about 3.7×10^4 conidia. Treated sclerotia were incubated either in the dark on dry or damp Whatman No. 1 filter paper or in pasteurized and nonpasteurized soil at 25 ± 2 C, for up to eight weeks. Colonization by *P. citrinum* of sclerotia incubated on damp or dry filter paper was 70 and 25%, respectively. Seventy four percent of sclerotia incubated in pasteurized soil were colonized and destroyed by *P. citrinum*, whereas 55% colonization and destruction occurred in sclerotia incubated in a nonpasteurized soil. Similarly treated sclerotia of *Sclerotinia sclerotiorum* variety *major* and *Sclerotium rolfsii* were incubated in pasteurized soil and colonized by *P. citrinum* at 45 and 5%, respectively, over the same period of time. Up to 50% colonization and destruction by *P. citrinum* has been observed on sclerotia of *S. minor* recovered from soil in a peanut field in Oklahoma. These findings suggest a potential use of *P. citrinum* as a biocontrol agent for *S. minor*.

Key Words: Groundnut, *Arachis hypogaea* L.

Sclerotia are the principal survival structures of several soilborne pathogens, and constitute an important link in many disease cycles (10,19). The rate of natural destruction of sclerotia in soil in the absence of a host plant varies with the different mycoparasites present (6,13,23), and is influenced by several other factors including soil moisture, temperature, aeration, and organic matter content (10). More that 30 species of fungi and bacteria have been implicated as parasites or antagonists of different *Sclerotinia* species (6,9,12,13,16, 18,20). For all but a few, tests for parasitic or antagonistic activity has been conducted exclusively in vitro.

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Proof of parasitic or antagonistic activity of most, under natural conditions, is just being explored (1,15,23). Huang and Hoes (16) showed that *Coniothyrium minitans* effectively controlled the population of *S. sclerotiorum* in sunflower fields. Huang (15) also observed the destruction of 58% of the sclerotia of *S. sclerotiorum* in soil infested with an isolate of *Trichoderma viride.* Turner and Tribe (22) reported up to 65% of sclerotia of *S. trifoliorum* were destroyed by *C. minitans* in field soil. Adams and coworkers (1,2,3) found three mycoparasites of *S. sclerotiorum* that appear to be involved in the natural destruction of sclerotia in soil. Tu (21) reported that *Gliocladium virens* parasitized both mycelia and sclerotia of *S. sclerotiorum* and inhibited the development of sclerotia.

Penicillium citrinum Thom, was isolated from field soil planted to peanuts in Oklahoma (17). Growth of *S. minor* was significantly inhibited on Czapek Dox agar medium amended with 5-15% (v/v) filtrate from cultures of *P. citrinum* and completely inhibited at 20% (17). Rai and Saxena (18) had previously reported the presence of *Penicillium* species among 24 other fungal species isolated from the surface of the sclerotia of *S. sclerotiorum.* In their study they found that *Penicillium citrinum,* P. *steckii, P. funiculosum* and *P. pallidum,* particularly caused heavy colonization and destruction of the sclerotia of *S. sclerotiorum. Aspergillus niger* and *A. ustus* were next to *Penicillium* species in causing such effects, thus showing that these organisms may play an important role in reducing the inoculum density of *Sclerotinia* species in soil. Ayers and Adams (4) also reported the occurrence of *Trichoderma* and *Penicillium* spp. on the surface of sclerotia of *Sclerotinia* spp.

The purpose of this study was to investigate the efficacy of *P. citrinum* on colonizing and destroying the sclerotia of *S. minor* and some other sclerotial-forming pathogens, incubated under varying laboratory conditions, and to explore the potential of *P. citrinum* as possible biocontrol agent for *S. minor.*

Materials and Methods

Production of sclerotia of S. minor

S. minor was isolated from infected peanut plants cv., Florunner and maintained on potato dextrose agar (PDA) at 25 ± 2 C. Sclerotia of *S. minor* used in the study were produced on oat seed. Fifty g of oat seed and 50 mL of deionized water were mixed in flasks, autoclaved for 1 h, allowed to cool, and then inoculated with two 6-mm discs of a 3-day old culture of *S. minor* grown on PDA. After incubation for five weeks at 25 ± 2 C, the flask contents were spread to dry in flat trays at room temperature. Sclerotia were then sieve separated from the oats (2).

Isolation of P. citrinum, and production of conidia

Penicillium citrinum was isolated on PDA from the sclerotia of *S. minor* recovered from field soil planted to peanuts (17). Sclerotia were separated from soil on mesh screen (.84 mm), surface sterilized in 0.5% sodium hypochlorite for 5 min, and then plated on PDA containing 100 μg/mL streptomycin sulfate (SPDA). Plates were incubated at 25 ± 1C under continuous light (800 lux) for 48 h. Conidia of *P. citrinum* which heavily colonized these sclerotia were picked using a sterile toothpick and placed on SPDA and incubated under the conditions described above. Isolate was maintained on SPDA in slants, and cultured regularly on SPDA plates. Five mL of sterile deionized water was pipetted into each plate of a 5-week old culture of *P. citrinum.* A small sterile brush was used to loosen and suspend conidia. There was about 1.3 x 10^7 conidia/mL of *P. citrinum* in the suspension as estimated by hemacytometer counts.

Treatment of sclerotia with conidia of P. citrinum.

Five grams of sclerotia were weighed into each of 4-50 mL beakers and surfaced sterilized with a 0.5% sodium hypochlorite solution for 4 min. Sclerotia were then washed with running tap water and rinsed twice with sterile deionized water. The beakers were labeled I, II, III, and IV for later treatments. Three hundred mg of carboxymethyl cellulose (CMC) was mixed into the sclerotial contents of beakers II and IV. Four mL of conidial suspension of *P. citrinum* were pipetted onto sclerotial contents of beakers III and IV. Contents of each of the four beakers were thoroughly mixed with a spatula and allowed to set for 1 h. From each of the 4 treatments, 10 sclerotia were randomly chosen and singly placed in a vial containing 1 mL of sterile deionized water containing Amway (Amway Corp., Michigan 49301) all purpose adjuvant at the rate of 1 mL/L of water. Vials were shaken vigorously on a vortex mixer for 1 min, and a hemacytometer was used to determine the approximate number of conidia of *P. citrinum* that were released from each sclerotium.

Soil Mix

Soil mix (Soil:sand:fine peat moss; 2:2:1, v/v) was sieved through a 2 mm mesh screen to remove large debris particles. Soil in this mix was river bottom soil obtained commercially from Stillwater Sand & Gravel Company in Stillwater, Oklahoma. About 40 g of soil mix were placed in 100 x 15 mm pyrex petri dishes, ten ml of deionized water added, the dishes pasteurized at 170 C for 1 h, and cooled overnight. Percent soil moisture content was determined gravimetrically from soil contained in 5 randomly chosen petri dishes.

Incubation conditions

Penicillium citrinum-coated sclerotia and nontreated controls were incubated on pasteurized damp or dry Whatman #1 filter papers in glass petri dishes (100 x 15 mm). All the dishes were kept at room temperature under diffused light. Also, *P. citrinum*-coated sclerotia and nontreated controls were incorporated into pasteurized and nonpasteurized soils contained in petri dishes, and incubated as described above. Three mL of deionized water were added to each soil plate weekly as needed. Each week, for 8 weeks, four petri dishes from each treatment were randomly selected, sclerotia retrieved, surface sterilized as described, and plated on SPDA to determine their viability. Forty sclerotia were picked out randomly from the sclerotial lot of each petri dish, plated in groups of 20 sclerotia per plate and incubated at room temperature for 3 days. Number of geminated sclerotia on each plate was recorded.

Effect of P. citrinum on other sclerotial-forming pathogens

Sclerotia of *Sclerotinia major* isolated from soybeans, and *Sclerotium rolfsii* isolated from peanuts, were produced on SPDA treated in a similar fashion with *P. citrinum* and incubated in pasteurized soil at room temperature. Non-coated sclerotia of both pathogens were also incubated in soil as controls. Plates were randomly selected weekly, and sclerotia were retrieved by sieving and plated on SPDA to determine their viability. The test was repeated twice and similar results were recorded each time.

Results

Maximum colonization and destruction of sclerotia of *S. minor* by *P. citrinum* occurred by the sixth week of incubation in pasteurized soil. Seventy four percent colonization occurred on sclerotia coated with conidia of *P.*

citrinum, whereas, 72% colonization occurred in sclerotia coated with both CMC and *P. citrinum* (Table 1). CMC did increase the adherence of more conidia of *P. citrinum* to the sclerotia of *S. minor*, as 4.6 x 10^4conidia/mL, were recovered when CMC was not used. *P. citrinum* was not recovered from sclerotia of treatments I and II. Seventy percent of the sclerotia were colonized and destroyed when coated with *P. citrinum* and incubated on damp filter paper, whereas, only twenty five percent colonization occurred on dry filter paper. Thus, moisture seemed to be a requirement for effective colonization and destruction of sclerotia of *s. minor* by *P. citrinum*. The percent moisture content of the pasteurized soil used was determined to be about 23%. About 56% of sclerotia of *S. minor* were colonized by *P. citrinum* when incubated for six weeks in nonpasteurized soil (Table 2).

Table 1. Percentages of *Sclerotinia minor* colonization by Penicillium citrinum in four treatments in pasteurized soil, over a period of six weeks.

Week	Treatment			
	Sclerotia only	Sclerotia and CMC	Sclerotia and P. citrinum	Sclerotia, CMC and P. citrinum
0	0.6	1.3	4.4 a	3.8 a
1	1.9	0.6	7.5 b	10.6 b
2	2.5	2.5	13.1 c	11.9 b
3	1.9	1.8	21.9 d	16.9 c
4	3.8	2.5	28.8 e	21.3 d
5	5.0	1.3	55.6 f	47.5 e
6	3.8	2.5	73.8 g	71.9 f

160 sclerotia were plated per treatment representing 4 replications.

Sclerotia of S. minor colonized by P. citrinum were recorded 3 days after plating on SPDA.

Means within a column followed by the same letter are not significantly different (P = .05) according to Duncan's multiple range test.

Table 2. Percentages of sclerotia of *Sclerotia minor* colonized by *Penicillium citrinum* in soil and on filter paper.

Week	Treatment			
	Damp filter paper	Dry filter paper	Pasteurized soil	Nonpasteurized soil
0	2.5 a	0.0 a	4.4 a	2.5 a
1	12.5 b	5.0 b	7.5 b	11.3 b
2	20.0 c	5.0 b	13.1 c	21.3 c
3	27.5 d	7.5 c	21.9 d	23.1 c
4	37.5 e	2.5 d	28.8 e	55.6 d
5	55.0 f	10.0 e	55.6 f	55.0 d
6	70.0 g	25.0 f	73.8 g	55.6 d

160 sclerotia of S. minor were plated from each treatment representing 4 replications.

Sclerotia colonized by P. citrinum were recorded 3 days after plating on SPDA.

Means within a column followed by the same letter are not significantly different (P = .05) according to Duncan's multiple range test.

Penicillium citrinum did not colonize sclerotia of *Sclerotium rolfsii* to a significant degree. Only 5% colonization occurred by the 4th week of incubation. A decline in colonization to 2.5% was noticed thereafter (Table 3). A substantial level of colonization (45%) was found when the sclerotia of *s. major* were coated and incubated in a pasteurized soil. In all comparisons with the other pathogens, sclerotia of *s. minor* were the most colonized by *P. citrinum*.

A significant positive linear correlation between the colonization of sclerotia of *S. minor* by *P. citrinum* and time of incubation was obtained under incubation conditions in pasteurized soil, nonpasteurized soil and damp filter paper (Figs. 1 and 2). Higher correlations

Table 3. Colonization of the sclerotia of three soilborne pathogens by *Penicillium citrinum* in pasteurized soil.

Pathogen	Weeks of incubation						
	0	1	2	3	4	5	6
S. minor[1]	4.4[3] a	7.5 a	13.1 a	21.9 a	28.8 a	55.6 a	73.8 a
S. major[2]	2.5 a	10.0 a	17.5 a	12.5 b	15.0 b	37.5 b	45.0 b
S. rolfsii[2]	0.0 a	2.5 b	2.5 b	0.0 c	5.0 c	2.5 c	2.5 c

[1] Percentages of sclerotia colonized by P. citrinum computed from 4 replications of 40 sclerotia in each.

[2] Percentages of sclerotia were computed from 4 replications of 10 sclerotia in each.

[3] Means within a column followed by the same letter are not significantly different (P = .05) according to Duncan's Multiple Range Test.

were obtained in pasteurized soil (r = .96) and damp filter paper (r = .98) than in nonpasteurized soil (r = .92). A higher linear correlation value (r = .96) was obtained in treatment where *P. citrinum* was coated directly to sclerotia of *S. minor*, than when CMC was used to enhance coating (r = .91) (Fig. 3). *Sclerotium rolfsii* had the lowest correlation value (r = .16) when sclerotia of 3 pathogens, *S. minor*, *S. sclerotiorum*, and *S. rolfsii* were compared in pasteurized soil for colonization by *P. citrinum* (Fig. 4).

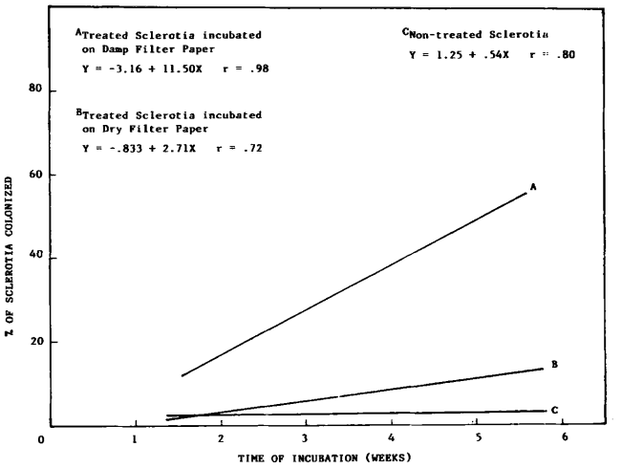

Fig. 1. Linear regression between percentages of sclerotia of *Sclerotinia minor* colonized by *Penicillium citrinum* and weeks of incubation on filter paper.

Discussion

The mechanism whereby antagonists control diseases caused by fungi producing sclerotia such as *Sclerotinia minor*, may involve a number of processes: interference with sclerotial germination by the antagonist that may or may not be followed by sclerotial degradation; inhi-

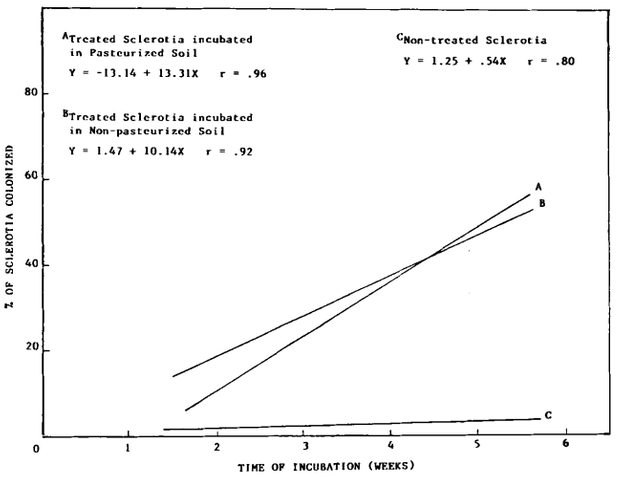

Fig. 2. Linear regression between percentages of sclerotia of *Sclerotinia minor* colonized by *Penicillium citrinum* and weeks of incubation in soil.

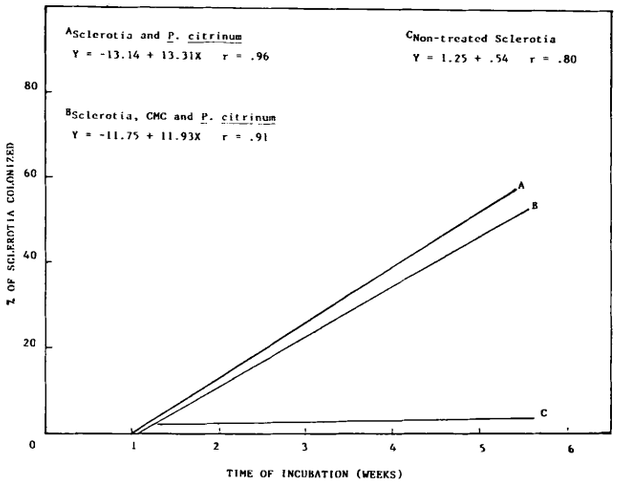

Fig. 3. Linear regression between percentages of sclerotia of *Sclerotinia minor* colonized by *Penicillium citrinum* under two different treatments and weeks of incubation.

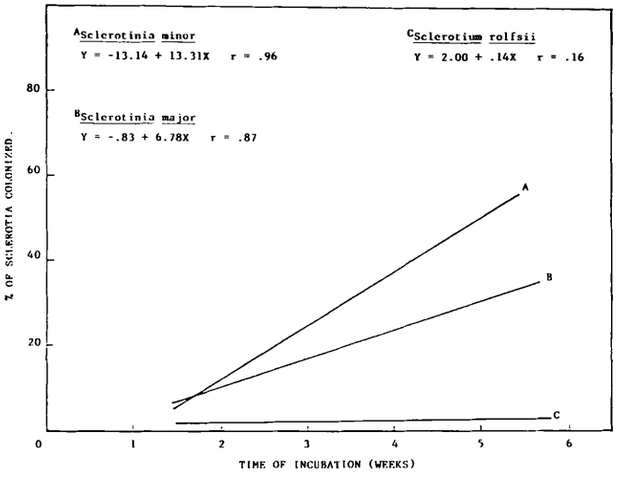

Fig. 4. Linear regression between percentages of sclerotia of three different pathogens colonized by *Penicillium citrinum* and weeks of incubation in pasteurized soil.

bition of the growth of the pathogen in the soil; and the prevention of host penetration by the pathogen (14). Resistance of sclerotia to microbial attack in the soil might be related at least in part to their resistance to penetration by segments of the soil microbiota (5,7). The properties of 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 (5,7). Mycoparasitism is a widespread phenomenon. The importance of this mechansim lies in whether it occurs in a natural or near natural environment in the presence of competing microorganisms and under the direct influence of the variable soil environment. Study of the ecology of mycoparasites will be required to fully understand biological control of soilborne pathogens by this mechanism, and to devise ways to increase its effectiveness. Substantial progress has been made with *Sporidesmium sclerotivorum,* a mycoparasite destructive to *Sclerotinia* spp. and related fungi (1). Ecological studies have shown that most mycoparasites have exacting nutritional requirements for growth and infection of the resistant structures such as sclerotia. There are still many aspects about mycoparasitism in relation to survival of fungal propagules in the soil that are largely unexplored and poorly understood.

The difference in the colonization level by *P. citrinum* with and without CMC suggests that CMC possibly acted as a barrier for effective colonization of sclerotia of *S. minor* by the antagonist. The lower level of colonization obtained in nonpasteurized soil was probably due to the effects of other microorganisms in the soil, whose activities may have hindered the interaction of *P. citrinum* and the sclerotia of *S. minor.* This agrees with the expected difference between the controlled environmental conditions, compared to the observed low level of performance by organisms when they are introduced into the natural environment (11).

The positive linear correlation obtained when regressing the percentages of sclerotia colonized by *P. citrinum* and the time of incubation gives an approximation of the predicted colonization rate, and can be calculated from the regression equations. The slope of the regression line is a measure of the rate of colonization of sclerotia by *P. citrinum* between different incubation periods. Thus, an increase in the time of incubation of treated sclerotia generally leads to higher colonization and destruction of sclerotia of *S. minor* by *P. citrinum.*

Baker and Cook (7), suggested that hyperparasites should be more effective against survival structures of pathogens, because these are generally less mobile and do not multiply rapidly. Consequently, the hyperparasite has the opportunity to penetrate and colonize its potential host. They also emphasized that hyperparasitism had only limited value in controlling pathogens present at high propagule densities and with rapid spreading characteristics. As much as 50% colonization and destruction of sclerotia by *P. citrinum* has been observed on sclerotia of *S. minor* recovered from soil planted to peanuts in Oklahoma.

Considering these characteristics, *P. citrinum* appears to be an effective antagonist of *S. minor.* It grows

rapidly, and sporulates profusely in agar media. Antagonists effective against a wide range of pathogens offer a greater potential for use in biological control (8). *P. citrinum* seems to be a selective antagonist as it effectively colonizes the sclerotia of *S. minor,* and *S. sclerotiorum,* but not the sclerotia of *S. rolfsii.* The high degree of destruction of sclerotia of *S. minor* brought about by coating the sclerotia with conidia of *P. citrinum,* under the conditions stated earlier, indicates that this fungus may have a potential as a biological control agent for reducing the inoculum density of this sclerotial-forming plant pathogen in soil. The exact mode of action of *P. citrinum* on sclerotia of *S. minor* still needs to be determined.

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