INHIBITION OF *SCLEROTINIA MINOR* BY *PENICILLIUM CITRINUM*; A POTENTIAL BIOLOGICAL CONTROL AGENT

By

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PREFACE

Many soilborne fungi which cause important plant diseases form sclerotia enabling them to survive in soil for long periods. Control of diseases caused by such fungi depends usually on eradication, or marked reduction of the population of sclerotia in soil. Sclerotia, however, are difficult to eliminate from soil because they are well adapted to survive under adverse environmental conditions.

The purpose of this study was to investigate the potential of one of many natural antagonists, in the soil, Penicillium citrinum Thom, as an inhibitor of mycelial growth and sclerotial germination of Sclerotinia minor. The potential of using this antagonist to inhibit other soilborne pathogens was also explored.

I wish to express my profound appreciation to my major advisor, Dr. Hassan A. Melouk, for his unlimited patience, assistance, and guidance throughout this study. My thanks are also due to Dr. Kenneth E. Conway, and Dr. Darold L. Ketring for serving on my advisory committee, and critical readings and valuable suggestions in the preparation of the final manuscript.

I would like to thank my fellow graduate students for their cordial friendship, and the spirit of togetherness shown to me during the course of my study.

Finally, I would like to thank my most beloved parents, Mr. Peter V. Akem, and Mrs. Grace M. Akem, for their complete support and continued love and encouragement, during my long stay away from home.

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CHAPTER I

INTRODUCTION

Sclerotinia blight of peanut (*Arachis hypogaea* L.) caused by the fungus *Sclerotinia minor*, has become a major problem in peanut producing areas of the United States including Oklahoma. The disease is difficult to detect in the early stages of development. Sclerotinia blight causes yield reduction of peanut. Most diseases caused by *Sclerotinia minor* have not been controlled consistently and economically. The rapid development of the fungus under favorable conditions, and the ability of its sclerotia to withstand adverse environmental conditions have allowed it to be a successful pathogen on many crops. At present, all peanut cultivars are susceptible to *S. minor* and fungicides provide only limited control. Other methods of control that have met with varying degrees of success include cultural practices, and materials that inhibit germination and/or destroy sclerotia. Biological control of the pathogen shows some potential. Effective control of other sclerotial forming pathogens using fungal antagonists have already been reported.

This thesis is comprised of two manuscripts written in a format that will facilitate immediate submission to a national scientific journal, and a third section dealing

with material covered in the study that is not included in the manuscripts. The manuscripts are presented as chapters, and each is complete in itself without additional supporting material.

Chapter II entitled, "Inhibition of Mycelial Growth of Sclerotinia minor and other Sclerotial-forming Pathogens by Inhibitor(s) from Penicillium citrinum", describes the effect of inhibitor(s) produced by broth cultures of Penicillium citrinum, on mycelial growth of the pathogens examined, and relates this to the potential for its use as a biological control agent effective through antibiosis. Chapter III entitled, "Colonization of sclerotia of Sclerotinia minor by Penicillium citrinum", reports on the efficacy of P. citrinum in colonizing and destroying the sclerotia of S. minor, and other sclerotial-forming pathogens thus exploring its potential use as a biological control agent for these sclerotial-forming pathogens. Both manuscripts are written for submission to PLANT DISEASE. The appendix of the thesis deals with aspects not covered in the manuscripts such as sclerotial germination of Sclerotinia minor on potato dextrose agar and Czapek-Dox agar media, and greenhouse trials to determine the number of sclerotia of S. minor needed to produce infection of peanut plants.

Approval for presenting the thesis in this manner is based upon the Graduate College`s policy of accepting a thesis written in manuscript form and is subject to the Graduate College`s approval of the major professor`s reguest for a waiver of the standard format.

CHAPTER II

INHIBITION OF MYCELIAL GROWTH OF *SCLEROTINIA MINOR* AND OTHER SCLEROTIAL- FORMING PATHOGENS BY INHIBITORS FROM THE FILTRATE OF *PENICILLIUM CITRINUM*

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ABSTRACT

Penicillium citrinum Thom, isolated from the sclerotia of *Sclerotinia minor*, was cultured in a broth of Czapek-Dox for 4 to 8 weeks. The filtrate obtained was incorporated into potato dextrose agar or Czapek-Dox agar at different concentrations (v/v). The amended media were tested for mycelial growth of *S. minor* and other pathogens. Mycelial growth of *S. minor* was completely inhibited on media amended with 20% (v/v) filtrate of *P. citrinum*, and considerable inhibition of *S. minor* occured at 10 and 15% concentrations. Mycelial growth of *S. major*, *Sclerotium rolfsii*, *Rhizoctonia solani* (AG-4) was inhibited by similar concentrations of filtrate of *P. citrinum*. Inhibitor(s) in the filtrate were extracted with ethyl acetate and tentatively identified as citrinin. Citrinin was shown to be an active component in the filtrate against mycelial growth of *S. minor*, *S. major*, and *Sclerotium rolfsii*.

INTRODUCTION

Penicillium citrinum has been reported to produce mycotoxin(s) that inhibit the mycelial growth of *Sclerotinia minor* and other pathogens (10). Mycotoxins are produced by several *Penicillium* species commonly isolated from grains and other sources (4,5,8). The mycotoxin, citrinin, is a toxic secondary metabolite of several fungal species belonging to the genera *Penicillium* and *Aspergillus* (2).

Citrinin was first isolated from the fungus *Penicillium citrinum* Thom by Hetherington and Raistrick (6). Although other species of *Penicillium* also produce citrinin (3,9,11,13,15), *P. citrinum* remains the primary source of citrinin. Rodig et al (12) found that yield of citrinin from *P. citrinum* reached a maximum after 35-45 days. They also discovered that the efficiency of citrinin produced, varied somewhat with different generations of *P. citrinum* cultures used. Decomposition studies of citrinin revealed that citrinin undergoes only limited decomposition in heat or light (12).

In this study, filtrate of *P.citrinum* at different concentrations (v/v) was tested for possible effects on the mycelial growth of some plant pathogens, and attempts were made to extract the inhibitor(s), and test their activity on the mycelia of *S. minor*. All tests were repeated twice and similar results were recorded each time.

MATERIALS AND METHODS

Culture of *Penicillium citrinum*

Penicillium citrinum was isolated from the sclerotia of *Sclerotinia minor* from a field planted to peanuts and maintained on potato dextrose agar slants (10). Fifty ml of Czapek broth in 300 ml bottles were autoclaved for 20 min, and after cooling were inoculated with two 5 mm diameter mycelial discs of *P. citrinum*, grown on Czapek-Dox agar (CDA). The broth cultures were incubated at room temperature, under direct flourescent light (800 lux) for six to eight weeks. The bottles were hand-agitated daily for 5 sec. to fascilitate conidial dispersal and rapid growth. Separation of the fungal biomass from the aqueous broth was accomplished by filtration through a glass fiber filter paper (Reeve Angel, Whatman, Inc. Clifton, New Jersey) in a 9cm-diameter Buchner funnel.

Incorporation of filtrate into potato dextrose agar and mycelial growth tests

Filtrate of *P. citrinum* culture was incorporated into streptomycin potato dextrose agar (SPDA), at 50C, at concentrations of 5, 10, 15, and 20% (v/v). SPDA with no filtrate amendment was included as controls. Forty petri dishes (15x100mm), containing 15 ml of media each, were made for each treatment. The pathogens tested for mycelial growth inhibition were: *Sclerotinia minor*, *Sclerotinia major*,

Sclerotium rolfsii, and Rhizoctonia solani (AG-4). Discs (6 mm diameter) of each of the pathogens from three-day old cultures were inoculated into the middle of ten petri dishes (15x100mm) at each concentration, and incubated at room temperature. After 48 hrs of incubation, in darkness, areas of mycelial growth were traced with a marker and transfered to paper. Using a Li-Cor model 3100 area meter (Lambda Instruments Corporation, Lincoln, NE 68504), the mycelial growth area in each dish was determined. Another test was conducted to determine the rate of growth of mycelia of S. minor, S. major and Sclerotium rolfsii at different filtrate concentrations between 24 and 42 hrs of incubation.

Extraction of inhibitor(s) with Ethyl acetate, and mycelial growth tests

Thirty ml of culture filtrate from 6 week-old broth cultures were placed in a 1000 ml separatory funnel, and 30 ml of 99.5% ethyl acetate was added and the funnel was vigorously shaken for 1 min. Solvent layers were then allowed to separate for 5 min. The lower aqueous layer and the upper ethyl acetate layer were collected. The aqueous layer was returned to the funnel and extracted three more times with 30 ml of ethyl acetate. The total ethyl acetate fraction was flash evaporated under reduced pressure at 48C to near dryness. The ethyl acetate fraction yielded a thin layer of a yellowish solid material. The aqueous fraction was placed in the flash evaporator to remove traces of ethyl acetate. Residue of the ethyl acetate fraction was dissolved

in 30 ml of deionized water. Ten and 15 ml portions were incorporated into Czapek-Dox agar at 50C to give concentrations of 10 and 15%, respectively. The amended media was autoclaved for 20 min, then 15 ml were dispensed in 10 (15x100) petri dishes. Corresponding concentrations of the aqueous fraction were incorporated into CDA. Dishes were inoculated with 3-day old 6 mm discs of S. minor, taken from the leading edge of 3-day old cultures grown on CDA. Mycelial growth areas in each dish was marked after 2 days of inoculation at 25±2C in darkness and the area of growth was determined. Crude filtrate of P. citrinum at concentrations of 10 and 15% (v/v) were used for comparison with the extract portions.

RESULTS AND DISCUSSION

When Czapek-Dox broth was inoculated with Penicillium citrinum, a progressive color change occured in the broth during incubation. The clear medium turned yellow, then light-brown, and finally dark-brown, by the 8th week of incubation. Determination of the levels of inhibitors in the filtrate at different culture ages revealed that maximum inhibitors were present in filtrates from six week old cultures of P. citrinum, as shown by the inhibition of mycelial growth of S. minor (Table 4). When filtrate from six week old cultures of P. citrinum was incorporated into PDA at a concentration of 20% (v/v), there was complete inhibition of the mycelial growth of S. minor. However, considerable growth occurred at 5%, but growth was greatly

reduced at both 10 and 15% concentration levels (Table 1). At 10 and 15% concentrations, the pattern of the limited mycelia growth was very irregular. In addition, the color of S. minor mycelia changed from tan-white to yellow after three days of incubation. Sclerotia were not formed from the discolored mycelia even after prolonged incubation. PDA amended at similar concentrations with filtrate of P. citrinum and tested for mycelial growth inhibition of S. major, Sclerotium rolfsii and Rhizoctonia solani, showed inhibition of growth of mycelia as filtrate concentration increased.

Mycelia of all four sclerotial-forming pathogens tested, were inhibited by filtrate of P. citrinum. Inhibition of mycelial growth on media amended with 15 and 20% filtrate of P. citrinum was greatest for S. minor and the least for R. solani (Table 1). Mycelial growth of S. rolfsii and S. major was inhibited more than R. solani but not as great as S. minor.

Comparison of mycelial growth between 24 and 42 hrs of incubation, of S. minor, S. major, and S. rolfsii, in media amended with 10,15, and 20% filtrate indicated that the greatest rate of growth occured in S. major, compared to the other fungi. The least growth occured in S. minor during the same time period (Table 2). In this rate comparison test, areas of mycelial growth were traced at 24 and 42 hrs of incubation because at 42 hrs, the petri dish was almost completely filled by mycelia of the pathogens in control treatments. Generally, the mycelia of S. minor was inhibited

most by the filtrate of P. citrinum as compared to the other pathogens tested.

The negative linear correlation values obtained when regressing the area of mycelial growth of the four pathogens and the filtrate concentrations give the approximate rates of the mycelial inhibition of the four pathogens as the filtrate concentration increases (Fig. 1).

Extraction of inhibitors from the filtrate of P. citrinum using 95% ethyl acetate, produced a yellow residue, that was tentatively identified with thin layer chromatography (TLC) as Citrinin. Information in the literature reveal this compound to be the main product of P. citrinum (6,12).

Incorporation of purified citrinin (Sigma Chemical, St. Louis, MO.) at 10 ppm into PDA, showed inhibition of mycelial growth of S. minor similar to that observed from incoporating the ethyl acetate fraction of the culture filtrate of P. citrinum into PDA. Thin layer chromatography of the ethyl acetate fraction using a procedure described by Hald and Krogh (2), produced a yellow spot with similar Rf value of citrinin.

Incorporation of citrinin extracted in the ethyl acetate fraction into PDA amended at 10 and 15% (v/v), produced considerable inhibition of the mycelial growth of S. minor. Marked inhibition of mycelial growth also occured at similar concentrations with the aqueous layer fraction (Table 3). This fraction was expected to have residual amounts of the inhibitor(s). It is likely that our extraction procedure did not completely extract all the citrinin in the filtrate. Or

perhaps, and even likely, there are other inhibitor(s) besides citrinin, produced by P. citrinum in Czapek-Dox broth culture that still need to be determined. For instance, Akem (1) has shown that Czapek-Dox agar inhibited sclerotial germination of S. minor. Perhaps, compounds in Czapek-Dox broth are also inhibitory to mycelial growth of S. minor.

Howell and Stipanovic (7) have isolated and characterized glioviren, an antibiotic compound from Gliocladium virens. They have shown that glioviren is highly toxic to Pythium ultimum, but inactive against other cotton seedling disease fungi tested. They have also shown that this antibiotic does not persist in nonsterile soil, as it is apparently inactivated by the soil microflora.

P. citrinum produces a compound which was tentatively identified as citrinin. Citrinin extracted from culture filtrate of P. citrinum showed almost complete inhibition of mycelial growth of S. minor. Investigation of the persistence of filtrate extracts in soil will determine the possible effects of other soil microflora on its activity.

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TABLE 1. Mycelial growth of four pathogens on potato dextrose agar (PDA), amended with the filtrate of *Penicillium citrinum* at different concentrations.

Pathogen	Concentration of filtrate (v/v) in PDA				
	0	5	10	15	20
	cm^2				
S. minor	35.54	8.14	2.89	0.63	0.00
S. major	44.08	11.34	1.64	1.14	0.51
S. rolfsii	18.57	4.50	1.69	1.09	0.42
R. solani	29.25	18.37	13.58	10.64	8.33

Area of growth (cm^2) was measured after 48 hrs.

Averages were determined from 10 replications representing each treatment.

TABLE 2: Mycelial growth of three pathogens on potato dextrose agar (PDA) amended with filtrate from six wk old cultures of *P. citrinum* at different periods of incubation.

Concentration of filtrate (v/v) in PDA medium	Incubation Period (hours)	Pathogen		
		S. minor	*S. major*	*S. rolfsii*
		cm^2		
0%	24	7.14	11.72	2.29
	42	31.61	45.18	7.88
5%	24	2.52	5.18	0.93
	42	8.98	19.12	3.31
10%	24	0.46	1.59	0.37
	42	1.78	6.09	1.69
15%	24	0.00	0.00	0.00
	42	0.23	0.57	0.98
20%	24	0.00	0.00	0.00
	42	0.00	0.48	0.56

Averages were determined from 10 replications of each treatment.

TABLE 3. Mycelial growth of *S. minor* on Czapek-Doth agar (CDA) amended with filtrate, ethyl acetate and aqueous layer fractions of *P. citrinum* at different concentrations (v/v).

Concentration of fraction (v/v) in CDA	Fraction		
	P. citrinum filtrate	Ethyl Acetate fraction	Aqueous layer fraction
	cm^2		
0	18.74	18.74	18.74
10	3.06	0.36	1.54
15	0.80	0.11	0.84

Averages were determined from 10 replications at each concentration per treatment.

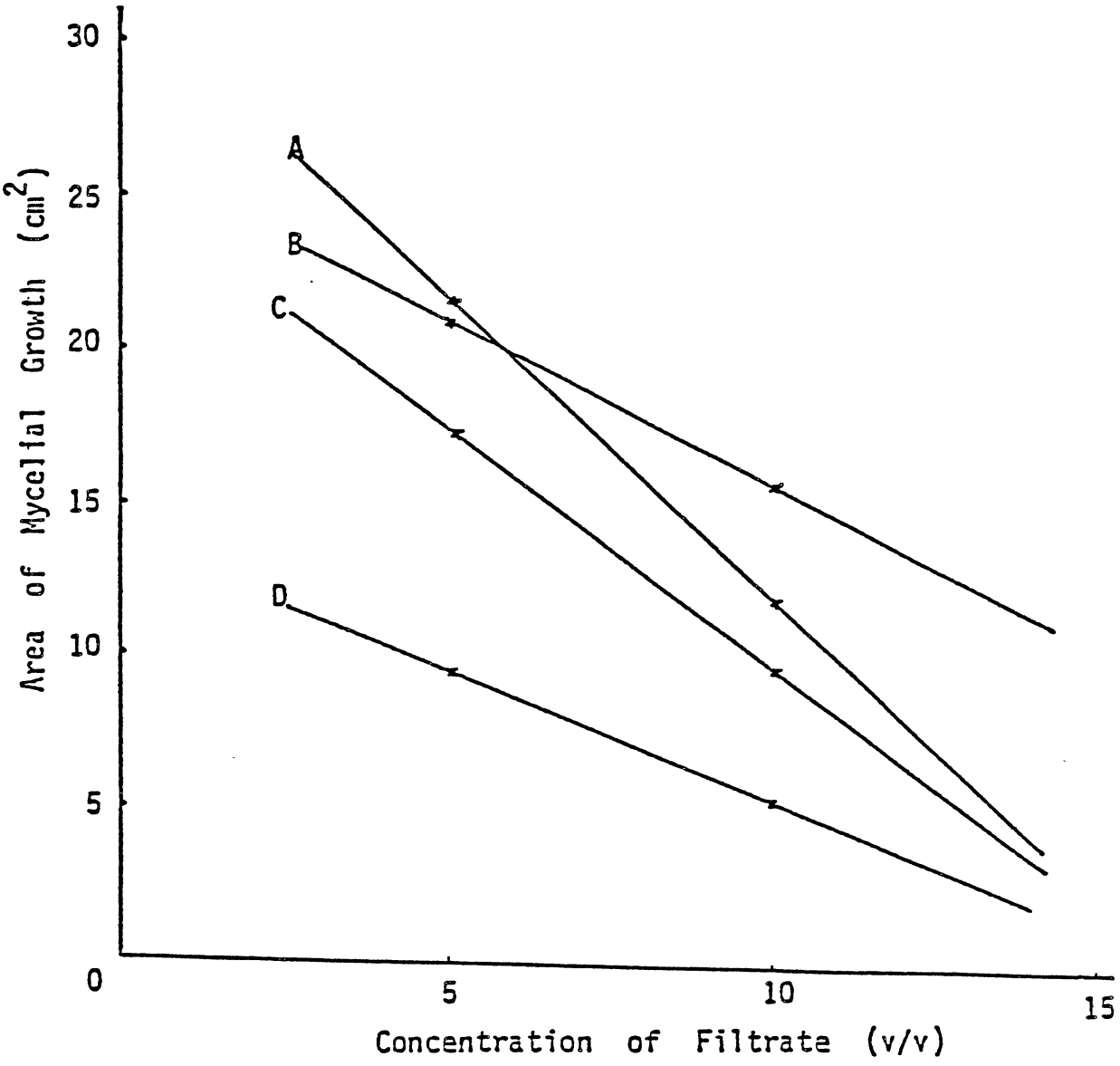
Mycelial growth (cm^2) was measured after 48 hrs.

TABLE 4. Mycelial growth of S. minor on potato dextrose agar (PDA), amended with different ages of filtrate of P.citrinum at different concentrations.

Age of filtrate (weeks)	Concentration of filtrate (v/v) in PDA				
	0	5	10	15	20
	cm^2				
2	28.62	24.30	21.52	21.10	18.30
3	27.57	21.62	20.41	18.74	13.36
4	29.37	19.41	16.37	11.46	10.28
5	26.82	12.89	7.24	3.13	1.47
6	27.34	7.21	2.42	0.23	0.00
8	28.41	9.48	3.26	1.27	0.29

Area of growth (cm^2) was measured after 42 hrs.

Averages were determined from 10 replications representing each treatment.



A Sclerotinia major
Y = 31.21 - 1.94 X
r = -.82

B Rhizoctonia solani
Y = 25.95 - .99 X
r = -.94

C Sclerotinia minor
Y = 25.16 - 1.57 X
r = -.83

D Sclerotium rolfsii
Y = 13.19 - .79 X
r = -.82

Fig. 1. Linear regression between the filtrate concentrations of Penicillium citrinum in potato dextrose agar and mycelial growth of four soilborne pathogens.

CHAPTER III

COLONIZATION OF SCLEROTIA OF *SCLEROTINIA MINOR* BY *PENICILLIUM CITRINUM*

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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 ± 2C for 1 hr. 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±2C, 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 major* and *Sclerotium rolfsii* and incubated in pasteurized soil were 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*.

INTRODUCTION

Sclerotia are the principal survival structures of several soilborne pathogens, and constitute an important link in many disease cycles (12,24). The rate of natural destruction of sclerotia in soil in the absence of a host plant varies with the different mycoparasites present (7,16,28), and is influenced by several other factors including soil moisture, temperature, aeration, and organic matter content (12). More than 30 species of fungi and bacteria have been implicated as parasites or antagonists of different *Sclerotinia* species (7,11,15,16,20,23,25,). For all but a few, tests for parasitic or antagonistic activity has been conducted exclusively in vitro. Proof of parasitic or antagonistic activity of most, under natural conditions, is just being explored (1,19,28). Huang and Hoes (20) showed that *Coniothyrium minitans* effectively controlled the population of *S. sclerotiorium* in sunflower fields. Huang (19) also observed the destruction of 58% of the sclerotia in soil infested with an isolate of *Trichoderma viride*. Turner and Tribe (27) reported up to 65% of sclerotia of *S. trifoliorum* were destroyed in field soil by *C. minitans*. Adams (1,2,3) found three mycoparasites of *S. sclerotiorium* that appear to be involved in the natural destruction of

sclerotia in soil. Tu (26) 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 (21). 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% (1). Rai and Saxena (23) 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 (5) also reported the occurence 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 a 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 ±2C. 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, and autoclaved for 1 hr, allowed to cool and then each 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 ±2C, the flask contents were spread to dry in flat trays at room temperature. Sclerotia were then sieved from the oats (2).

Isolation and maintainance of P. citrinum, and preparation of conidial suspension

Penicillium citrinum was isolated on PDA from the sclerotia of S. minor recovered from field soil planted to peanuts (21), maintained on PDA containing 100ug/ml Streptomycin sulfate (SPDA), 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 1.3×10^7 conidia/ml of P. citrinum in the suspension as estimated by hemacytometer counts.

Treatment of sclerotia and estimating the number of conidia of *P. citrinum* coating each sclerotium

Five grams of sclerotia were weighed into each of 4-50ml 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 labelled I,II,III, and IV for later treatments. Three hundred milligrams 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 hr. 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 1ml/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 2mm mesh screen to remove large debris particles. About 40g of soil mix were placed in 100x15mm pyrex petri dishes, ten ml of deionized water added, and the

dishes pasteurized at 170C for 1 hr and cooled overnight. soil moisture content was determined from soil contained in 5 randomly chosen petri dishes by computations involving the fresh and dry weights of the soil. It was expressed as % moisture content in the soil on a dry weight basis.

Incubation conditions

Penicillium citrinum-coated sclerotia and nontreated controls were incubated on pasteurized damp and dry Whatman #1 filter papers in glass petri dishes (100x15mm). 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, and up to 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 germinated sclerotia on each plate was recorded.

Effect of P. citrinum on other sclerotial-forming pathogens:

Sclerotia of Sclerotinia major and Sclerotium rolfsii, produced on SPDA were 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* occured by the sixth week of incubation in pasteurized soil. Seventy four percent colonization occured on sclerotia coated with conidia of *P. citrinum*, whereas, 72% colonization occured in sclerotia coated with CMC and *P. citrinum* (Table 1). CMC did increase the adherence of more conidia of *P. citrinum* to the sclerotia of *S. minor*, as 4.6×10^4 conidia/ml, were recovered from each sclerotium when CMC was used and 3.7×10^4 conidia/ml were recovered when CMC was not used. *P. citrinum* was not recovered from sclerotia of treatments I and III. 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 occured on dry filter paper. Thus, moisture seem 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 a nonpasteurized soil (Table 2).

P. citrinum did not colonize sclerotia of Sclerotium rolfsii to a significant degree as only 5% colonization occured by the 4th week of incubation, and a decline in colonization to 2.5% was noticed thereafter (Table 3). A substantial level of colonization (45%) was recorded with the sclerotia of S. major, 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 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 a very low correlation value ($r = .16$) when sclerotia of 3 pathogens, S. minor, S. major, and S. rolfsii (Fig. 4) were compared in pasteurized soil for colonization by P. citrinum.

DISCUSSION

The mechanisms 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; inhibition of the growth of the pathogen in the soil; and the prevention of host penetration by the pathogen (17). 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 (6,8). The properties of a successful antagonist which have been suggested as neccessary for an efficient disease control agent include strong competitive ability, antibiotic production, direct parasitism, and lysis (6,8). Mycoparasitism is a widespread phenomenon. The importance of this mechanism 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.

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 (8), 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 (22).

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 (10). P. citrinum seems to be a selective antagonist as it effectively colonizes the sclerotia of S. minor, and S. major, 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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TABLE 1. Percentage of sclerotia of *Sclerotinia minor* colonized by *Penicillium citrinum* in four treatments 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 followed by the same letter were not significant at the P=.05 level.

TABLE 2. Percentages of sclerotia of Sclerotinia minor colonized by Penicillium citrinum in Soil and on Filter paper.

Week	Incubation conditions			
	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.

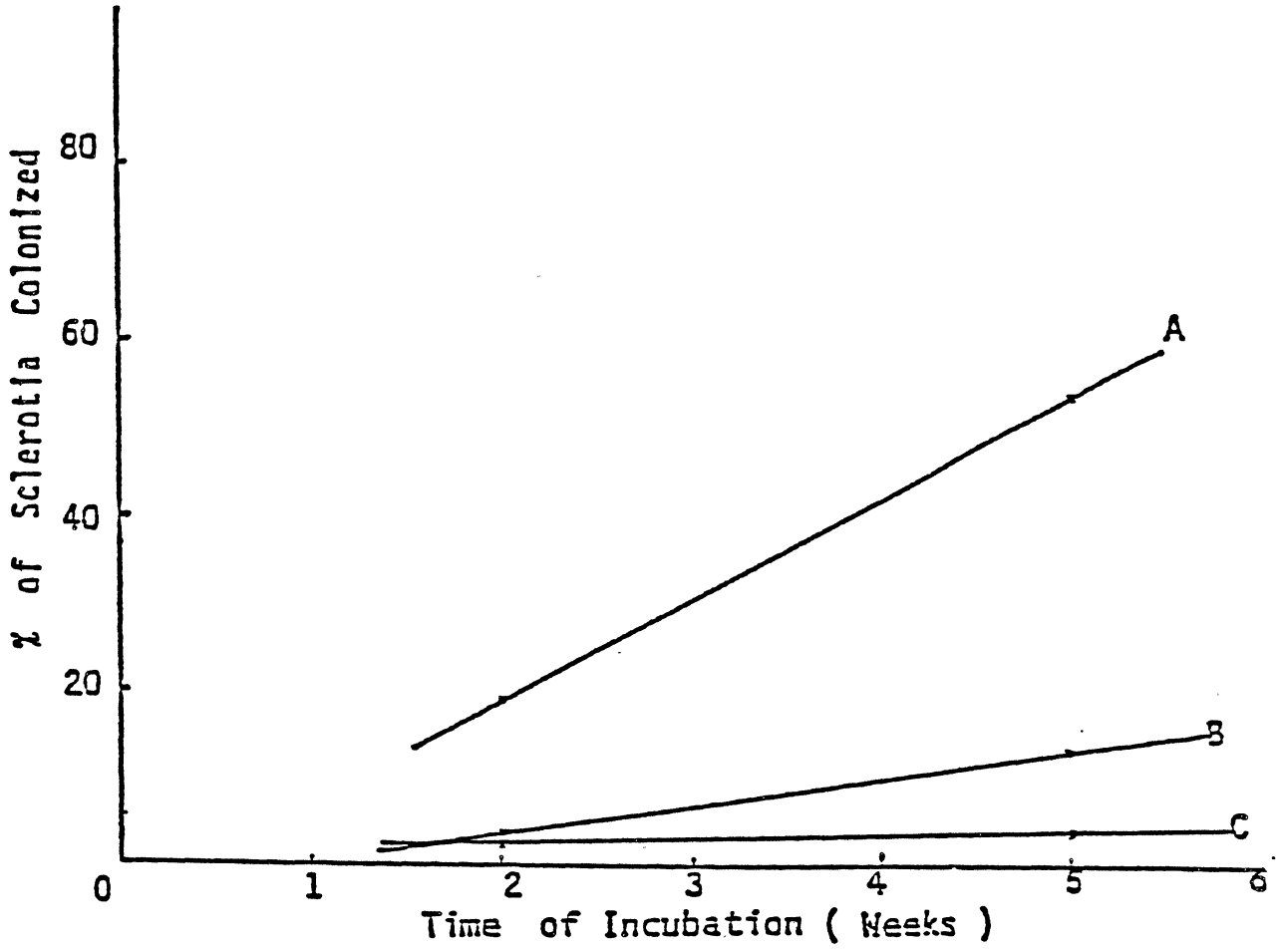
Means followed by the same letter were not significant at the P=.05 level.

TABLE 3. Colonization of the sclerotia of three soil-borne pathogens by *Penicillium citrinum* in pasteurized soil.

	Pathogen		
Week	*S. minor*[1]	*S. major*[2]	*S. rolfsii*[2]
0	4.4	2.5	0.0
1	7.5	10.0	2.5
2	13.1	17.5	2.5
3	21.9	12.5	0.0
4	28.8	15.0	5.0
5	55.6	37.5	2.5
6	73.8	45.0	2.5

[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.



A Treated Sclerotia incubated on Damp Filter Paper

Y = -3.16 + 11.50X

r = .98

B Treated Sclerotia incubated on Dry Filter Paper

Y = -.833 + 2.71X

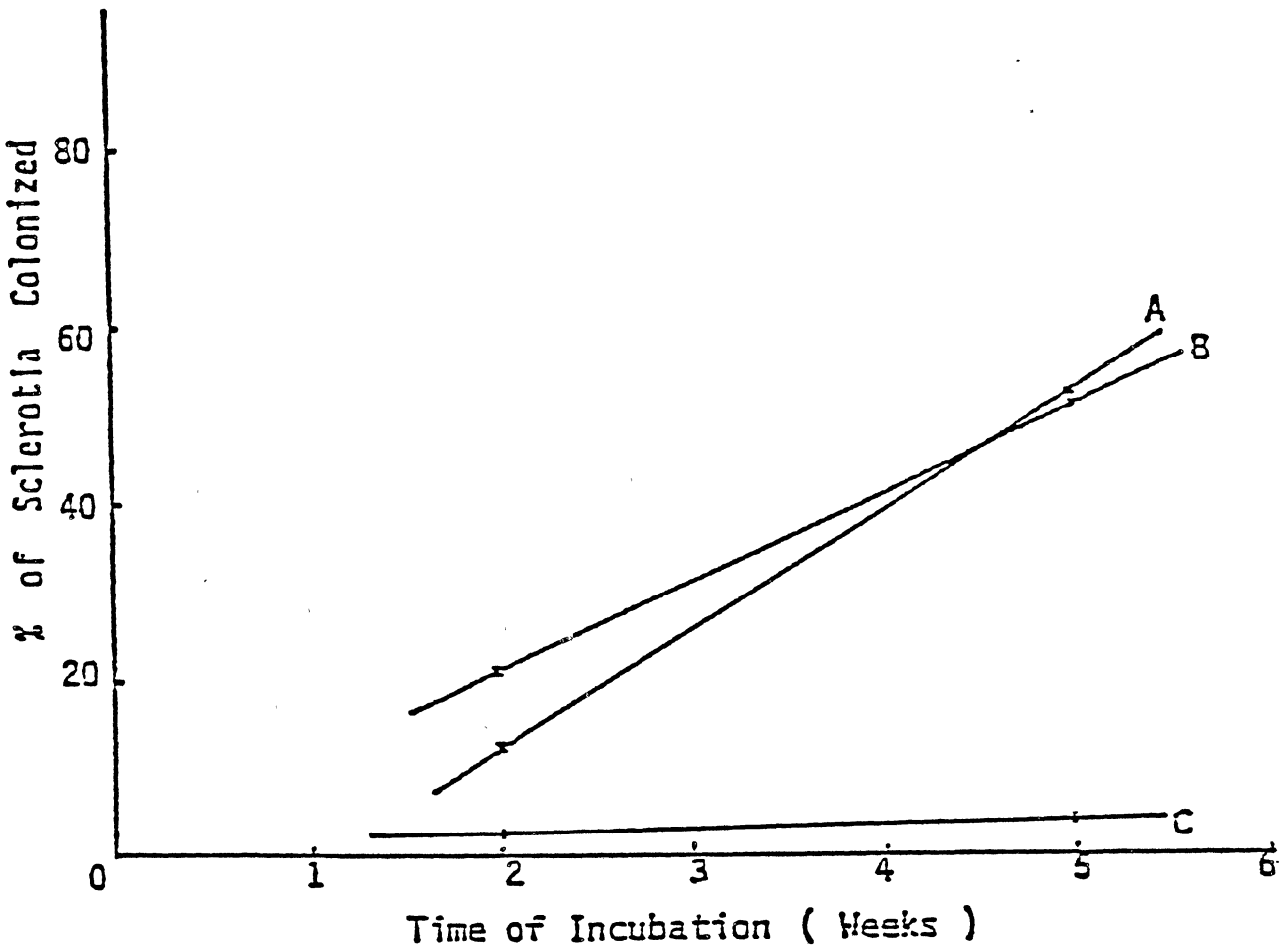
r = .72

C Non-treated Sclerotia

Y = 1.25 + .54X

r = .80

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



A Treated Sclerotia incubated in Pasteurized Soil

Y = -13.14 + 13.31X

r = .96

B Treated Sclerotia incubated in Nonpasteurized Soil.

Y = 1.47 + 10.14X

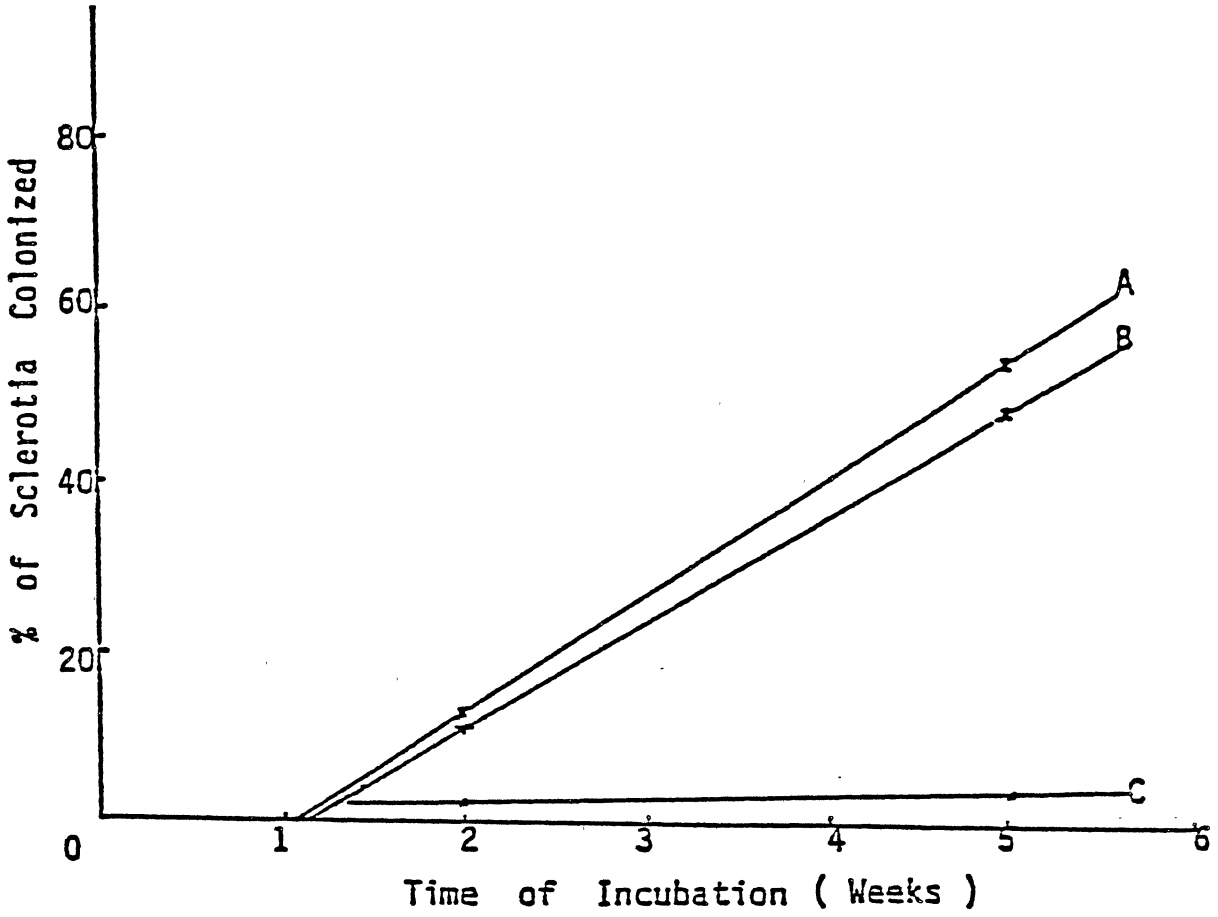
r = .92

C Non-treated Sclerotia

Y = 1.25 + .54X

r = .80

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

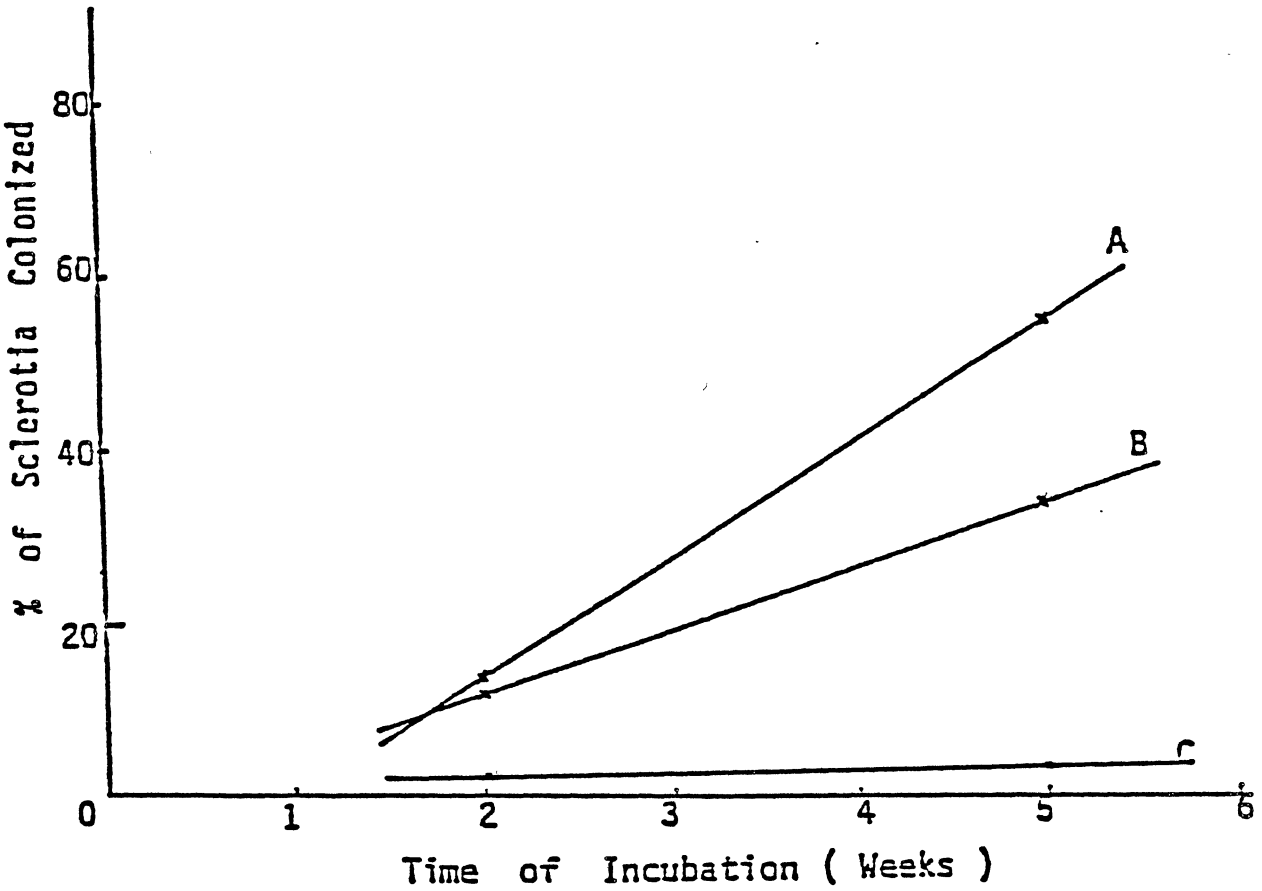


A Sclerotia and P. citrinum
Y = -13.14 + 13.31 X
r = .96

B Sclerotia, CMC and P. citrinum
Y = -11.75 + 11.93 X
r = .91

C Non-treated Sclerotia
Y = 1.25 + .54
r = .80

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



A Sclerotinia minor

Y = -13.14 + 13.31X

r = .96

B Sclerotinia major

Y = -.83 + 6.78X

r = .87

C Sclerotium rolfsii

Y = 2.00 + .14X

r = .16

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

APPENDIX A

GREENHOUSE STUDIES TO DETERMINE THE NUMBER OF SCLEROTIA OF *S. MINOR* NEEDED TO PRODUCE INFECTION ON PEANUT PLANTS.

Although much has been reported about the biology and ecology of *Sclerotinia* species, very little has been documented on natural populations of sclerotia in the soil. Henderson (9) reported finding from zero to twenty sclerotia of *S. sclerotiorum* per 929 cm of field soil to a depth of 5.1cm. This is the equivalent of about 0 to 3 sclerotia per Kg of soil. Working with sunflower, Hoes and Huang (10) found approximately 2 to 3 sclerotia of *S. sclerotiorum* per Kg of nonrhizosphere soil. However, they found about 24 sclerotia per Kg of soil in the rhizosphere of diseased plants. Abawi and Grogan (1) determined similar rates for field soil in New York and Nebraska bean fields.

On the basis of this limited amount of information, it appears that the levels of sclerotia in natural soils range from zero to less than ten sclerotia per Kg of soil in a field ready for planting. There is very little data on the natural sclerotium densities of *S. minor*. Adams (4) determined the inoculum densities of a number of fields in Eastern U.S with a history of severe lettuce drop and found that the inoculum density of *S. minor* ranged from 160 to 820

sclerotia per Kg of soil. In New Jersey, the inoculum density of S. minor ranged from zero to 230 sclerotia per Kg of soil. In a field with a history of severe peanut sclerotinia blight in Virginia, they found the inoculum density range from 35 to 100 sclerotia per Kg of soil. Thus in fields with a history of losses due to Sclerotinia species, the inoculum density of S. minor was found to be 10 to 100 times greater than that of S. sclerotiorum.

All publications of crop losses due to Sclerotinia spp. show the importance of the host in the production of sclerotia. Adams (4) reported that as many as 1000 sclerotia of S. minor formed on a single diseased romaine lettuce plant. Weed hosts were sighted as a significant factor in the production of sclerotia in the field, a factor often overlooked. All these accounts present figures that estimate the sclerotium density of Sclerotinia in naturally infested fields.

Under greenhouse conditions, a close correlation has been reported between the incidence of lettuce drop and inoculum density of sclerotia of S. minor (8). An inoculum density of 2 to 7 sclerotia per 100g of soil caused about 10% lettuce drop. The incidence of lettuce drop was about 20 and 80% at inoculum densities of 31 and 250 sclerotia per 100g of soil respectively. Results like this have not yet been documented with sclerotinia blight of peanuts.

It has also been reported that the infection of lettuce by S. minor results from mycelial germination of sclerotia (6,11,12); thus, inoculum density of germinable sclerotia in

soil and the prevalence of conditions that favor sclerotial germination influence the incidence of the disease. It has been suggested that drying of sclerotia at or near the soil surface stimulates germination and infection when soil moisture is again adjusted to near capacity. This observation by Abawi and Grogan (2), agrees with reports by Smith (14) and Beach (7) that incidence of lettuce drop was greatly increased in crops grown in infested soil that had been allowed to become dry prior to planting.

Adams and Tate (5) reported infection of lettuce directly from sclerotia (via mycelium) without an intermediate source of nutrients like organic matter. In contrast, Purdy (13) reported that to achieve infection of lettuce from sclerotia of the large-sclerotia type, nonliving or detached lettuce leaves must be in contact with sclerotia and the lettuce stem. It has also been suggested that infection of lettuce by S. sclerotiorum can originate directly from sclerotia in the soil (8,13,15), but Coley-Smith and Cooke (8) disagree.

The purpose of this study was to investigate the infection of peanut plants by the sclerotia of S. minor. This was necessary since most of the early studies along these lines were done with lettuce, a rather classic host for Sclerotinia spp. It was also necessary to establish the levels of sclerotial inoculum needed to produce infection for future tests, and to follow up with the symptomatology produced when peanut plants are infected by S. minor in the greenhouse.

MATERIALS AND METHODS

Sixty plastic pots (12cm in diameter) were filled with soil mix (soil:sand:fine peat moss; 2:2:1, v/v/v) to within 5 cm. of the rims. Peanut leaves from 9-week old plants were collected and autoclaved for 20 minutes. Autoclaved leaves were spread on a greenhouse bench to dry for 2 days. The dried leaves were crushed, weighed and incorporated into some pots at 2g/100g soil weight. This was necessary since Purdy (30) had reported the need for organic matter of some sort in the soil to induce sclerotial germination.

Sclerotia of S. minor grown on oat seeds and sieve separated after drying were used in the experiment. More than 95% of the sclerotial inoculum produced in this fashion was viable, and germinated on potato dextrose agar within 48 hrs of incubation. Ten sclerotia were incorporated into the top 100g of soil amended with peanut organic matter in 10 pots, and 8 sclerotia were incorporated and mixed into the top 100g of amended soil in another set of 10 pots. Into the top 100g of soil in another set of pots not amended with the organic matter, were incorporated 10 and 8 sclerotia and again labelled. Organic matter was incorporated into the top 100g of soil in 10 other pots with no sclerotia added, and the remaining 10 pots were left untreated with organic amendment or sclerotia. The last two sets served as controls. Tamut 74 peanut cultivar seeds were germinated in an incubator at 33C.

The germinated seeds with uniform radicles were planted

into the pots at a rate of one seed per pot. A thin layer of sand was uniformly spread on the surface of the pots to reduce moisture loss. Pots were placed on greenhouse benches and greenhouse temperature was maintained at about 28C during the day and 23C during the night. Pots were watered and plants examined daily to detect infection by S. minor. Each plant received 400 ml of 0.2% ammonium nitrate solution six weeks after planting.

RESULTS AND DISCUSSION

After 96 days of plant growth, the expected typical symptoms for Sclerotinia minor infection were not observed on inoculated plants. However, control plants were healthier than the inoculated ones. Poor root development was observed on inoculated plants, and the crown areas had dark-brown discolorations, and were crookedly developed. Isolations of the pathogen (S. minor) were made on potato dextrose agar from the crown tissues of inoculated plants.

From symptoms observed and the isolation of the pathogen, it was established that peanut plants were infected by S. minor in pots with and without organic matter amendment (Table 1). There was an 80% infection on plants in pots amended with organic matter and inoculated with 10 sclerotia. Fifty percent of the plants without organic matter also amendment showed infection. It would appear that peanut plants can be infected directly from sclerotia, via mycelia without an intermediate source of organic matter nutrients. This agrees with Adam and Tate's (5) ascertion,

in the case of lettuce and disagrees with Purdy's report (13) in another study.

Infection of plants was observed at both sclerotial levels used. These levels, abitrarily choosen, fall within the large range of inoculum levels reported to cause infection of lettuce plants. Stress seem to accelerate the infection of peanut plants by Sclerotinia minor. This was observed in a few plants deliberately stressed by watering less frequently than the others. The stressed plants showed infection symptoms sooner than the others. This is not unexpected since in field conditions, severe infection and possible death of plants usually show up late in the season, and often first on the older plants. At this time of the season, temperatures are usually high and the soil so dry that plants become stressed. Thus, besides inoculum density of pathogen propagules, and organic matter requirements, environmental factors may play a vital part in the infection of peanut plants by S. minor.

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TABLE 1. Infection of peanut plants by S. minor at different inoculum treatments in the greenhouse.

Treatment[1]	No. of plants	No. of plants infected
Organic matter, 10 sclerotia	10	10
Organic matter, 8 sclerotia	10	8
No organic matter, 10 sclerotia	10	5
No organic matter, 8 sclerotia	10	4
Organic matter, no sclerotia	10	0
No organic matter, no sclerotia	10	0

[1] Sclerotia and organic matter were incorporated in the top 100g layer of soil in pots. Organic matter was added at the rate of 2 g for each 100 g of soil mix.

APPENDIX B

SCLEROTIAL GERMINATION OF *SCLEROTINIA MINOR* ON POTATO DEXTROSE AGAR AND CZAPEK DOX AGAR AMENDED WITH FILTRATES OF *PENICILLIUM CITRINUM*

Filtrate of *Penicillium citrinum* was shown to inhibit the mycelial growth of *Sclerotinia minor* at various concentrations on potato dextrose agar (PDA) or Czapek-Dox agar (CDA). There was complete inhibition of mycelial growth at filtrate concentration of 20% (v/v). The following test was performed to investigate the effects of *P. citrinum* filtrate on the germination and growth of sclerotia of *S. minor*.

Sclerotia of *S. minor* produced on oat seeds were used in this test. Sclerotia were washed and surface sterilized with a 10% chlorox for 3 min. They were then rinsed with deionized water and spread to dry. CDA and PDA were amended with filtrate of *P. citrinum* from six week old cultures at concentrations of 0, 10, 20, and 30% (v/v). Sclerotia were placed onto CDA and PDA amended media in 9 cm petri plates. Another set of media plates were inoculated with 2-day old mycelial discs (5mm) of *S. minor*. Inoculated plates were incubated at 25±2C in the dark.

No germination of sclerotia was observed on the CDA media at all treatments after three days of incubation.

However, in the PDA amended media, sclerotia germinated at the 0, 5 and 10% levels after three days of incubation, and by the fifth day there was considerable sclerotial germination at all concentrations tested. Area of mycelial growth in PDA amended media plates inoculated with mycelial discs of S. minor were marked out and measured on the third day of incubation (Table 2). Mycelial growth in these plates was completely inhibited at 20% (v/v) filtrate concentration. Similar results of inhibition with mycelial discs were observed on CDA amended media. No changes on the sclerotia were observed for an additional two days on the CDA amended media. In the PDA amended media, sclerotia just initiated germination at the 20% filtrate concentration level, and the sprouting mycelia never extended to any appreciable area. Close examination of germinating sclerotia in the plates amended with filtrate at the 15 and 20% levels, showed a yellowish discoloration of the limited mycelia sprouting from the sclerotia. Growth of the mycelia was uneven, sparse and restricted around the germinating sclerotia. Further growth of the mycelia did not occur after the yellowish discoloration was observed. The limited growth areas were marked out and measured (Table 2). Possible reasons for the failure of sclerotia to germinate on CDA were investigated. The pH of both media was checked for its possible role in sclerotial germination. The industrial pH of PDA as recorded is 6.98, while that of Czapek-Dox is 8.50. The pH of CDA was adjusted by 1N HCl to equal that of PDA. Both media at the same pH levels were amended with broader P. citrinum

filtrate concentrations of 0, 10, 20, 30, 40 and 50% (v/v), to study the possible germination and growth of the sclerotia of S. minor in them. The petri dishes were inoculated with surface sterilized sclerotia and incubated for up to 5 days at room temperature. On the amended PDA sclerotia germinated at concentrations up to 30%. At 40 and 50% concentrations, there was complete inhibition of germination. As before, no appreciable sclerotial germination occured on the CDA even at the control level. From these results it could be concluded that pH was not the factor hindering sclerotial germination of S. minor on CDA.

Germination of sclerotia of S. minor on potato dextrose agar and Czapek-Dox agar

Sclerotia of S. minor produced on oat seeds were used in this test and treated as previously described. Fifty petri plates of CDA and 50 of PDA were prepared. Nine sclerotia were plated in each of the petri dishes and incubated in the dark at room temperature for germination. The first germination count was taken one day after incubation, when sclerotial germination was first observed. Sclerotial germination in the two media were recorded daily for five days and fraction of sclerotia germinated was computed (Table 3). During this period most of the PDA plates became completely covered with mycelial growth of S. minor.

Generally, on PDA, once germination was initiated, rapid mycelial growth followed, almost completely filling the plates by the 5th day, and making it difficult for further

observations of any ungerminated sclerotia. However, in CDA, sclerotial germination was initiated in most cases, but no appreciable growth of mycelia in the media followed.

TABLE 2: Mycelial growth of Sclerotinia minor from sclerotia and mycelia on potato dextrose agar (PDA) amended with Penicillium citrinum filtrate .

Concentration of filtrate (v/v) (v/v) in PDA	Mycelial discs[1]	Sclerotia[2]
	cm^2	
0	35.54 a	41.13 a
5	8.14 b	8.91 b
10	2.89 c	5.09 c
15	0.63 d	4.21 c
20	0.00 d	0.43 d

[1] Area of mycelial growth was measured after 48 hours of incubation.

[2] Mycelial growth was measured after 5 days of incubation to allow for sclerotial germination and growth.

Means within columns followed by the same value are not significantly different (P = .05) level.

TABLE 3. Germination of sclerotia of Sclerotinia minor on potato dextrose agar (PDA), and Czapek-Dox agar (CDA).

Sample #	Incubation time (days)	Fraction of sclerotia germinated on PDA	Fraction of sclerotia germinated on CDA
1	1	0.13	0.02
	2	0.33	0.04
	3	0.75	0.10
	4	0.93	0.13
	5	0.96	0.20
2	1	0.21	0.01
	2	0.42	0.03
	3	0.86	0.08
	4	0.96	0.11
	5	0.97	0.23
3	1	0.12	0.02
	2	0.31	0.04
	3	0.66	0.07
	4	0.91	0.15
	5	0.98	0.22

TABLE 3 (Continued)

Sample #	Incubation time	PDA	CDA
4	1	0.18	0.02
	2	0.35	0.04
	3	0.58	0.06
	4	0.87	0.12
	5	0.98	0.20
5	1	0.20	0.01
	2	0.44	0.05
	3	0.71	0.10
	4	0.92	0.13
	5	0.97	0.20

450 sclerotia of S. minor were plated on each media in 5 replications of 90 sclerotia in each.

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