EFFECTS OF VOLATILES FROM SOIL AMENDED WITH MEAL AND GREENS OF *BRASICCA NAPAS* TO SELECTED SOILBORNE PATHOGENS OF PEANUT AND CHARACTERIZATIONS OF ISOLATES OF *SCLEROTINIA MINOR*

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
IPM	Integrated pest management
LSD	Least significant difference
MIC	Minimum inhibition concentration
MW	Molecular weight
OA	Oxalic acid
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PPM	Parts per million
RH	Relative humidity
RSG	Rapeseed greens
RSM	Rapeseed meal
SDS	Sodium dodecyl sulfate

INTRODUCTION

Several soilborne diseases limit the growth, productivity and quality of peanut. Some of the most damaging ones include southern blight (*Sclerotium rolfsii*), sclerotinia blight (*Sclerotinia minor*), verticillium wilt (*Verticillium dahliae*), and pod rot (*Pythium myriotylum*). All commercial cultivars including runner, spanish and virginia types are susceptible in varying degrees to one or more of these diseases.

The content of this dissertation is composed of four chapters written in the form for journal publication. The first two chapters examine the effects of rapeseed meal (RSM) and rape greens (RG) as soil amendments on the growth of four soilborne pathogens of peanut: *S. minor*, *S. rolfsii*, *V. dahliae* and *P. myriotylum*. Two mechanisms are possibly involved: 1) the release of biocidal volatiles from soil amended with RSM or RG, and 2) the enhancement of activity of microorganisms in RSM or RG amended soil which are antagonistic to these soilborne pathogens. The last two chapters describe the characterizations of isolates of *Sclerotinia minor* from peanut in Oklahoma.

Chapter I, titled "BIOCIDAL ACTIVITY OF VOLATILES FROM SOIL AMENDED WITH RAPESEED MEAL OR RAPE GREENS AGAINST FOUR SOILBORNE PATHOGENS OF PEANUT," tests the biocidal activity of volatiles released from soil amended with RSM or RG. Four soilborne pathogens of peanut

including *S. minor*, *S. rolfsii*, *V. dahliae*, and *P. myriotylum*, were exposed to the volatiles. All except *P. myriotylum* exhibited reduced mycelial growth, and delayed and reduced sclerotial or microsclerotial formation when exposed to volatiles released from RSM or RG-amended soil.

Chapter II, titled "INHIBITION OF *SCLEROTINIA MINOR* AND *SCLEROTIUM ROLFSII* BY MICROORGANISMS IN SOIL AMENDED WITH RAPESEED MEAL OR RAPE GREENS," describes the recovery of several microorganisms that colonized sclerotia of these two fungi in soil amended with RSM or RG. The antagonists included *Mucor* spp., *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp., *Chaetomium* spp., *Gliocladium* spp., *Erwinia* spp. and *Bacillus* spp. One isolate of *Gliocladium* spp. and four isolates of *Bacillus* spp. produced significant amounts of substances that inhibited the mycelial growth of both *S. minor* and *S. rolfsii* on potato dextrose agar (PDA) as determined by minimal inhibitory concentration (MIC) procedure.

Chapter III, titled "CHARACTERIZATIONS OF ISOLATES OF *SCLEROTINIA MINOR* FROM PEANUT IN OKLAHOMA," describes the characterizations of 62 isolates of *S. minor* from Oklahoma based on their growth on PDA and virulence on detached Romaine lettuce leaves. The production of oxalic acid by 10 isolates were measured in potato dextrose broth (PDB), on lettuce leaves, and on peanut stems. There was no correlation between production of oxalic acid by different isolates and their pathogenicity on peanut or their virulence on lettuce. This suggests that oxalic acid is not the sole pathogenicity factor in *S. minor* on peanut or

lettuce.

Chapter IV, titled "FORMATION OF SCLEROTIA OF *SCLEROTINIA MINOR* IN MIXED CULTURES," describes the formation of white sectors in growth of *S. minor* that occurred from germinating sclerotia which were produced in mixed cultures and paired cultures. Formation of apothecia from different sclerotia was investigated too. The results of these tests were discussed. No extrachromosomal genetic material was detected in any of the isolates studied. The results of these tests suggest that sclerotia of *S. minor* are not homogenous genetically. Isolates of *S. minor* are able to entrap mycelia of other isolate into their sclerotia.

CHAPTER I

BIOCIDAL ACTIVITY OF VOLATILES FROM SOIL AMENDED WITH RAPESEED MEAL OR RAPE GREENS AGAINST FOUR SOILBORNE PATHOGENS OF PEANUT

ABSTRACT

A closed chamber system was constructed to assess the biocidal activity of volatiles released from soil amended with rapeseed meal (RSM) or rape greens (RG). Four major soilborne pathogens of peanut, including *Sclerotinia minor*, *Sclerotium rolfsii*, *Verticillium dahliae*, and *Pythium myriotylum*, were exposed in the closed chamber to these volatiles for several days. The volatiles reduced mycelial growth, delayed and reduced sclerotial formation of *S. minor* and *S. rolfsii*. Growth of *P. myriotylum* was not affected. In volatiles released from soil amended with RSM (containing about 36 μM glucosinolates/g meal) at 55,000 ppm, mycelial growth and sclerotial formation of *S. minor* and *S. rolfsii* were reduced by more than 30%. Mycelial growth of both fungi was reduced by about 50% by volatiles released from soil amended with RG (harvested from plants grown from seed the meal of which contained about 73 μM glucosinolates/g meal) at the same rate of the RSM. Volatiles released from soil amended with RSM at 60,000 ppm totally inhibited mycelial

growth and microsclerotial formation of *V. dahliae*. These results demonstrate that RSM and/or RG amendment in soil is able to reduce growth and sclerotial or microsclerotial formation of several soilborne pathogens of peanut.

INTRODUCTION

Several soilborne diseases limit the growth, productivity, and quality of peanut (*Arachis hypogea* L.) in Oklahoma. The most damaging of these include southern blight caused by *Sclerotium rolfsii* Sacc. (3), sclerotinia blight caused by *Sclerotinia minor* Jagger (16, 22), Verticillium wilt caused by *Verticillium dahliae* Kleb. (16, 21), and pod rot caused in part by *Pythium myriotylum* Drechs. (4). Because of the ability of these pathogens to form survival structures such as sclerotia, microsclerotia, or thick-walled oospores in soil, they are very difficult to control in fields where they have become established.

Few chemicals are available for control of these diseases, especially sclerotinia blight (3, 16, 20). Some chemicals do not provide acceptable level of disease control and the potential problems of chemical toxicity, environmental contamination, and pesticide residues in food are an increasing concern to consumers (10, 14, 17). Therefore, alternative control methods, especially integrated pest management (IPM) systems which allow farmers to control diseases using minimum amounts of chemicals are of interest to researchers. In Oklahoma, Tamspan 90 and Southwest Runner, which are resistant to sclerotinia blight, are used in problem fields (16). Amending soil with organic matter is a strategy that has been successful for controlling *S. minor*

on lettuce and *S. rolfsii* (6, 9, 14, 15). Adamsen *et al.* (1) reported that rapeseed (*Brassica napus* L.) meal has the potential to reduce the viability of microsclerotia of *Cylindrocladium crotalatiae* Bell & Sobers, the causal agent of cylindrocladium black rot of peanut. The control effect was probably due to the sulfur-containing antimicrobial volatiles released from the breakdown of glucosinolates in the rapeseed meal (1, 5). The volatiles include mercaptans, sulfides of various types, and isothiocyanates. Isothiocyanates include methyl isothiocyanate, a breakdown product of metham sodium (5). Amending soil with rape green manure and other Cruciferae members was also reported to be effective against *Rhizoctonia solani*, *Aphanomyces euteiches*, nematodes, and other soilborne pathogens (7, 17, 18).

Rapeseed meal (RSM) and/or rape greens (RG) not only have the advantage of other organic amendments such as stimulating antagonistic microorganisms against pathogens, they also are able to release biocidal volatiles from glucosinolates against soilborne pathogens. The objective of this study was to assess the biocidal activity of volatiles released from soil amended with RSM or RG against *S. minor*, *S. rolfsii*, *V. dahliae* and *P. myriotylum*, using a closed chamber system. Brief reports of this research were presented earlier (11, 12). The other portion of this study assessing the effects of RSM or RG amendment on the viability of sclerotia of *S. minor* and *S. rolfsii* in soil, and on the stimulation of antagonists to these two pathogens, are reported in Chapter II.

MATERIALS AND METHODS

RSM which contained about 36 μM glucosinolates/g meal was obtained from a commercial source (Calgene Chemical Inc., Springfield, IL). Seeds of cultivar Emerald Rape (Calgene Inc.), which produced meal containing about 73 μM glucosinolates/g meal, were planted at the Plant Pathology Farm in Stillwater on Sept. 9, 1992. The greens or the entire above-ground parts of the plants (RG) were harvested in March 1993, dried on a greenhouse bench at 28 C, and chopped in a blender. The soil used in these tests from Fort Cobb, OK, was a Menofine sandy loam with pH 6.0. The soil was pulverized to a fine structure before use. The pathogens used in this study, including *S. minor*, *S. rolfsii*, *V. dahliae*, and *P. myriotylum*, were maintained on potato dextrose agar (PDA) at 22$\pm$1 C.

Growth of *S. minor*, *S. rolfsii*, and *P. myriotylum* were tested in volatiles released from soil amended with RSM at 55,000 ppm. Only *S. minor* and *S. rolfsii* were tested in volatiles released from soil amended with RG at 55,000 ppm. RSM or RG were mixed with air-dried soil at the rate of 55,000 ppm. Six hundred g of amended soil was placed in a 22x12x8 cm Pyrex glass baking pan to which 200 ml of water was added to induce the microbial activity in the mixture, which was necessary for the release of the volatile compounds from the degradation of glucosinolates (19). Baking pan each containing 600 g of air-dried soil alone to which 200 ml of water were used as control in each test. Each pan was then placed in a 32x16x10 cm sealed plexiglass box (Figure 1). After incubation for 2 days at 22$\pm$1 C, the test organisms were introduced into the closed chamber system as follows: Tissue culture flasks (75

cm^2, canted neck, phenolic style cap, Corning, NY) each containing 40 ml of PDA were each inoculated with a mycelial plug (0.17 cm^2) of the test organism taken from the periphery of a colony. The caps of the flasks were loosened before placing the flasks onto the surface of the soil mix in the closed plexiglass boxes, so that the volatiles released could enter flasks without contamination. Colony growth area of the test organisms was measured periodically using an area meter (Delta-T Devices, Cambridge, England), and the time required for formation of sclerotia was recorded. The percentage inhibition of growth caused by the volatiles from soil amended with RSM or RG was calculated as (100 * (1 -Growth area in treated/Average growth area in control)). After two weeks, the number of sclerotia formed was also counted for *S. minor* and *S. rolfsii* and the average number of sclerotia formed on 1 cm^2 was calculated. Each test was performed twice, except the test with *P. myriotylum* which was conducted once. Each plexiglass box constituted an experimental unit. Each treatment and control had four replications of experimental units.

In addition, *S. minor* was exposed to volatiles released from soil amended with RSM at 55,000 ppm at 10, 15, 25 and 30 C using the same procedures described above. This test was conducted once.

In a preliminary experiment, *V. dahliae* grew poorly when exposed to volatiles released from soil amended RSM at 55,000 ppm, thus RSM at concentrations of 20,000, 40,000, and 60,000 ppm, were tested using the same procedures described above. Colony growth area of the pathogen was measured periodically and microsclerotia production was quantified by measuring the amount of melanin formed

by the colonies, as melanin is required for the melanization of microsclerotia. Melanin was extracted by mixing 4x1.5 cm^2 plugs of fungal culture with 35% HCl, boiling for 10 minutes and filtering through Whatman glass microfiber filter paper. After filtration, the filter paper was washed with water, dried in an oven at 70 C overnight, and the extracted melanin was weighed. This test was conducted once.

Growth of *S. minor* was also tested in volatiles released from soil amended with RSM minus glucosinolates (RSM-G) at 55,000 ppm. RSM-G was prepared as follows: 300 g of RSM was mixed with 500 ml of 1N HCl. The mixture was boiled and filtered through Whatman #1 filter paper. The extracted RSM was then washed twice with 1,000 ml of boiling water and dried in an oven at 70 C overnight. Colony growth of *S. minor* in volatiles released from 600 g of air-dried soil alone to which 200 ml of water was added, and in volatiles from soil amended with RSM at 55,000 ppm, were used as controls. Each treatment had three replicates and the test was conducted once.

Data of sclerotial formation by *S. minor* and *S. rolfsii*, and data of melanin formation by *V. dahliae* were analyzed using ANOVA, and treatment means were compared by LSD. Dummy variable regression was used to determined the inhibition effects of volatiles released from soil amended with RSM or RG on the growth of *S. minor* and *S. rolfsii*, and appropriate linear models were fitted. Linear regression was used to compare the inhibition effects of volatiles released from soil amended with different concentrations of RSM on the growth of *V. dahliae*. Linear regression was also used to compare the inhibition effects of volatiles from soil amended with RSM

at 55,000 ppm on the growth of *S. minor* at different temperatures. Models were evaluated based on the *F* test, lack-of-fit, and coefficient of determination. Effect of volatiles from soil amended with RSM on the growth of *P. myriotylum* was analyzed using 2x5 factorial arrangement of treatment (FAT) analysis. Effect of volatiles from soil amended with RSM minus glucosinolates was analyzed using 2x3 FAT analysis. Data of repeated tests were pooled as no significant effects of test or test by time interaction were observed in all repeated tests. Only significant (P=0.05) data are discussed unless stated otherwise. All analysis were conducted with SAS (SAS Institute, Cary, NC).

RESULTS

Mycelial growth of *S. minor* when exposed to volatiles released from soil amended with RSM or RG at 55,000 ppm was inhibited as compared with that of the control. There were significant effects of time, type of organic amendment (RSM and RG), and time by type of organic amendment interaction on the percent inhibition of the pathogen. More inhibition of mycelial growth was observed when *S. minor* was exposed to volatiles released from soil amended RG at 55,000 ppm than when it was exposed to volatiles from soil amended with RSM at 55,000 ppm (Figure 2). After 50 hours incubation, mycelial growth of *S. minor* was reduced by 40% by volatiles released from soil amended with RSM. In volatiles released from soil with RG at the same rate, 78% inhibition was observed. Fewer sclerotia were formed by *S. minor* in volatiles from soil with RSM than that in volatiles from soil alone, and the fewest

sclerotia were formed in volatiles released from soil with RG (Table 1). The sclerotial formation of *S. minor* was also delayed three days under the influence of volatiles released from soil amended with RSM, and three more days in volatiles released from soil with RG (Table 1).

When *S. minor* was subjected to volatiles from soil with RSM at 55,000 ppm at different temperatures, mycelial growth was inhibited. There were significant effects of time, temperature, and time by temperature interaction on the percent inhibition of the pathogen. After 40 hours, more than 35% inhibition was observed at 10 C and 25 C. At 15 C, less inhibition effects was observed (Figure 3). At 15 C, less than 25% inhibition was observed after 24 hours. Sclerotia formed after 10 days in volatiles from soil at 10 C and 15 C. Sclerotial formation was delayed five days at 10 C and 3 days at 15 C in volatiles from soil amended with RSM. At 30 C, *S. minor* grew poorly and it was impossible to observe the inhibitory effects of the volatiles.

Volatiles from soil amended with RSM or RG at 55,000 ppm reduced the mycelial growth and number of sclerotia formed by *S. rolfsii*. There were significant effects of time, type of organic amendment (RSM and RG), and time by type of organic amendment interaction on the percent inhibition of the pathogen. Better inhibition was observed when *S. rolfsii* was exposed to volatiles from soil amended with RG at 55,000 ppm than when it was exposed to volatiles from soil amended with RSM at 55,000 ppm (Figure 4). After 96 hours incubation, mycelial growth of *S. rolfsii* was reduced by 48% in volatiles from soil amended with RSM, but more than

80% inhibition was achieved in volatiles released from soil amended with RG. Fewer sclerotia were formed in volatiles from soil amended with RSM and no sclerotia formed in volatiles from soil amended with RG. Sclerotial formation was delayed 13 days in volatiles from soil amended with RSM (Table 2).

Volatiles from soil amended with RSM were able to reduce the mycelial growth of *V. dahliae*. There were significant effects of time, RSM concentration, and time by RSM concentration interaction on the percent inhibition of the pathogen. The higher the concentration of RSM in soil, the more inhibition observed. More than 40% inhibition was observed when *V. dahliae* was growing in volatiles from soil amended with RSM at 20,000 ppm. More than 60% inhibition was observed in volatiles from soil amended with RSM at 40,000 ppm. Mycelia growth was totally inhibited when exposed to volatiles from soil amended with RSM at 60,000 ppm (Figure 5). Less microsclerotia were formed by *V. dahliae* in volatiles from soil amended with RSM based on the amount of melanin produced (Table 3). No melanin was extracted when the fungus was grown in volatiles from soil amended with RSM at more than 40,000 ppm.

No inhibition was observed in the mycelial growth of *P. myriotylum* in volatiles released from soil amended with RSM (Figure 6).

No significant difference was observed between the mycelial growth of *S. minor* in volatiles released from soil alone and soil amended with RSM minus glucosinolates (Figure 7). Growth of the pathogen in volatiles from soil amended with RSM at 55,000 ppm was significantly different from that in volatiles released

from soil alone or in RSM-G amended soil.

DISCUSSION

This study demonstrated that volatiles from soil amended with RSM or RG inhibited mycelial growth and formation of sclerotia or microsclerotia of *S. minor*, *S. rolfsii* and *V. dahliae*, but did not inhibit growth of *P. myriotylum*. Volatiles released from RSM or RG-amended soil probably have different activities against different types of microorganisms. The closed system described in this paper can be used to test inhibitory effects of other volatiles released in the system to the growth and other growth habits of microorganisms.

No inhibition was observed when *S. minor*, *S. rolfsii*, or *V. dahliae* was exposed to volatiles from air-dried soil amended with RSM at 55,000 ppm without the addition of water to the mix (Data not presented). The addition of water to the soil amended with RSM or RG is crucial for the decomposition of the glucosinolates present in RSM or RG and the release of volatiles, as water is needed for the growth and reproduction of RSM or RG-decomposing microbes. Thus it is necessary to add water to the soil when RSM or RG is intended for field application.

The inhibitory volatiles were also released at 10 and 15 C, although *S. minor* grew slower at lower temperatures. More inhibitory effects were observed when *S. minor* was exposed to volatiles from soil amended with RSM at 10 C and 25 C than when it was exposed to volatiles released from soil amended with the same amount of RSM at 15 C. The reason for this might be that at 15 C, the microorganisms

involved in volatile releasing do not grow as well as *S. minor*, because *S. minor* is a pathogen that favors cool weather. At 10 C, these microorganisms grow as slowly as *S. minor*, and the volatiles accumulate in time to cause inhibition of the pathogen. At 25 C, these microorganism grow fast and more biocidal volatiles are released. When temperature is above 30 C, *S. minor* does not grow much, and it was impossible to test the effects of the volatiles. However, the system can be used if the microorganism being tested grows at higher temperatures.

For both *S. minor* and *S. rolfsii*, more inhibitory effect of the volatiles were observed in soil amended with RG than in soil amended with the same amount of RSM. This is probably because of the difference in glucosinolates concentration between the two kinds of amendment used. The RSM contained 36 μM glucosinolates/g meal, but the cultivar of rape from which the RG was obtained had seed meal containing about 73 μM glucosinolates/g meal. As the concentration of glucosinolates in greens is similar to that in meal (8, 19), the RG probably contained more glucosinolates than the RSM. However, the exact concentration of glucosinolates in the RG used was not determined because of the lack of proper chemicals and equipment.

No inhibition was observed when *S. minor* was grown in volatiles released from soil amended with RSM minus glucosinolates at 55,000 ppm. This indicates that the extraction removed the active ingredients (glucosinolates) that contributed to the release of inhibitory volatiles from soil amended with RSM at 55,000 ppm.

The effects of the RSM amendment in soil on the sclerotial viability of *S.*

minor and *S. rolfsii* were also tested in an open system (13). The details are written in Chapter II of this dissertation. The amendments stimulated the microorganisms which were able to either colonize the sclerotia of both pathogens or produce antibiotics against one or both of the pathogens. The more amendment added, the less the viability of the sclerotia. The microorganisms involved also contribute to the control effects of RSM or RG.

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Table 1. The effect of volatiles released from soil amended with rapeseed meal (RSM) or rape greens (RG) on the formation of sclerotia of *S. minor*.

Treatment	DAI*	No. Sclerotia formed/cm^2 **
Unamended soil	5 a	19.5 a
RSM at 55,000 ppm	8 b	13.0 b
RG at 55,000 ppm	11 c	5.7 c
$LSD_{0.05}$	0	0.9

* DAI: Days after inoculation when mature sclerotia of *S. minor* were formed. Values are the means of two tests. Each test had four replicates. Means in the column followed by the same letter do not differ significantly ($P<0.05$) according to Fisher's LSD test.

** The values are the mean numbers of sclerotia formed per cm^2 on the plate area. Values are the means of two tests. Each test had four replicates.

Table 2. The effect of volatiles released from soil amended with rapeseed meal (RSM) or rape greens (RG) on the formation of sclerotia by *S. rolfsii*.

Treatment	DAI*	No. Sclerotia formed/cm^2 **
Unamended soil	12 a	3.55 a
RSM at 55,000 ppm	25 b	0.23 b
RG at 55,000 ppm	N/A***	0 c
$LSD_{0.05}$	0	0.19

* DAI: Days after inoculation, when mature sclerotia of *S. rolfsii* were formed. Values are the means of two tests. Each test had four replicates. Means in the column followed by the same letter do not differ significantly ($P<0.05$) according to Fisher's LSD test.

** The values are the mean numbers of sclerotia formed per cm^2 on the plate area. Values are the means of two test. Each test had four replicates.

*** Data not available because no sclerotia were observed in this treatment.

Table 3. The effect of volatiles released from soil amended rapeseed meal (RSM) on the melanin formation of *V. dahliae*.

Treatment	Melanin (mg)*
Unamended soil	15.74 a
RSM at 20,000 ppm	0.06 b
RSM at 40,000 ppm	0 b
RSM at 60,000 ppm	0 b
$LSD_{0.05}$	1.7

* Amount of melanin produced by 4x1.5 cm^2 colony. Values are the means of four replicates. Means in the column followed by the same letter do not differ significantly ($P<0.05$) according to Fisher's LSD test.

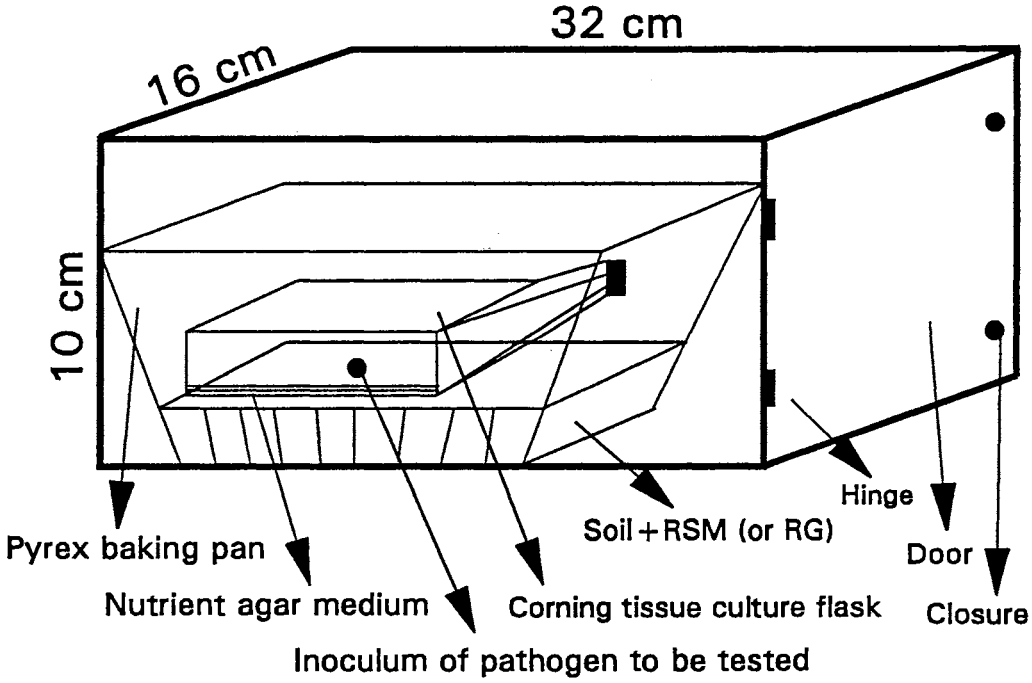

Figure 1. Schematic drawing of a closed chamber system unit used to test the effects of volatiles, released from soil amended with rapeseed meal (RSM) or rape greens (RG), on growth of soilborne pathogens.

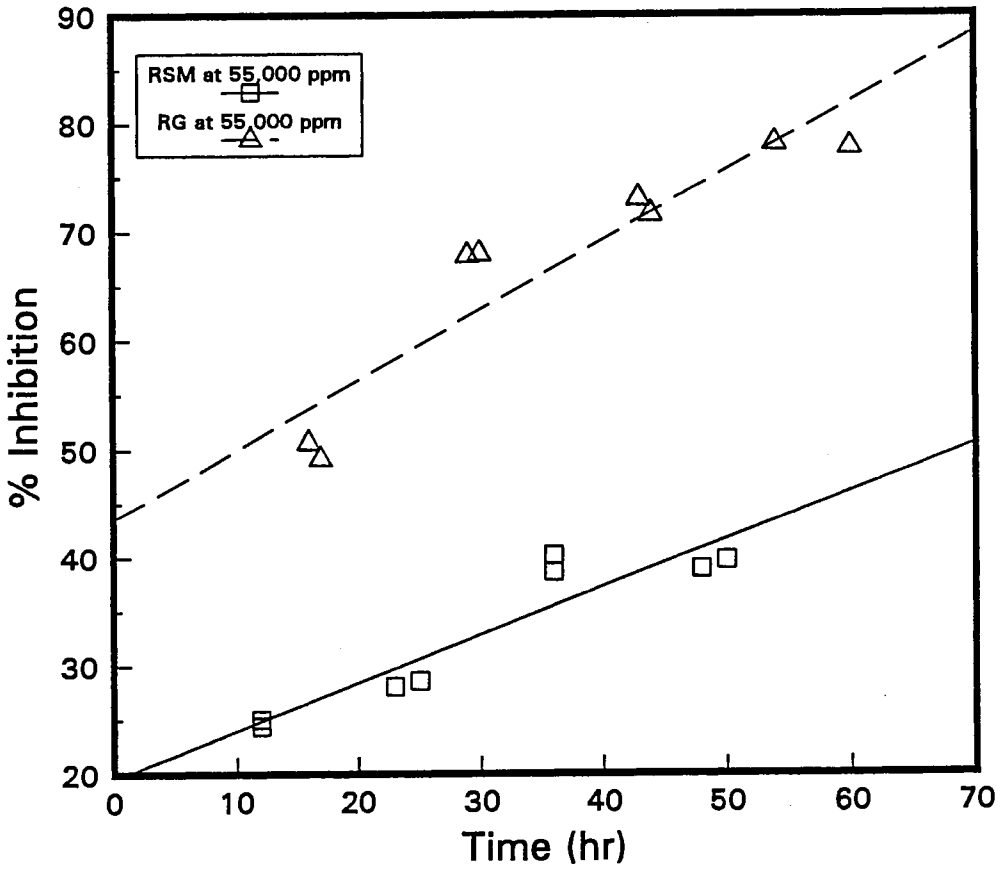

Figure 2. Effect of volatiles from soil amended with rapeseed meal (RSM) or rape greens (RG) on mycelial growth of *S. minor* on potato dextrose agar. Data points represent the percent inhibition of colony area, caused by the volatiles released from soil amended with RSM or RG at 55,000 ppm at different periods of time, compared to colony area of control when exposed to volatiles from unamended soil. Data points are means of two tests. Each test had four replicates. Regression lines are of the form $\hat{y}=b_0+b_1x+b_2(dv)+b_3(dv)x$ (dv=dummy variable, dv=0 for RSM amendment, dv=1 for RG amendment; $b_0=19.54$, $b_1=0.44$, $b_2=23.97$, $b_3=0.20$). Lines have r^2 value of 0.95.

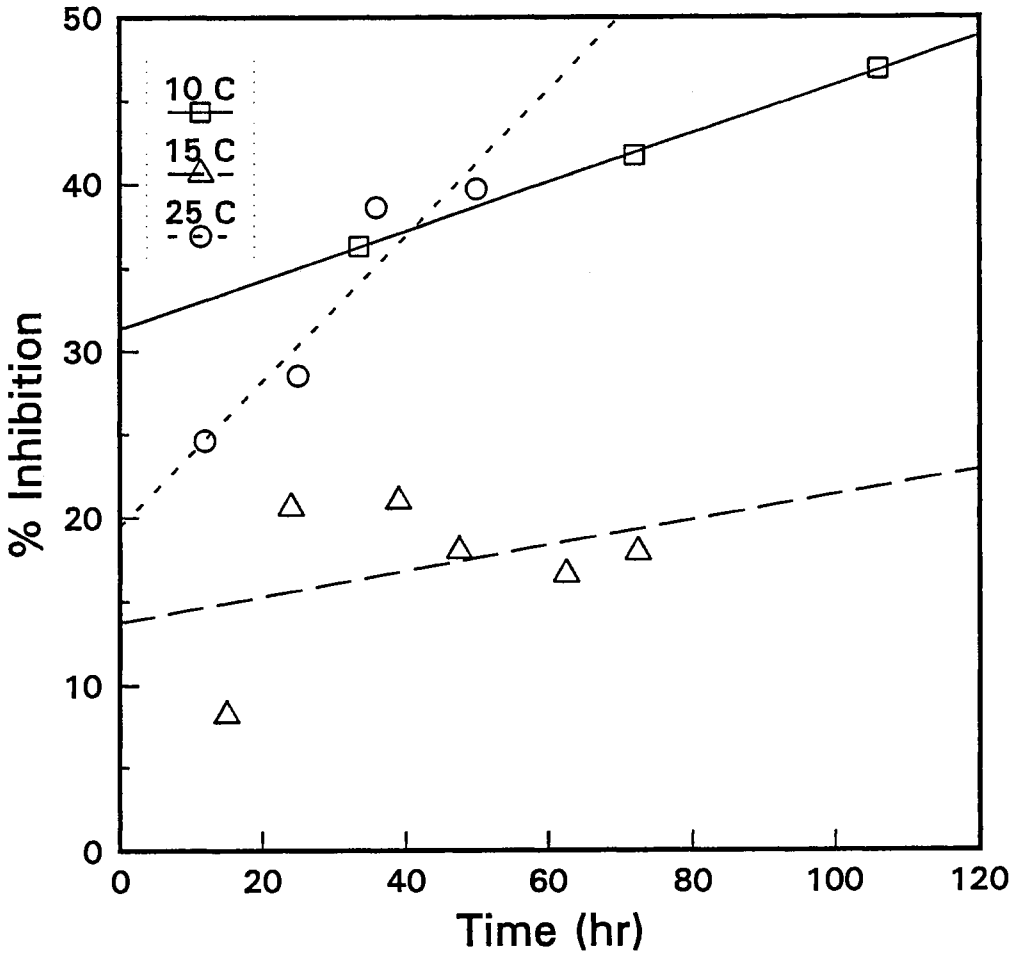

Figure 3. Effect of volatiles from soil amended with rapeseed meal at 55,000 ppm on mycelial growth of *S. minor* at different temperatures on potato dextrose agar. Data points represent the percent inhibition of colony area. Data points represent means of four replicates. Equations of the lines derived from linear regression of the data are $y=31.3+0.15x$, $y=13.7+0.08x$ and $y=19.4+0.43x$ for tests at 10 C, 15 C and 25 C, respectively. Lines have r^2 values of 0.99, 0.13 and 0.89 for tests at 10 C, 15 C and 25 C, respectively.

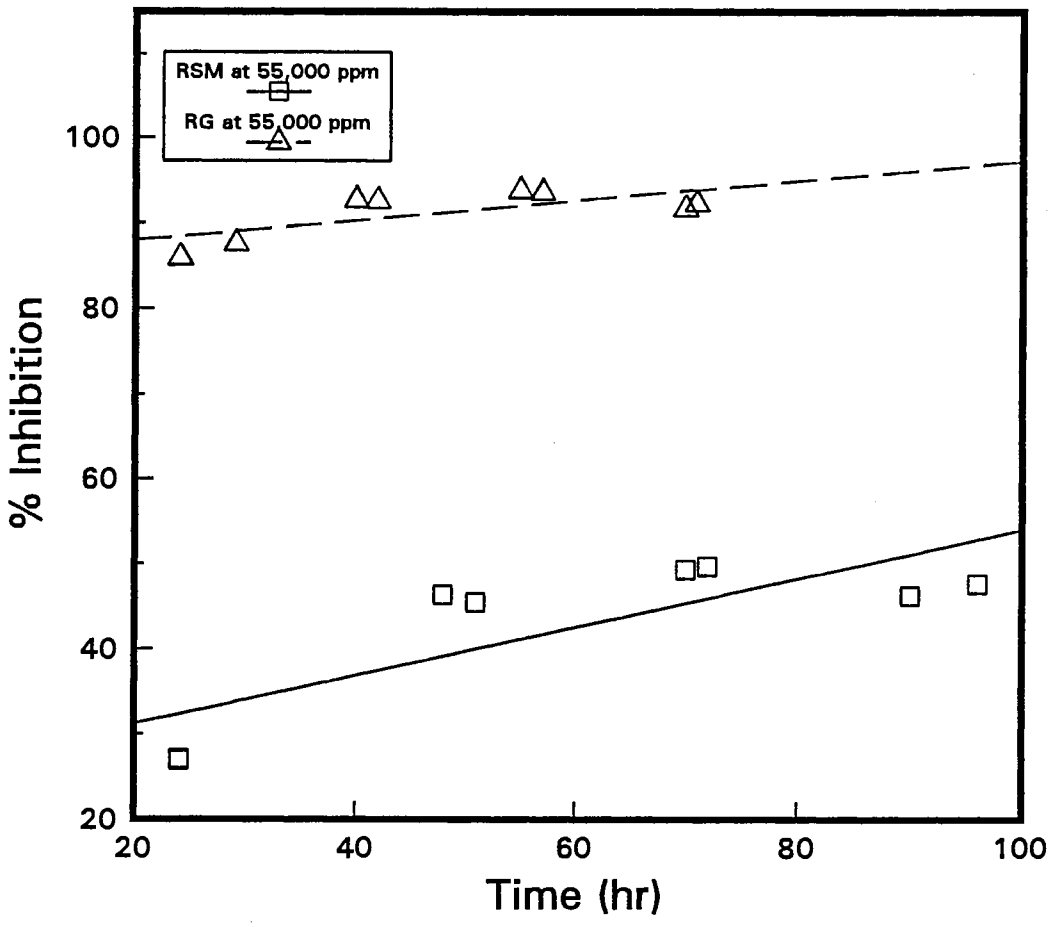

Figure 4. Effect of volatiles from soil amended with rapeseed meal (RSM) or rape greens (RG) on mycelial growth of *S. rolfsii* on potato dextrose agar. Data points represent the percent inhibition of colony area, caused by the volatiles released from soil amended with RSM or RG at 55,000 ppm at different periods of time, compared to colony area of control when exposed to volatiles from soil. Data points are means of two tests. Each test had four replicates. Regression lines are of the form $\hat{y}=b_0+b_1x+b_2(dv)+b_3(dv)x$ (dv=dummy variable, dv=0 for RSM amendment, dv=1 for RG amendment; $b_0=25.43$, $b_1=0.28$, $b_2=60.30$, $b_3=-0.17$). Lines have r^2 values of 0.97.

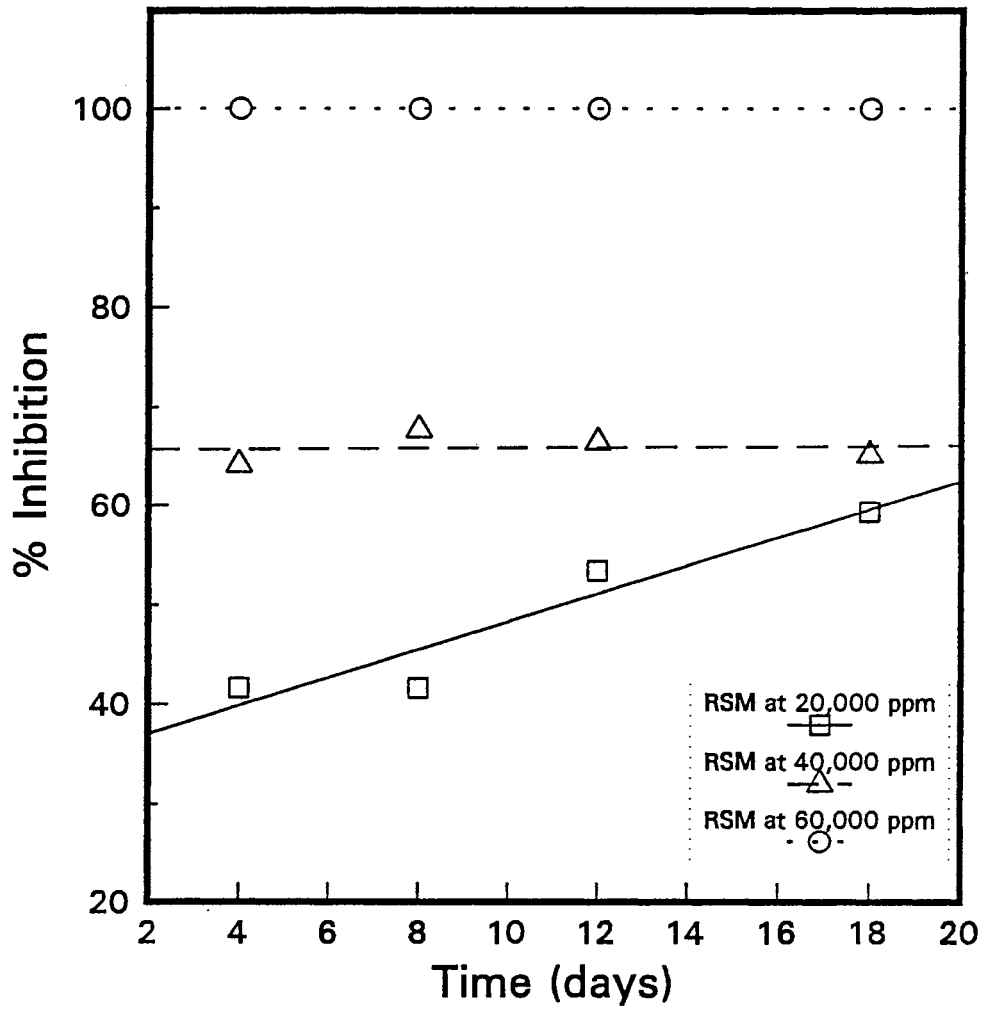

Figure 5. Effect of volatiles from soil amended with different rates of rapeseed meal (RSM) on mycelial growth of *V. dahliae* on potato dextrose agar. Data points represent the percent inhibition of colony area, caused by the volatiles released from soil amended with RSM at 20,000, 40,000 or 60,000 ppm at different periods of time, compared to colony area of control when exposed to volatiles from soil. Data points are means of four replicates. Equations of the lines derived from linear regression of the data are $y=34.1+1.41x$, $y=65.6+0.022x$ and $y=100$ for tests with RSM at 20,000, 40,000 and 60,000 ppm, respectively. Lines have r^2 values of 0.90, 0.08 for tests with RSM at 20,000 and 40,000 ppm, respectively.

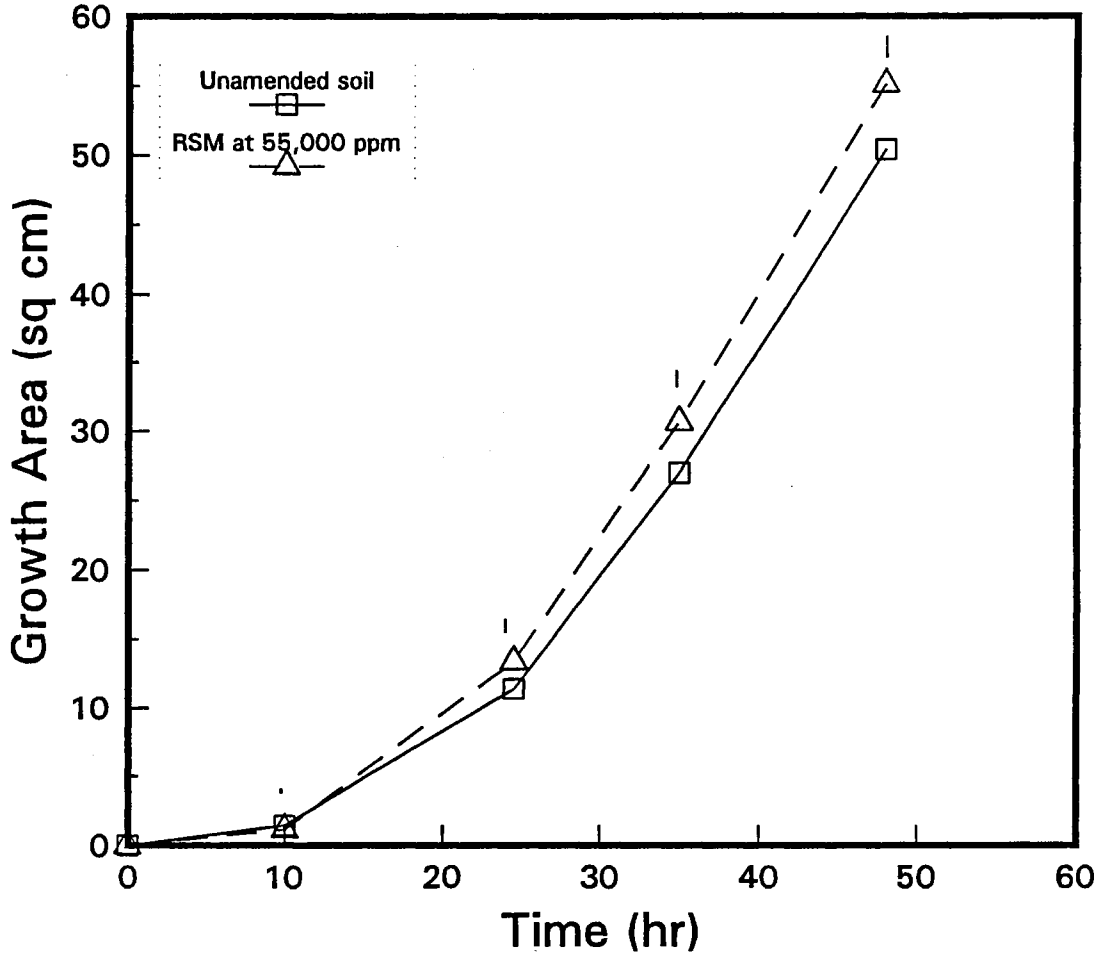

Figure 6. Effect of volatiles from soil amended with rapeseed meal (RSM) on mycelial growth of *P. myriotylum* on potato dextrose agar. Data points represent the areas of the colony formed by the pathogen when exposed to volatiles released from soil amended with RSM at 55,000 ppm at different periods of time. Data points are means of four replicates. Vertical lines above the data points represent the $LSD_{0.05}$ values of the data at different hours of incubation. The $LSD_{0.05}$ values are 0, 0.13, 0.54, 0.65, 1.18 for colony growth area after 0, 10, 24, 35, 48 hours of incubation, respectively.

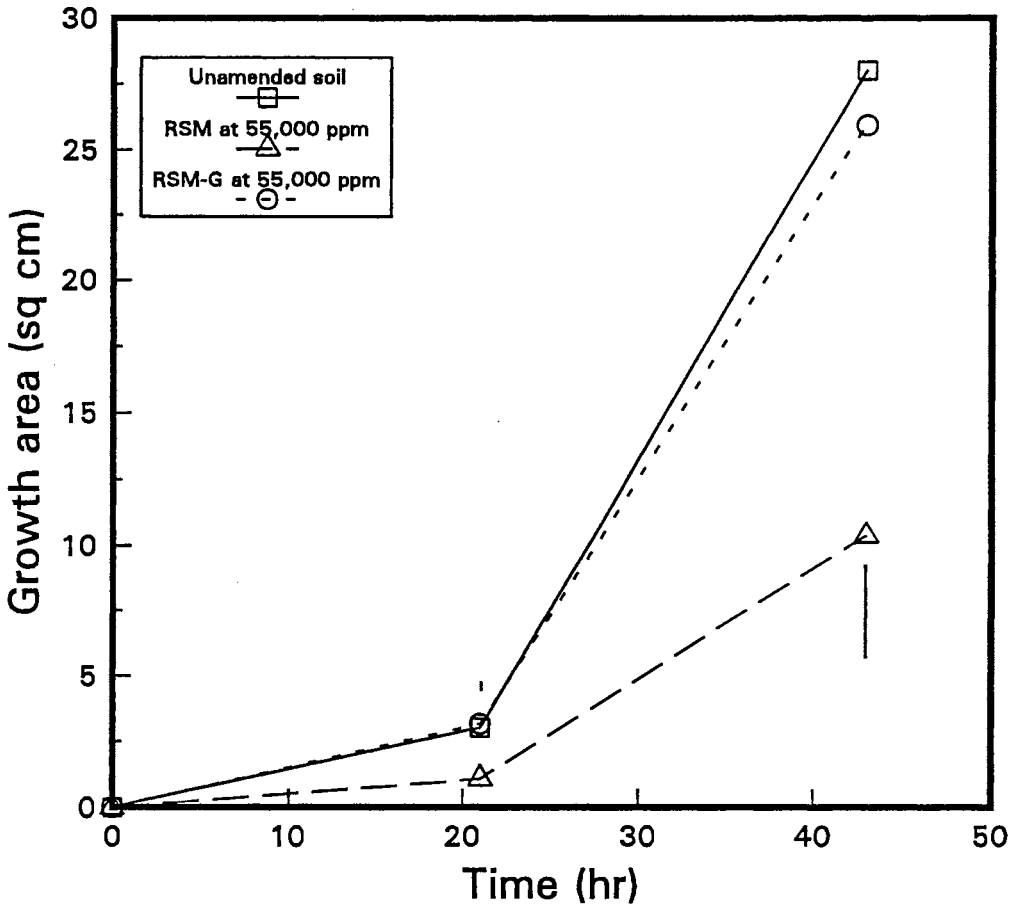

Figure 7. Effect of volatiles from soil amended with rapeseed meal (RSM) or RSM minus glucosinolates (RSM-G) on mycelial growth of *S. minor* on potato dextrose agar. Data points represent the areas of the colony formed by the pathogen when exposed to volatiles released from soil amended with RSM at 55,000 ppm at different periods of time. Data points are means of three replicates. Vertical lines close to the data points represent the $LSD_{0.05}$ values of the data at different hours of incubation. The $LSD_{0.05}$ values are 0, 0.20, 3.64 for colony growth area after 0, 21, 43 hours of incubation, respectively.

CHAPTER II

INHIBITION OF *SCLEROTINIA MINOR* AND *SCLEROTIUM ROLFSII* BY MICROORGANISMS IN SOIL AMENDED WITH RAPESEED MEAL OR RAPE GREENS

ABSTRACT

Cloth pouches containing sclerotia of *Sclerotinia minor* or *Sclerotium rolfsii* were buried in soil amended with rapeseed meal (RSM) or rape greens (RG) at varying concentrations from 0 to 30,000 ppm and incubated for up to 44 days at 22±1 C. Sclerotia were retrieved at various times, surface sterilized, and plated on potato dextrose agar (PDA) to assess their viability. Viability of sclerotia decreased and colonization of sclerotia by microorganisms increased with the concentration of RSM or RG, and with incubation time. In soil amended with RSM or RG at 20,000 or 30,000 ppm, no sclerotia of *S. minor* or *S. rolfsii* survived after 40 days of incubation at 22±1 C. Several microorganisms that colonized the sclerotia of both *S. minor* and *S. rolfsii* were identified, that included *Mucor* spp., *Fusarium* spp., *Trichoderma* spp., *Penicillium* spp., *Chaetomium* spp., *Gliocladium* spp., *Erwinia* spp. and *Bacillus* spp. Among these microorganisms, *Fusarium* spp., *Penicillium* spp., *Chaetomium* spp., *Gliocladium* spp. and *Bacillus* spp. formed inhibition zones

with either *S. minor* or *S. rolfsii* on PDA. One isolate of *Gliocladium* sp. and four isolates of *Bacillus* spp. secreted antimicrobial substances that inhibited the mycelial growth of both *S. minor* and *S. rolfsii* on PDA as determined by minimal inhibitory concentration (MIC) procedures. These results suggests that RSM or RG amendment in soil is able to reduce the sclerotial population of both pathogens. Such amendment stimulates antagonistic microorganisms which are able to colonize the sclerotia, and some of them are able to produce antibiotics against both pathogens.

INTRODUCTION

Sclerotinia blight, caused by *Sclerotinia minor* Jagger, is a major disease of peanut (*Arachis hypogea* L.) in Oklahoma, Virginia, Texas and North Carolina with annual losses of 7% or more (19, 27). The disease has also caused yield losses in excess of 50% in research plots and commercial fields (9). *S. minor* has a broad host range that includes species in 64 plant families worldwide. The fungus is capable of surviving in field soils as sclerotia for several years in the absence of host plants.

Southern blight, or stem rot, caused by *Sclerotium rolfsii* Sacc. which is also a sclerotial-forming fungus, is another damaging disease of peanut. It annually accounts for 5-10% loss in peanut yields despite crop management practices that decrease disease incidence (4, 19). It is known to be favored by high temperature, i.e., 30-35 C, and has a host range of more than 100 families of plants (4, 6).

Epidemics of sclerotinia blight and southern blight are monocyclic and disease incidence depends mainly on the level of sclerotia in the field (4, 27). Thus control

of these two pathogens focuses on strategies which reduce levels of sclerotia in the soil or exclude their introduction into fields.

Few fungicides are available for control of these two diseases, especially sclerotinia blight (4, 9, 26). Some fungicides do not provide acceptable level of disease control, and problems of chemical toxicity, environmental contamination, and pesticide residues in food are an increasing concern to consumers (9, 16). Therefore, alternative control methods, especially integrated pest management (IPM) systems which allow farmers to control diseases using minimum amounts of chemicals, are needed. In Oklahoma, Tamspan 90 and Southwest Runner which are resistant to sclerotinia blight are used in fields with sclerotinia problems (19). Organic amendment as a control strategy for soilborne pathogens has been successful for *S. minor* on lettuce and *S. rolfsii* (5, 6, 7, 8, 10, 16, 17). Amending soil with rape green manure and other Cruciferae members was also reported to be effective against *Rhizoctonia solani*, *Aphanomyces euteiches*, nematodes and other soilborne pathogens (10, 22, 23). Adamsen *et al.* (2) reported that rapeseed (*Brassica napus* L.) meal amendment in soil reduced the viability of microsclerotia of *Cylindrocladium crotalatiae*, the causal agent of cylindrocladium black rot of peanut. It was suggested that the control effect was caused by volatile compounds released from breakdown of glucosinolates which exist in almost all commercial lots of rapeseed meal. Mechanisms of control with organic amendments also include an indirect effect of predisposition of sclerotia to antagonistic microorganisms and a direct effect on increasing populations of soil microflora or shifting microfloral population in soil,

which in turn reduces the activity of the pathogens (14). Biological mechanisms such as antibiosis, parasitism, or nutrient competition by antagonistic microorganisms are probably responsible for the prevention of disease development and disease build-up (3, 14, 16). The use of organic amendments have several major advantages over chemical control including a lack of pesticide residues in soil, the mode of action relies on a shift in microbial ecology, not on chemical toxicity, and broad spectrum control may be achieved (3). One disadvantage of using organic amendments is the potentially high application rates needed to achieve disease control in the field (3).

In previous work, we demonstrated that volatiles released from soil amended with rapeseed meal (RSM) or rape greens (RG) had biocidal activity against both *S. minor* and *S. rolfsii* (11, 12, Chapter I of this dissertation). This research had two objectives: 1) to evaluate the effects of RSM and RG on the survival of sclerotia of *S. minor* and *S. rolfsii* in amended soil; and 2) to investigate possible mechanisms for these organic amendment such as stimulating the activity of beneficial organisms and antibiosis in amended soil. A brief report of this research was presented earlier (13).

MATERIALS AND METHODS

RSM was obtained from a commercial source (Calgene Chemical Inc., Springfield, IL) which contained 36 μM glucosinolates/g meal. Seeds of variety Emerald Rape (Calgene Inc.), which produced meal containing about 73 μM glucosinolates/g meal, were planted at the Plant Pathology Farm in Stillwater on Sept 9, 1992. The greens or the entire above-ground parts of the plants (RG) were

harvested in March 1993, and dried on a greenhouse bench at 28 C. The dried greens were then chopped in a blender. The soil used in these tests from Fort Cobb, OK, was a Menofine sandy loam with pH 6.0. The soil was pulverized to a fine structure before use.

An open system was used to determine the effects of amending soil with various rates of RSM or RG from 0 to 30,000 ppm on the viability of sclerotia of either *S. minor* or *S. rolfsii* buried in amended soil. RSM or RG were mixed with aired-dried soil at 0, 10,000, 20,000 or 30,000 ppm. In each 8-cm dia plastic pot, 600 g of amended soil was placed and 150 ml of water was added. Acrylic fiber cloth pouches were fabricated, and 30 sclerotia of either pathogen were placed inside each pouch before the pouches were closed by stitching. Four pouches were buried into the soil mixture in each pot for incubation at 22 ± 1 C. Water was added periodically to the pots to keep the soil moist during incubation. Each treatment had four pots (replicates). After about 10, 20, 30 or 40 days of incubation, one pouch from each replicate in each treatment was retrieved and washed under running cold tap water. Sclerotia inside the pouches were then surface disinfected in 1% sodium hypochlorite for two minutes and plated on potato dextrose agar (PDA). The number of sclerotia which germinated after four days of incubation at 22 ± 1 C was recorded, and the percentage of viable sclerotia was calculated. The number of sclerotia colonized with microorganisms was also recorded, and the percentage of sclerotia colonized was calculated. The test was performed twice.

The microorganisms commonly found colonizing the sclerotia of both

pathogens were isolated and identified. Identification of fungi were based on microscopic examination. Identification of the bacteria was based on Bergey's Manual (8a).

Inhibition zone tests in paired cultures were used to assay the antagonistic effects of these microorganisms to either *S. minor* or *S. rolfsii* (10a). PDA was used as a standard medium throughout. For fungi, a 0.17 cm^2 plug of 2-day-old mycelium of each pathogen was inoculated in paired culture with the various antagonistic microorganisms. For bacteria, the pathogens were paired with streaks of the bacteria. Paired cultures were incubated for 7 days at 22$\pm$1 C. A clear zone between the pathogen and the antagonistic microorganism indicated the production of antimicrobial substances from the antagonistic microorganism.

A minimum inhibition concentration (MIC) test was used to compare the ability of microorganisms to produce antibiotics (10a). Each microorganism was inoculated into 200 ml potato dextrose broth (PDB) in 500 ml flasks, and incubated on a rotary shaker at 200 rpm at 22$\pm$1 C. After 4 days, contents in the flasks were centrifuged at 10,000 rpm for 20 minutes, and the supernatant was filtered through 0.45μm Millipore filter (Gelman Sciences, Ann Arbor, MI). The pH of the filtrate was measured to ensure it would not change the pH of the PDA medium that would be used in the MIC test. The filtrate was incorporated into PDA at rates of 0, 0.25, 0.5, 1, and 2 ml per 5 ml of total volume. Four plates were prepared for each filtrate concentration. Plates were inoculated with one 0.17 cm^2 plug of 2-day-old mycelium of either *S. minor* or *S. rolfsii*. Plates were observed for growth of the pathogens

after 1, 2, and 3 days incubation at 22±1 C. The minimum filtrate concentration which totally inhibited growth was recorded as the MIC of the microorganism. The test was performed twice.

Data of viability of sclerotia of either pathogens, and data of colonization of these sclerotia by microorganisms in soil amended with RSM or RG at different rates were analyzed using ANOVA. Linear regression by rate of amendment was used to test the viability or colonization of sclerotia in soil amended with different rates of RSM or RG over time, and appropriate linear models were fitted. Models were evaluated based on the *F* test, lack-of-fit, and coefficient of determination. Data of repeated tests were pooled as no significant effects of test, test by rate of amendment or test by time interaction were observed in all repeated tests. Only significant (P=0.05) data are discussed unless stated otherwise. All analysis was conducted with SAS (SAS Institute, Cary, NC).

RESULTS

Viability of sclerotia of either *S. minor* or *S. rolfsii* was reduced in soil amended with RSM or RG than in unamended soil. Viability of sclerotia of both pathogens decreased either when the rate of RSM or RG was increased or when the incubation time was lengthened. There were significant effects of amendment rate, incubation time, and rate by time interaction on the viability of sclerotia of either pathogens in soil amended with RSM or RG. When the data were analyzed at each amendment rate, the effect of time on the viability of sclerotia were all significant.

Viability of sclerotia decreased with time. Viability of sclerotia of *S. minor* remained at more than 60% in soil without amendment. No sclerotia were viable in soil amended with RSM or RG at 20,000 ppm after 30 days of incubation at 22±1 C (Figure 1, 2). Viability of sclerotia of *S. rolfsii* remained at more than 70% in soil without amendment. No sclerotia survived in soil amended with RSM at 10,000 ppm after 46 days of incubation (Figure 3), and no sclerotia survived in soil amended with RG at 10,000 ppm after 37 days at 22±1 C (Figure 4).

In contrast, the total number of microorganisms colonizing the sclerotia increased either when the amendment rate increased or when the incubation period was lengthened. No sclerotia of either *S. minor* or *S. rolfsii* were colonized with microorganisms at the beginning of the different treatments. There were significant effects of incubation time, amendment rate, and time by rate interaction on the colonization of sclerotia of either pathogens in soil amended with RSM or RG. When the data were analyzed at each rate, the effect of time on the colonization of sclerotia were all significant. Colonization of sclerotia increased with time. In soil without any amendment, less than 30% of the sclerotia of *S. minor* were colonized with microorganisms after 50 days of incubation at 22±1 C (Figure 5). In soil amended with RSM at 20,000 or 30,000 ppm, all the sclerotia were colonized after 50 days (Figure 5). In soil amended with RG at 20,000 or 30,000 ppm, all the sclerotia were colonized after 40 days of incubation (Figure 6). In soil without any amendment, only 5% of sclerotia of *S. rolfsii* were colonized with microorganisms after 50 days of incubation at 22±1 C. In soil amended with RSM at 20,000 or 30,000 ppm, all the

sclerotia were colonized after 40 days of incubation (Figure 7). In soil amended with RG at 20,000 or 30,000 ppm, all sclerotia were colonized after 45 days of incubation at 22±1 C.

Microorganisms frequently colonizing the sclerotia of both pathogens were collected, isolated and identified. These antagonistic microorganisms included *Mucor* spp., *Fusarium* spp., *Trichoderma* spp., *Penicillium* spp., *Chaetomium* spp., *Gliocladium* spp., *Erwinia* spp. and *Bacillus* spp. Only four isolates of *Bacillus* spp. were further identified to species level. Isolates m11, m12 and r9 were identified as *Bacillus subtilis*. Isolate m14 was identified as *B. megaterium*.

As determined by inhibition zone test, microorganisms which inhibited growth of *S. minor* included *Gliocladium* spp., *Bacillus* spp., *Fusarium* spp., *Erwinia* spp., *Penicillium* spp. and *Chaetomium* spp. Microorganisms which inhibited growth of *S. rolfsii* included *Gliocladium* spp., *Bacillus* spp., *Penicillium* spp. and *Chaetomium* spp.

Among all the microorganisms which formed inhibition zones with either pathogens, only one isolate of *Gliocladium* spp. and four isolates of *Bacillus* spp. were able to produce antibiotics which exist in the filtrates at low MICs (Table 1). Filtrates of *Bacillus* isolates r9 and m14, and *Gliocladium* isolate YE were able to totally inhibit growth of *S. minor* at 10% concentration of the total volume in the PDA medium. Filtrates of *Bacillus* isolates r9 and m14 were able to totally inhibit growth of *S. rolfsii* at 20% concentration of the total volume in the PDA medium (Table 1).

DISCUSSION

The results of the tests have shown that it is possible to reduce the population of sclerotia of *S. minor* and/or *S. rolfsii* in soil by using RSM or RG as an organic amendment. The sclerotial populations of both pathogens decreased in soil amended with RSM or RG at different rates. Better control effects were observed on the reduction of sclerotial populations of *S. rolfsii* than *S. minor*, probably because the sclerotia of *S. rolfsii* are more vulnerable to soil microorganisms compared with that of *S. minor*, as sclerotia of *S. rolfsii* only have a thin melanized rind, which is only two to four cell layers thick, and sclerotia of *S. minor* has a thicker melanized rind (15, 25). Other kinds of organic amendments, such as cattle manure and composted grape marc, were reported to be effective in controlling *S. rolfsii* in the field (8).

From results of this study, amending soil with RSM or RG at 10,000 ppm (about 11.3 t/ha in top 7.5 cm soil) is able to kill all sclerotia of *S. rolfsii* after 50 days at 23 C. Amending soil with RSM or RG at 20,000 ppm (about 22.6 t/ha in top 7.5 cm soil) is able to kill all sclerotia of *S. minor* after 50 days at 23 C. Amending soil with RSM or RG at 10,000 ppm is able to kill more than 70% of the sclerotia of *S. minor* in soil after 50 days at 23 C. Thus 12 t/ha is probably the best economical rate for future field application of RSM or RG.

From previous study which is reported in Chapter I, volatiles from soil amended with RSM or RG have biocidal effects against *S. minor* and *S. rolfsii*. These volatiles, generated from the degradation of glucosinolates in RSM or RG, contribute to the control effects by RSM or RG. The other mechanism involved in

the effects of RSM or RG amendment on the decrease of sclerotial population is probably similar to that in the other systems where different organic amendments were used, which is due to the shift of population of microorganisms in soil (6). The RSM or RG organic amendment stimulates the growth of microorganisms which are antagonistic to both soilborne pathogens. Among the microorganisms identified which were able to colonize the sclerotia of either *S. minor* or *S. rolfsii*, *Trichoderma* spp. were used previously to control collar rot of glass house lettuce caused by *S. minor* and southern blight of peanut caused by *S. rolfsii* (18, 24). *Trichoderma* spp., which were reported to be hyperparasites of *S. rolfsii*, are able to form appressorium-like structure in the sclerotia (21). Both *Trichoderma* spp. and *Gliocladium* spp. were reported to be effective as biocontrol agents against *S. rolfsii* (18). *Penicillium citrinum* was also reported to produce antibiotics in vitro against *S. minor* (20). From our results, one isolate of *Gliocladium* spp. and four isolates of *Bacillus* spp. were able to produce antibiotics in vitro at low MICs against both pathogens. However, their ability to produce antibiotics in soil was not tested.

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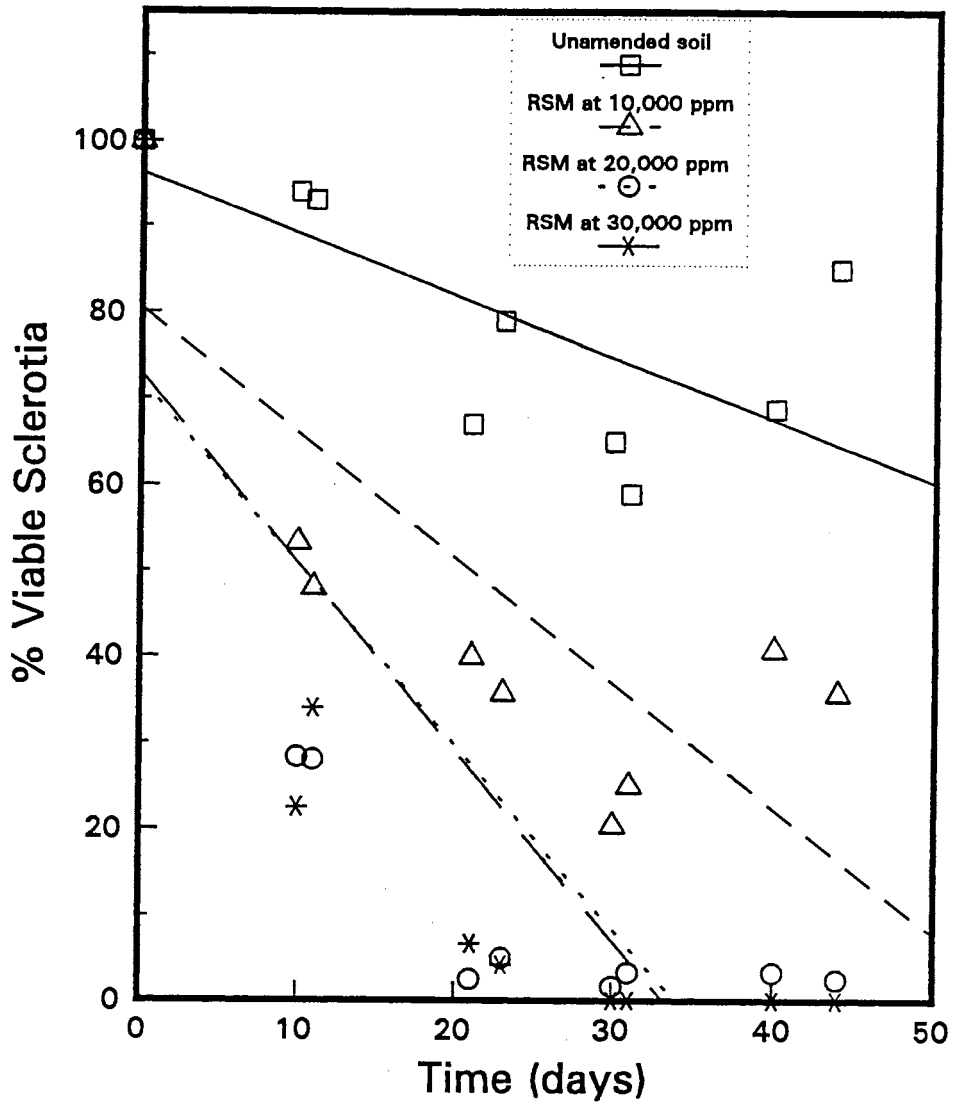

Figure 1. Effect of incubation time on the viability of sclerotia of *S. minor* in soil amended with different rates of rapeseed meal (RSM). Equations of the lines are: y=96.3-0.72x, y=80.5-1.45x, y=72-2.12x, and y=72.8-2.2x, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.15, 0.47, 0.66, and 0.67, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Slopes for the lines are all significantly different (P=0.05) from each other except for lines for soil amended with RSM at 20,000 and 30,000 ppm.

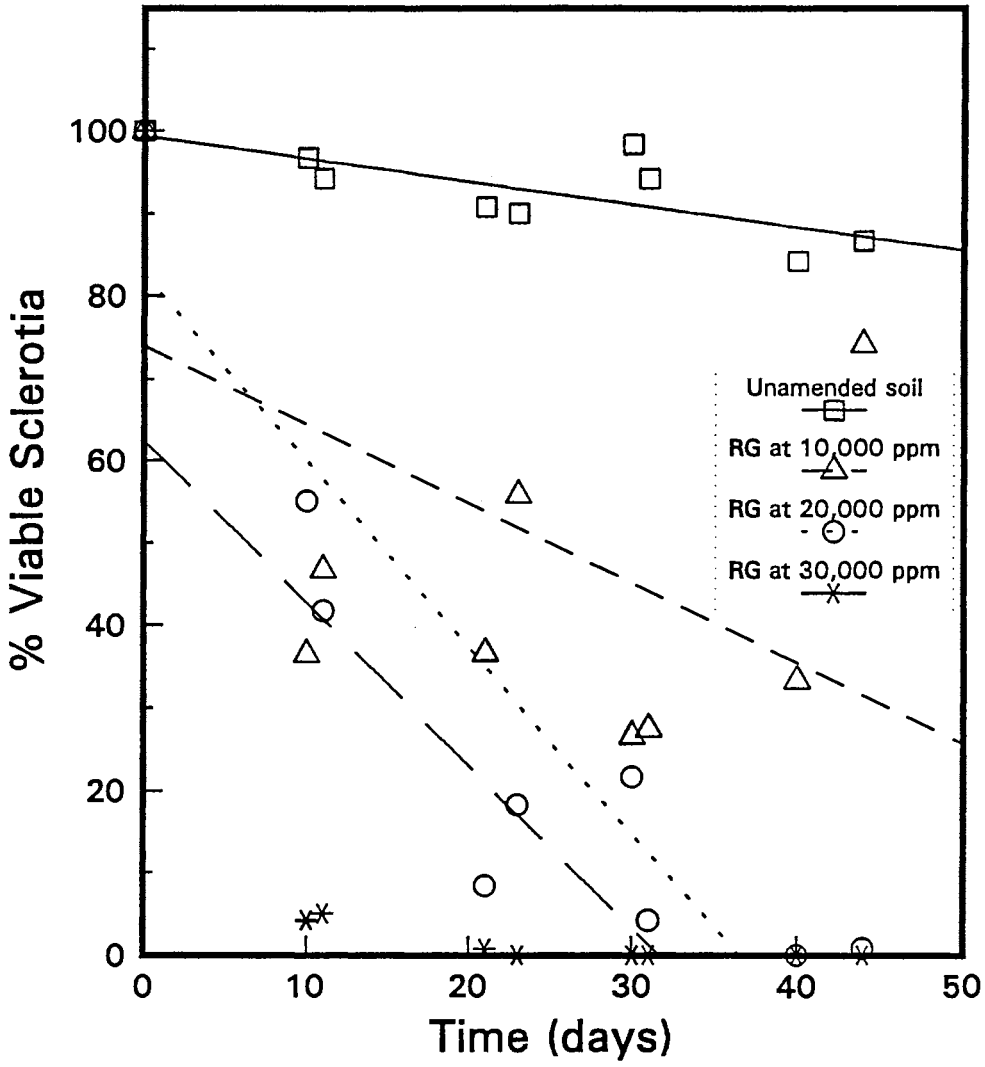

Figure 2. Effect of incubation time on the viability of sclerotia of *S. minor* in soil amended with different rates of rape greens (RG). Equations of the lines are: y=99.3-0.28x, y=74-0.97x, y=82.5-2.26x, and y=62.4-1.97x, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.17, 0.14, 0.73, and 0.54, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Slopes for the lines are all significantly different (P=0.05) from each other except for lines for soil amended with RG at 20,000 and 30,000 ppm.

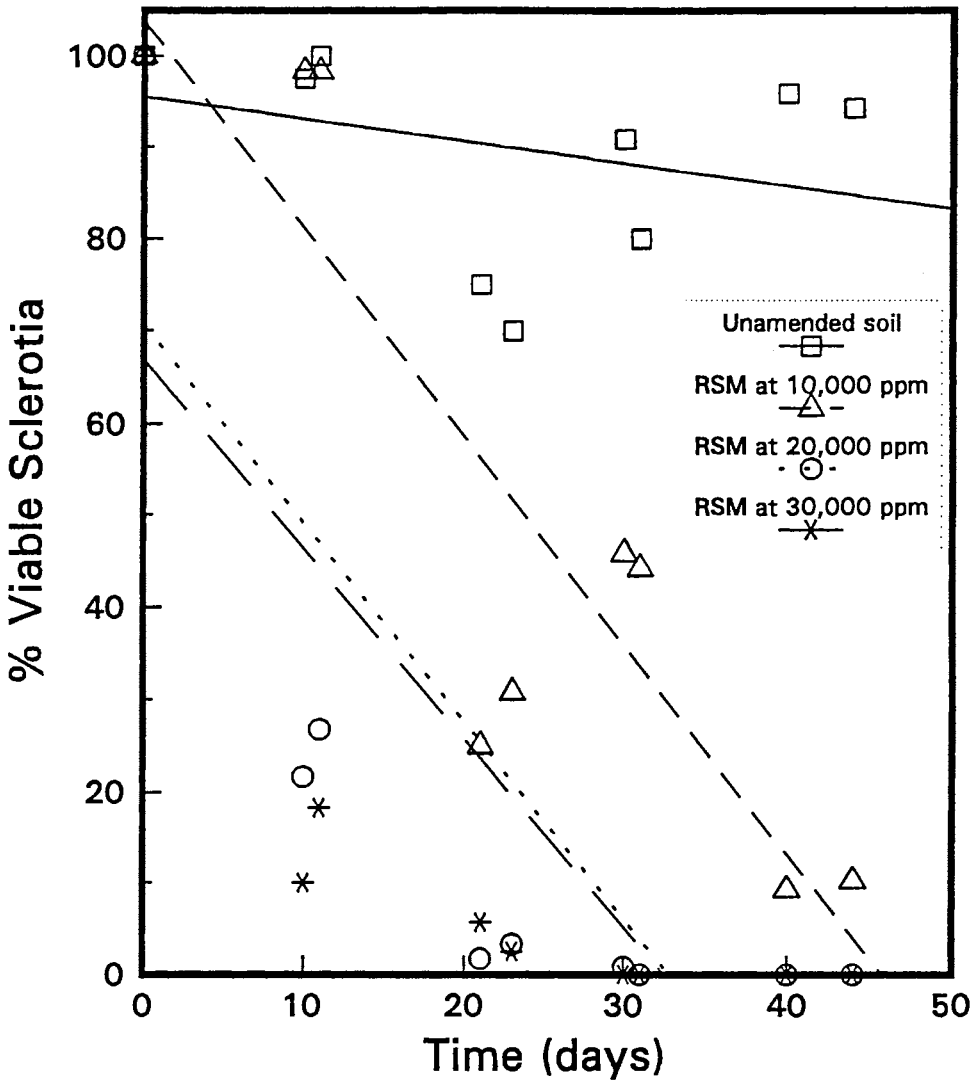

Figure 3. Effect of incubation time on the viability of sclerotia of *S. rolfsii* in soil amended with different rates of rapeseed meal (RSM). Equations of the lines are: y=95.4-0.24x, y=103.8-2.27x, y=70.6-2.15x, and y=66.9-2.06x, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.05, 0.62, 0.62, and 0.60, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Only the slope for unamended soil is significantly different (P=0.05) from that of all others.

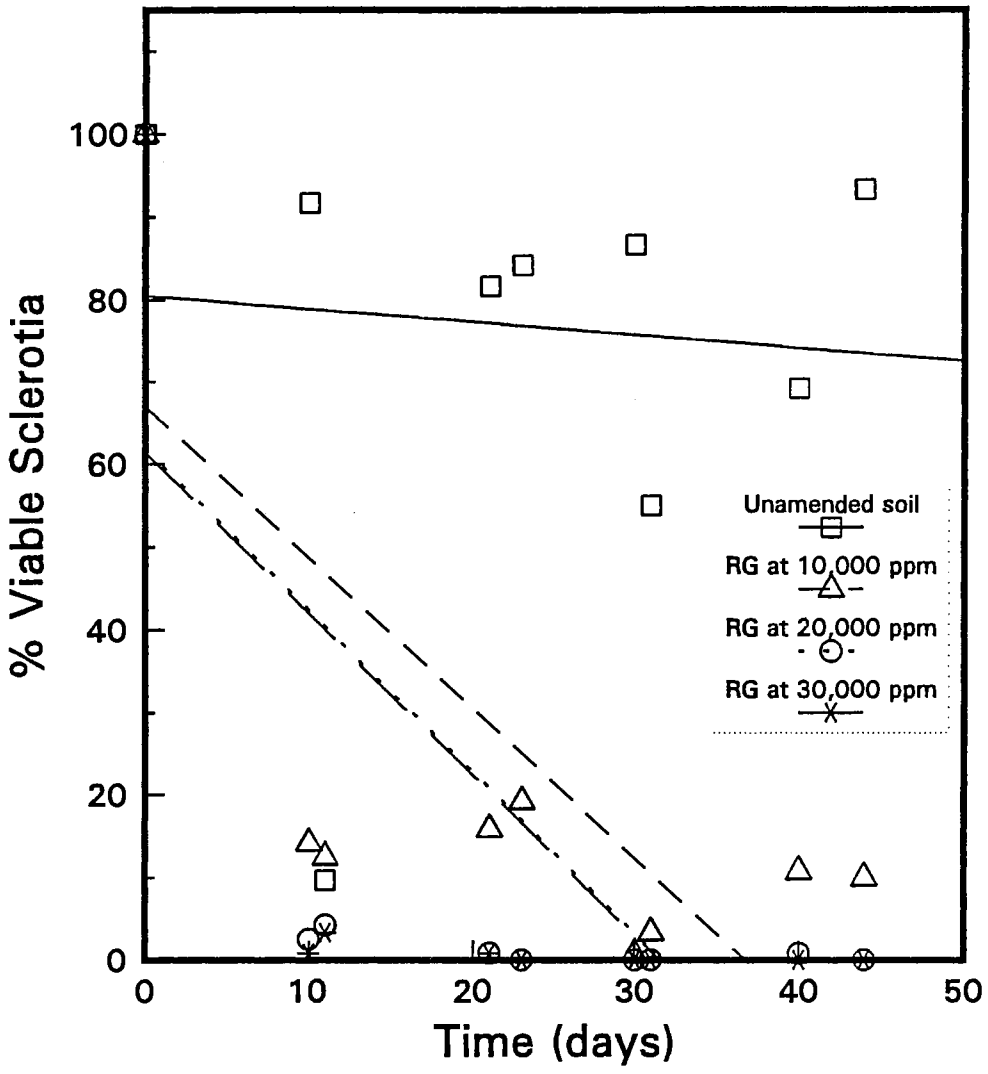

Figure 4. Effect of incubation time on the viability of sclerotia of *S. rolfsii* in soil amended with different rates of rape greens (RG). Equations of the lines are: y=96.6-0.54x, y=66.9-1.82x, y=61.8-1.95x, and y=61.4-1.95x, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.16, 0.46, 0.53, and 0.52, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Only the slope for unamended soil is significantly different (P=0.05) from that of all others.

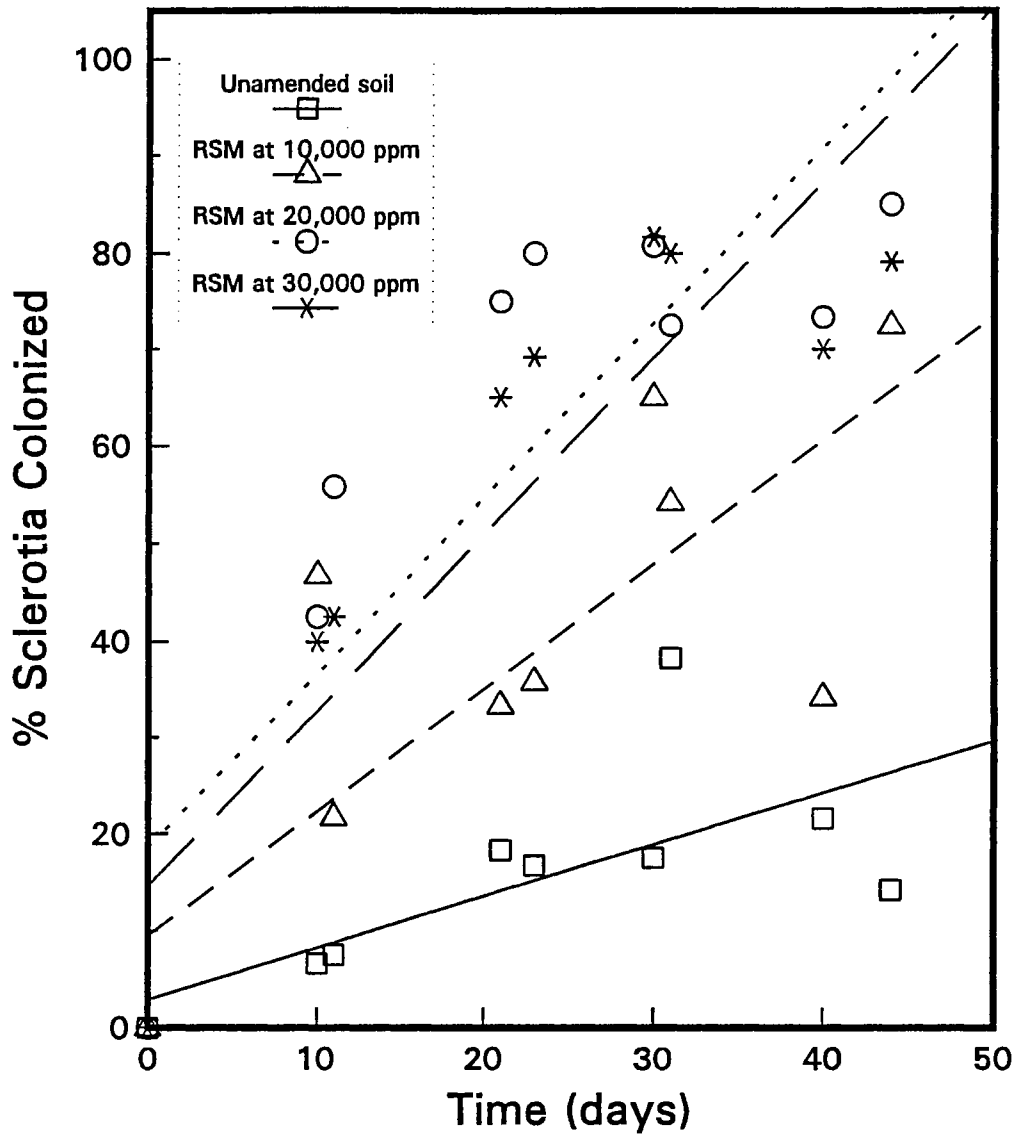

Figure 5. Effect of incubation time on the colonization of sclerotia of *S. minor* in soil amended with different rates of rapeseed meal (RSM). Equations of the lines are: $y=2.86+0.53x$, $y=9.48+1.28x$, $y=18.56+1.81x$, and $y=14.57+1.82x$, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.16, 0.51, 0.68 and 0.70, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Slopes for the lines are all significantly different from ($P=0.05$) each other except for lines for soil amended with RSM at 20,000 and 30,000 ppm.

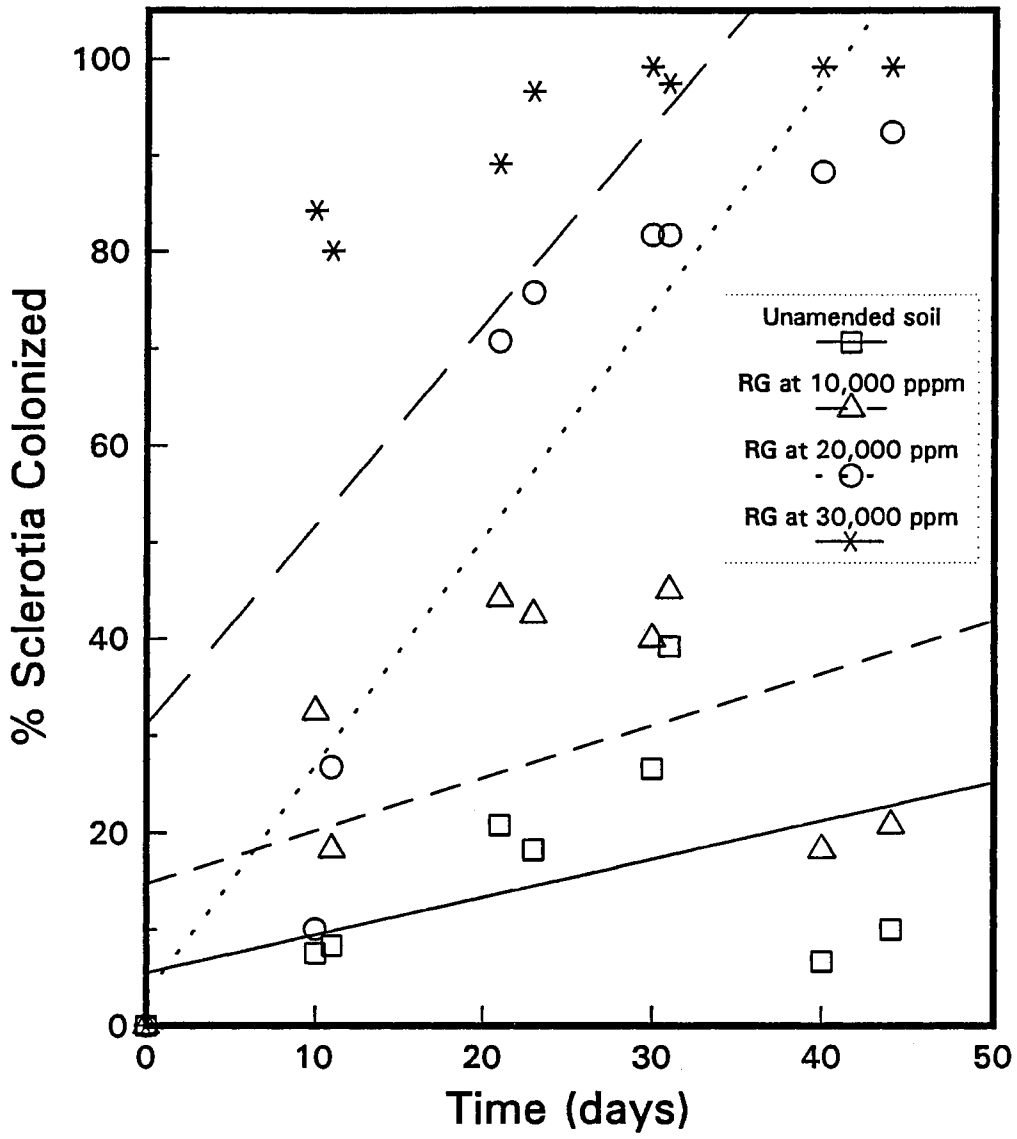

Figure 6. Effect of incubation time on the colonization of sclerotia of *S. minor* in soil amended with different rates of rape greens (RG). Equations of the lines are: y=5.46+0.33x, y=14.77+0.54x, y=3.26+2.26x, and y=31.1+2.07x, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.18, 0.09, 0.78 and 0.63, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Slopes for the lines are all significantly different (P=0.05) from each other except for lines for soil amended with RG at 20,000 and 30,000 ppm.

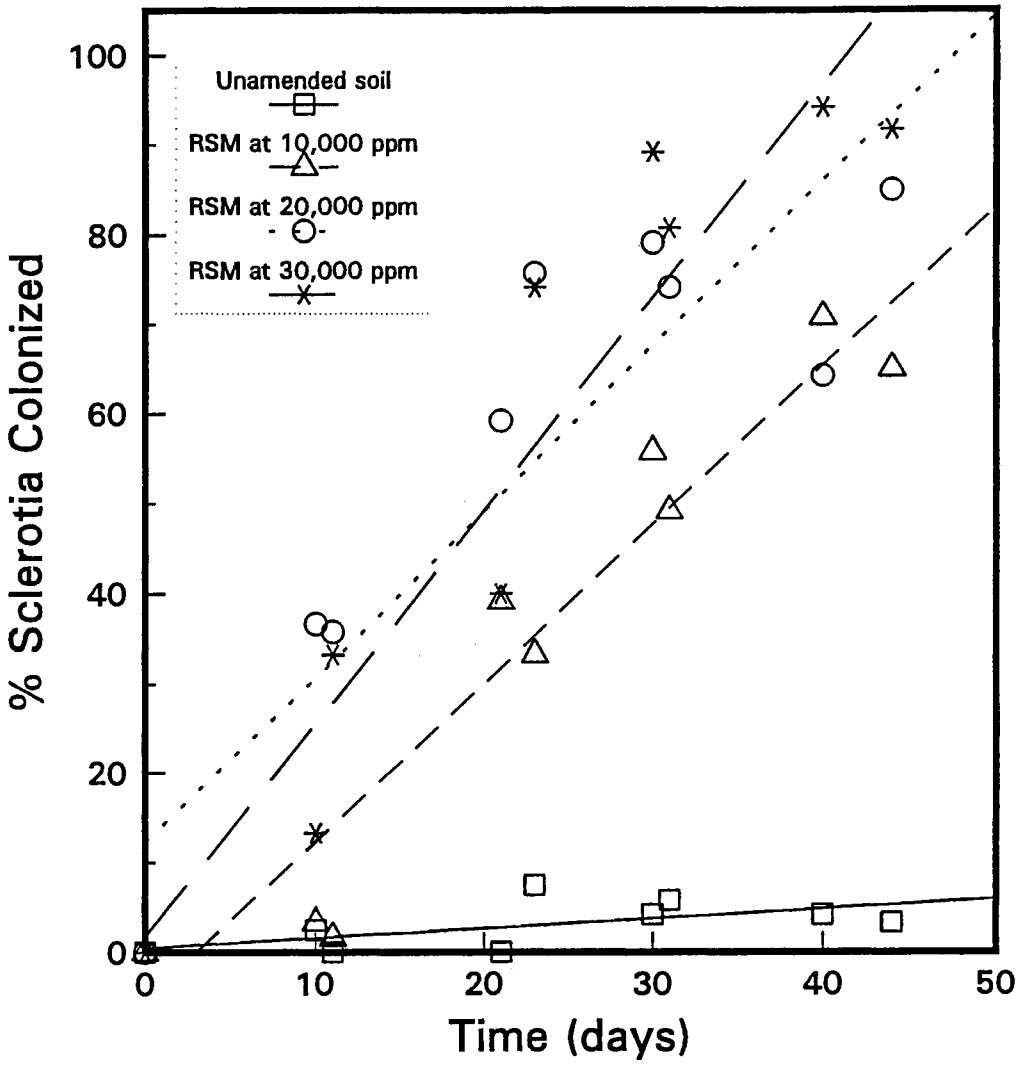

Figure 7. Effect of incubation time on the colonization of sclerotia of *S. rolfsii* in soil amended with different rates of rapeseed meal (RSM). Equations of the lines are: $y=0.45+0.11x$, $y=-5.27+1.77x$, $y=12.26+1.84x$, and $y=1.75+2.38x$, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.11, 0.57, 0.63 and 0.81, for soil amended with RSM at 0, 10,000, 20,000, and 30,000 ppm, respectively. Only the slope for unamended soil is significantly different from ($P=0.05$) that of all others.

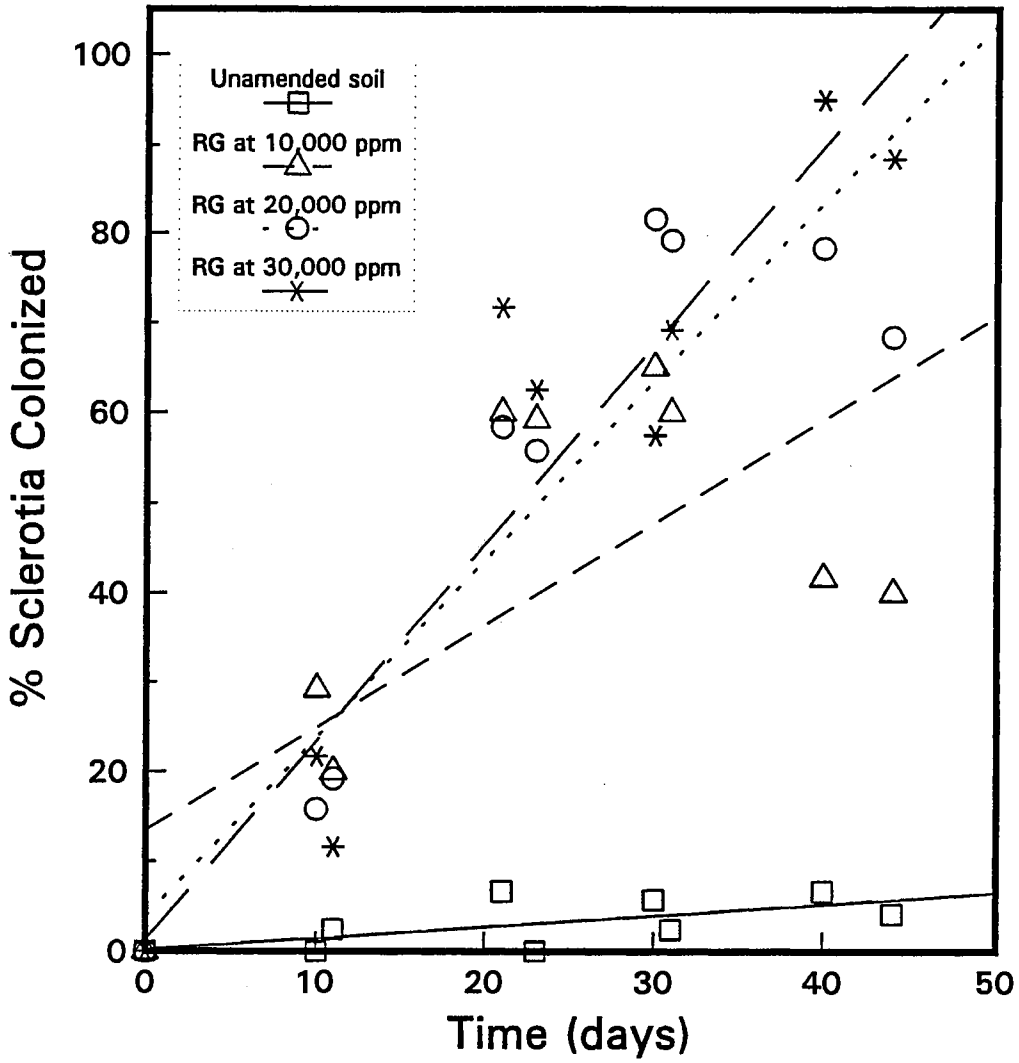

Figure 8. Effect of incubation time on the colonization of sclerotia of *S. rolfsii* in soil amended with different rates of rape greens (RG). Equations of the lines are: $y=0.18+0.13x$, $y=13.44+1.15x$, $y=3.89+1.98x$, and $y=1.3+2.21x$, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Linear regression lines have r^2 values of 0.23, 0.29, 0.79 and 0.79, for soil amended with RG at 0, 10,000, 20,000, and 30,000 ppm, respectively. Only the slope for unamended soil is significantly different from ($P=0.05$) that of all others.

Table 1. Minimum inhibition concentrations for the filtrates of microorganisms which produced antibiotics against either *S. minor* or *S. rolfsii*.

Antagonistic microorganism	Minimum Inhibition Concentration (MIC)***	
	S. minor	*S. rolfsii*
Bacillus m11*	0.2**	0.4
Bacillus m12	0.4	0.4
Bacillus m14	0.1	0.2
Bacillus r9	0.1	0.2
Gliocladium spp. (YE)	0.1	0.4

* Isolates m11, m12 and r9 were identified as *Bacillus subtilis*. Isolate m14 was identified as *B. megaterium*.

** All the data points represent means of two tests. Each test had four replicates.

*** The concentrations indicate the proportion of filtrate in the total volume of the PDA medium.

CHAPTER III

CHARACTERIZATIONS OF ISOLATES OF *SCLEROTINIA MINOR* FROM PEANUT IN OKLAHOMA

ABSTRACT

Sixty-two isolates of *Sclerotinia minor* collected from peanut grown in four counties in Oklahoma were evaluated for growth on potato dextrose agar (PDA) and pathogenicity on detached Romaine lettuce leaves. All isolates, except one (subisolate 9M-N), formed colonies on PDA with an average colony diameter of 7.5 cm after two days at 22±1 C, and formed black sclerotia. The colony diameter of 9M-N was 4.2 cm under the same conditions. All isolates were pathogenic on Romaine lettuce leaves, and formed lesions with an average size of 15.8 cm^2 after two days of incubation at 22±1 C. However, lesions on lettuce leaves caused by subisolate 9M-N were significantly smaller than that of the other isolates, averaging 6.3 cm^2 under the same conditions. Sclerotia were formed by all isolates on lesions on lettuce leaves except 9M-N. Ten isolates, randomly selected, were further evaluated for pathogenicity on cv. Okrun, a sclerotinia-susceptible peanut. All isolates except 9M-N were pathogenic on Okrun. Oxalic acid (OA) production of these ten isolates was measured using a quantitative enzymatic oxalate assay (Sigma Diagnostics, St. Louis,

MO). All isolates tested produced OA in potato dextrose broth, on lettuce leaves and on peanut stems. Subisolate 9M-N produced similar amounts of OA as other more virulent isolates. These data suggest that OA is not the sole pathogenicity factor in *S. minor* on either peanut or lettuce.

INTRODUCTION

Sclerotinia minor Jagger is a pathogen with a broad host range that includes lettuce (*Lactuca* spp.) and peanut (*Arachis hypogaea* L.) in the United States (5, 15, 23). In Oklahoma, sclerotinia blight of peanut has become a widespread disease causing annual yield losses in excess of 10% (15, 22). **S. minor** is able to survive in soil as sclerotia for several years in the absence of host plants (23). It characteristically forms numerous, small (0.5-2 mm in dia), black sclerotia over the entire surface of mature colonies.

Diseases caused by this pathogen are difficult to control with chemicals and/or cultural practices (2, 15). Resistant or tolerant cultivars are not available for most crops. In peanut, cultivars Tamspan 90, Va 93B, and Southwest Runner possess partial resistance to *S. minor* (4, 15, 20).

Pathogenecity in *Sclerotinia* spp. is complex and not well understood. Oxalic acid (OA) has been found to be related to pathogenecity of several fungal pathogens and has been documented as an important factor in the pathogenesis of *Sclerotinia* spp. (7, 8, 9, 13, 14, 16, 17, 21). Disease incidence and severity of sclerotinia blight varies between field locations in Oklahoma. Reasons for this variation may be due to

differences in sclerotial density and distribution of the pathogen, and pathogenic variability among isolates of *S. minor*. No previous research addressed the variability in growth, OA production, and pathogenicity among isolates of *S. minor* from different locations in Oklahoma. Therefore the objective of this study was to investigate the variability among isolates of *S. minor* in regards to: 1) growth on a nutrient medium, 2) virulence on detached Romaine lettuce leaves, 3) pathogenicity on peanut, and 4) production of OA in nutrient medium, and on infected lettuce and peanut tissues. Brief reports of this research were presented earlier (10, 11).

MATERIALS AND METHODS

Collection of isolates. Sixty-one isolates of *Sclerotinia minor* were collected from four counties in Oklahoma (Caddo, Hughes, Payne, and Pottawatomie) (Table 1). Each isolate originated from a sclerotium collected from one diseased peanut plant in the field. Sclerotia were surface disinfected for two minutes in an aqueous solution containing 0.5% sodium hypochlorite and 10% ethanol, then plated on potato dextrose agar (PDA). Sclerotia of one isolate, 9M, formed white sectors on PDA, void of sclerotia. After several transfers, two subisolates, 9M-S and 9M-N, were obtained (S = sclerotial forming; N = nonsclerotial forming). All isolates were maintained on PDA. No sectors were observed in the other isolates collected. Sclerotia formed by the original isolates on PDA were collected and stored at 22±1 C in a desiccator containing anhydrous calcium sulfate.

Growth of isolates on nutrient agar. Mycelial plugs (0.17 cm^2) from the

edge of a two-day-old colony of each isolate of *S. minor* were each transferred to four separate 15-cm petri plates containing PDA. Colony diameters were measured after 46 hours at 22±1 C. This test was performed twice.

Virulence of *S. minor* on detached Romaine lettuce leaves. This test is similar to that used by Boland *et al.* (1). Romaine lettuce leaves were washed in cold running tap water and then blot-dried. Square segments (about 25 cm^2) from the distal end of the leaves were excised. Mycelial plugs (0.17 cm^2) from the edge of a 2-day-old colony on PDA of each isolate were inoculated onto the centers of four leaf segments. Each inoculated leaf segment was placed in a 15-cm petri plate containing a 9-cm Whatman No. 1 filter paper to which 1.5 ml of deionized water was added. The lesions that formed on the lettuce leaves were excised after 46 hours of incubation at 22±1 C under 12 hr of alternating dark and indirect light (5 $\mu E\ m^{-2}s^{-1}$). Lesion areas were measured using an area meter (Delta-T Devices, Oxford, England). This test was performed twice.

Pathogenicity of *S. minor* on peanut. To prepare the fungal inoculum, 100 ml of potato dextrose broth (PDB) in 250 ml flask were inoculated with four mycelial plugs (0.6 cm dia) from the edge of a 2-day-old colony of each of ten isolates. After 3 days of incubation on a rotary shaker (200 rpm) at 22±1 C, the mycelial mats were collected by filtration through Whatman No. 3 filter paper. For every 2 g of fresh mycelia, 100 ml of water was added, and the mycelia were fragmented using a Tissumizer (Tekmar Company, Cincinnati, OH) for 30 sec to make the inoculum.

Four to six-week-old peanut plants (cv. Okrun, grown in 10 cm dia pots

containing 1V of soil, 1V of peat moss and 2V of sand) were used in the pathogenicity tests. Ten ml of the prepared inoculum was applied to the soil around the crown area of each plant. For 48 hr after inoculation, plants were maintained in a dew chamber (Percival, Boone, Iowa) at 20 C at 100% relative humidity (RH) in darkness. Thereafter, the conditions in the dew chamber were maintained at 100% RH at 20 C in the dark cycle (16 hr), and at 70% RH at 25 C in the light cycle (55 μE $m^{-2}s^{-1}$; 8 hr). Plants were removed from the dew chamber after two weeks, and the number of dead plants was recorded. Uninoculated plants were used as control. This test was performed twice. In the first test, isolates C, 9M-N, and 9M-S were tested. Sixteen plants (representing 4 replicates) were inoculated by each isolate. In the second test, ten isolates were used. Eight plants (representing 4 replicates) were inoculated by each isolate.

Quantification of oxalic acid. Production of oxalic acid (OA) by isolates of *S. minor* in PDB, on lettuce leaves, and on peanut stems was determined using an enzymatic analysis test kit (Sigma Diagnostics, Sigma Chemical Co., St. Louis, MO). Solutions with known concentrations of OA were used as standards. The liquid samples were diluted accordingly to obtain assay values within the linear range of the standards. The amount of OA produced was expressed in terms of OA per gram dry weight of material. This test was performed twice.

Production of OA in PDB was determined as follows: One hundred ml of PDB in a 250 ml flask was inoculated with a mycelial plug (1.48 cm^2) from the edge of a 2-day-old colony of *S. minor*. Four replicate flasks were used for each isolate.

Flasks were incubated on a rotary shaker (200 rpm) at 22±1 C. After 3 days, the mycelial mats were removed by filtration through Whatman No. 3 filter paper. The filtrate was saved for the quantification of OA. The mycelial mats were dried in an oven at 70 C for 24 hr, and the weights were recorded. The production of OA was expressed as mg OA per g dry weight of mycelia. The production of OA by three isolates, C, 9M-S and 9M-N, was also measured over an 8-day period using the same procedure. PDB inoculated with PDA plug was used as blank control.

Production of OA on Romaine lettuce was determined as follows: Romaine lettuce leaf segments were inoculated with fresh mycelial plugs (0.17 cm^2) from the edge of a 2-day old culture of *S. minor* grown on PDA. Four leaf segments were inoculated per isolate. Inoculated leaf segments were incubated as stated previously. Two days after inoculation, two 1.48 cm^2 sections were taken from each lesion, using a cork-borer cylinder, and 1 ml of deionized water was added. The mixture was then vortexed, frozen, thawed and vortexed again, and centrifuged at 10,000 g for 10 min. One half ml of the supernatant was taken to determined the amount of OA. Leaf material left in the centrifuge tube was dried and the weight was recorded. The production of OA was expressed as mg OA per g dry weight of lettuce leaf tissue. Healthy leaves inoculated with PDA plugs, without the fungus, were used as blank control.

Production of OA on peanut stems was determined as follows: Six-week old peanut plants (cv. Okrun, grown in 10 cm dia pots containing 1V of soil, 1V of peat moss and 2V of sand) were used in the test. Central stems of four peanut plants were

inoculated in the middle with mycelial plugs (0.17 cm^2) from the edge of a 2-day-old culture of *S. minor* grown on PDA. Plants were incubated in the dew chamber under the same conditions stated previously in the pathogenicity test. Isolate 9M-N formed a superficial small lesion <0.5 cm long on the surface of the inoculated stem, while other isolates formed lesions >2.0 cm long which girdled the stems after 3 days of incubation in the dew chamber. The lesion formed on the stem was excised and 1 ml of deionized water was added after three days. The mixture was then vortexed, frozen, thawed and vortexed again, and centrifuged at 10,000 g for 10 min. The amount of OA was determined in 0.5 ml of supernatant. Stem material left in the centrifuge tube was dried and the dry weight was determined. The production of OA was expressed as mg OA per g dry weight of peanut stem tissue. Healthy stems inoculated with PDA plugs, without the fungus, were used as blank control.

All the data were analyzed with analysis of variance using SAS (SAS Institute, Cary, NC) and the figures were done on Freelance (Version 4.0, Lotus Development Corporation, Redwood City, CA). Fisher's protected least significant difference (LSD) test and/or Tukey's test were used for mean separation. Only significant ($P=0.05$) data are discussed unless stated otherwise.

RESULTS

Growth of isolates of *S. minor* on PDA. All isolates, except subisolate 9M-N, formed similar sized colonies averaging 7.5 cm in dia after two days of incubation at 22 ± 1 C (Table 2). Subisolate 9M-N formed smaller colonies averaging 4.2 cm in

dia under the same conditions (Table 2). Furthermore, all except 9M-N formed sclerotia on PDA after 5 days of incubation at 22±1 C. Subisolate 9M-N did not form sclerotia on PDA.

Virulence of isolates of *S. minor* on Romaine lettuce leaves. All isolates produced water-soaking lesions 46 hr after incubation at 22±1 C (Table 3). The sizes of the lesions formed by subisolate 9M-N averaging 6.3 cm^2 were smaller than those of the other isolates averaging 15.8 cm^2 (Table 3). All the isolates, except 9M-N, formed sclerotia on detached lettuce leaves after 5 days of incubation at 22±1 C. Subisolate 9M-N produced white fluffy mycelia throughout the lesions on the lettuce leaves.

Pathogenicity of isolates of *S. minor* on Okrun peanut. All isolates, except 9M-N, caused typical blight symptoms and death on inoculated plants after 7 days of incubation in the dew chamber (Table 4). Pathogenic isolates exhibited typical sclerotinia blight symptoms as flagging and wilting on Okrun peanut after three or four days of incubation in the dew chamber.

Production of oxalic acid by isolates of *S. minor* in PDB, on lettuce leaves and on peanut stems. Production of OA by C, 9M-S and 9M-N in PDB over time is shown in Figure 1. Production of OA by 9M-N in PDB followed a similar trend as 9M-S and C did (Fig. 1). The production of OA by ten isolates in PDB, on lettuce leaves and on peanut stems, respectively, is shown in Figures 2, 3 and 4. Subisolate 9M-N produced similar amount of OA as some other isolates did in PDB, on lettuce leaves and on peanut stems. Subisolate 9M-N produced 240 mg OA per g of dry

mycelia in PDB after three days of incubation (Figure 2). Subisolate 9M-N also produced 18.8 mg of OA per g dry weight of lettuce leaf tissue after two days (Figure 3), and 1.9 mg of OA per g dry weight of peanut stem tissue after 3 days of incubation (Figure 4).

DISCUSSION

The results presented in this study provide an in-depth examination and characterization of isolates of *Sclerotinia minor* collected from peanut in Oklahoma. Woodard and Simpson characterized five isolates of *S. minor* collected from diseased peanut plants in Texas, and all isolates showed similar growth and sclerotial production under different cultural conditions (24). The isolates investigated here were similar in terms of growth on PDA, pathogenicity on detached Romaine lettuce leaves, and pathogenicity on peanut cv. Okrun. Only one subisolate, 9M-N, which was obtained from white sectors produced by isolate 9M, was significantly different from all the others in these aspects. It formed significantly smaller colonies on PDA, and it formed significantly smaller lesions on lettuce leaves than the other isolates. Subisolate 9M-N did not cause disease on Okrun peanut, and it did not form sclerotia on either PDA or on lettuce leaves (10).

Cultures originated from sclerotia of isolate 9M frequently exhibited white sectors void of sclerotia. *S. minor* does not usually sector on plates as some other fungi. It was found out later that the reason why these sclerotia sectored was because the sclerotial forming isolates were able to entrap the 9M-N mycelia into their

sclerotia (12). Mycelia of subisolate 9M-N may be able to survive in the sclerotia of other isolates of *S. minor* in field soils. Subisolate 9M-N probably was a mutant which occurred in the field and survived in sclerotia of isolate 9M.

The vegetative growth of three isolates of *S. minor* was investigated further in PDB where dry weight of the mycelial mats were compared. It was shown that subisolate 9M-N actually had a similar vegetative growth ability as isolates C and 9M-S (Figure 5). Subisolate 9M-N grows slower on PDA probably because it forms fluffier aerial mycelia on PDA plates than other isolates. Although it forms similar amount of mycelia as other isolates, its colony occupies less area than others on the plate.

Use of oxalic acid (OA) as a selective agent to obtain resistant germ plasm has been proposed by several researchers (6, 18, 19). But there are still disagreements about the role of OA in *Sclerotinia* spp. Godoy *et al.* used mutants of *Sclerotinia sclerotiorum* specifically deficient in OA production to demonstrate that it is a pathogenicity determinant on *Phaseolus vulgaris* (6). But Callahan and Rowe reported later that OA is not the sole pathogenicity determinant in the exudate of *Sclerotinia trifoliorum* on alfalfa (3). From the results of our study, since subisolate 9M-N produced a similar amount of OA as the pathogenic isolates on diseased tissues, and it is nonpathogenic on peanut and less virulent on lettuce, OA can not be functioning as the sole pathogenicity factor for *S. minor* on either lettuce or peanut (11). These findings disagree with the conclusions drawn by other investigators about the role of OA in the pathogenicity of *Sclerotinia* (6). In the studies by Godoy *et al.*,

they concluded that OA is a pathogenecity determinant in *S. sclerotiorum* on beans by using UV-induced OA-minus mutants (6). Further, they found that their OA-minus mutants do not produce sclerotia (6). Since 9M-N does produce OA and does not produce sclerotia, we suspect that genes involved with development of sclerotia in *S. minor* may be more likely be related to pathogenicity.

Oxalic acid probably contributes to some of the symptoms such as water-soaking, flagging and wilting in diseases caused by *S. minor*, because it is a strong chelator of divalent cations (14). OA facilitates the lytic enzyme function which induces water-soaking and wilting.

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Table 1. Isolates of *S. minor* obtained from four counties in Oklahoma.

Isolates Designation	Total	County of Origin
1*A, 1B, 2A, 2B, 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3I, 3J, 4A, 4B, 4C, 5A, 5B, 5C, 5E, 9C, 9E, 9F, 9G, 9M, 10D, 11A, 11C, 11D, 11E, 14A, 14C, 14D, 14E O, W	36	Caddo
6A, 6B, 6C, 6E, 6F, 6G	6	Hughes
8A, 8B, 8C, 12C, 12D, 13A, 13B, 13D	8	Pottawatomie
7A, 7B, 7C, 7D, 15B, 15C, 15D, 15E, 15F, T90, C	11	Payne

* Numbers refer to individual field plot.

Table 2. Growth of isolates of *S. minor* on potato dextrose agar after 46 hours at 22±1 C.

Isolate	Colony diameter* (cm) Test 1	Test 2	Mean**
All others***	8.1	7.9	8.0 a
9M-S	6.9	7.3	7.1 a
9M-N	4.2	4.2	4.2 b
Minimum significant difference$_{0.05}$			1.9

* The colony diameter is the average of four different plates.

** Mean of the two tests. Values followed by the same letter are not significantly different (P=0.05) according to Tukey's test. Isolate effect was determined by an F test on a one-way ANOVA.

*** The data of all other isolates represents the other 60 isolates; there was no significant difference between the 60 isolates.

Table 3. Virulence of isolates of *S. minor* on detached Romaine lettuce leaves after 46 hours at 22±1 C.

Isolate	Lesion size* (cm^2) Test 1	Test 2	Mean**
All others***	16.8	19.6	18.2 a
9M-S	14.4	13.1	13.7 a
11E	13.4	12.1	12.8 ab
6E	11.4	13.1	12.3 ab
15C	12.0	12.0	12.0 ab
9M-N	6.4	6.2	6.3 b
Minimum significant difference$_{0.05}$			6.6

* The lesion size is an average of four lesions on four different lettuce leaf segments.

** Mean of the two tests. Values followed by the same letter are not significantly different (P=0.05) according to Tukey's test. Isolate effect was determined by an F test on a one-way ANOVA. Value of 9M-N is significantly different from all the other values according to LSD test ($LSD_{0.05}$=0.9).

*** The data of all other isolates represents 57 the other isolates; there was no significant difference between the 57 isolates.

Table 4. Pathogenicity of 10 isolates of *S. minor* on Okrun peanut plants.

Isolate	Test #1 Percentage of Dead Plants*	Test #2 Percentage of Dead Plants*
9M-N	0 a	0 a
9M-S	100 b	100 b
C	100 b	100 b
3D		87.5 b
11E		87.5 b
2B		100 b
3J		100 b
6C		100 b
5B		100 b
7C		100 b
$LSD_{0.05}$	0	16.2

* Values followed by the same letter are not significant different (P=0.05) according to LSD test. Isolate effect was determined by an F test on a one-way ANOVA. In test #1, the numbers are the averages of four replications with four plants in each replicate. In test #2, the numbers are the averages of four replicates with two plants in each replicate.

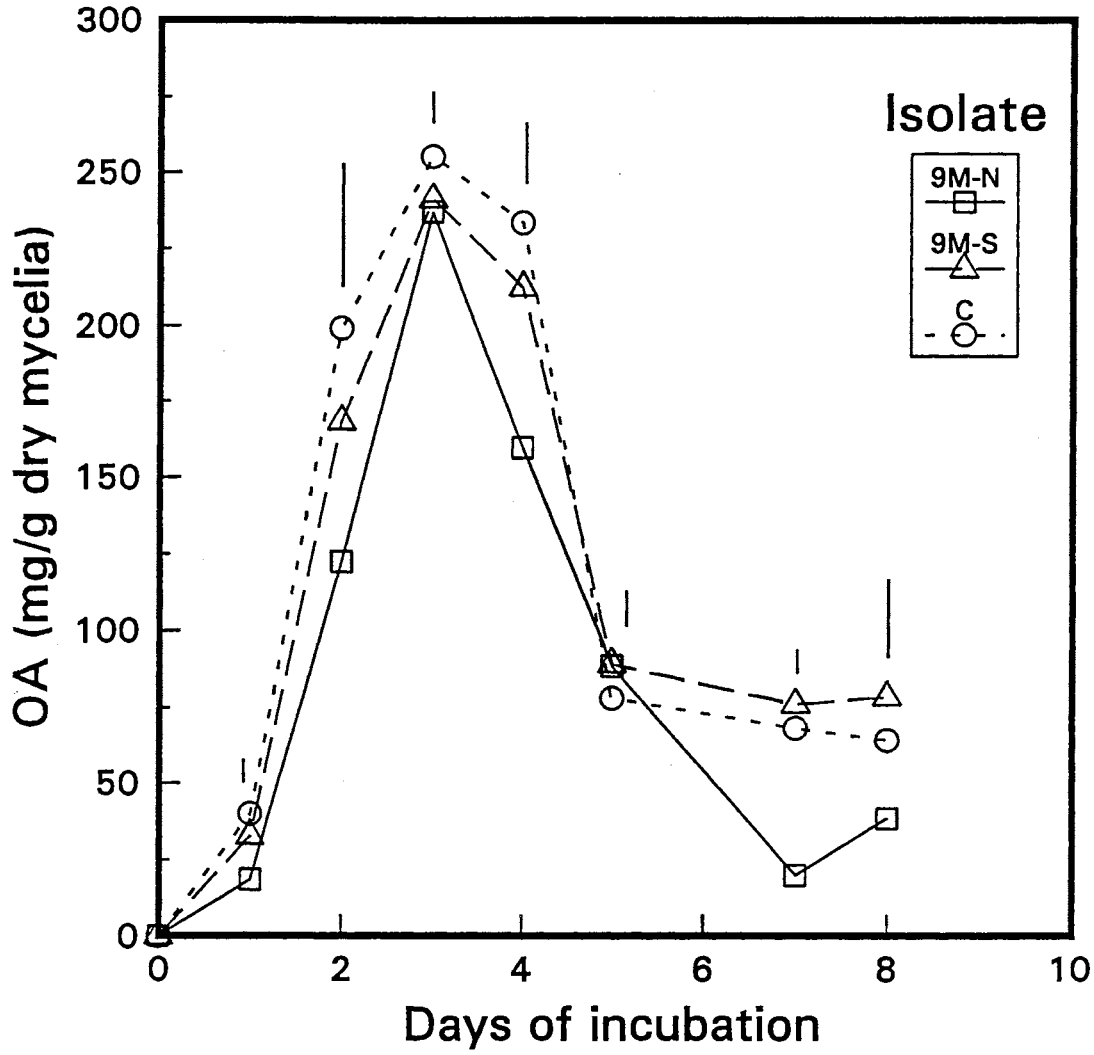

Figure 1. Production of oxalic acid (OA) by three isolates of *S. minor* in potato dextrose broth. Vertical lines above the data points represent the $LSD_{0.05}$ values of the data at different days of incubation. The $LSD_{0.05}$ values are 0, 6.3, 41.8, 11.6, 21.1, 21.8, 8.4, and 27 for OA production after 0, 1, 2, 3, 4, 5, 7, and 8 days of incubation, respectively.

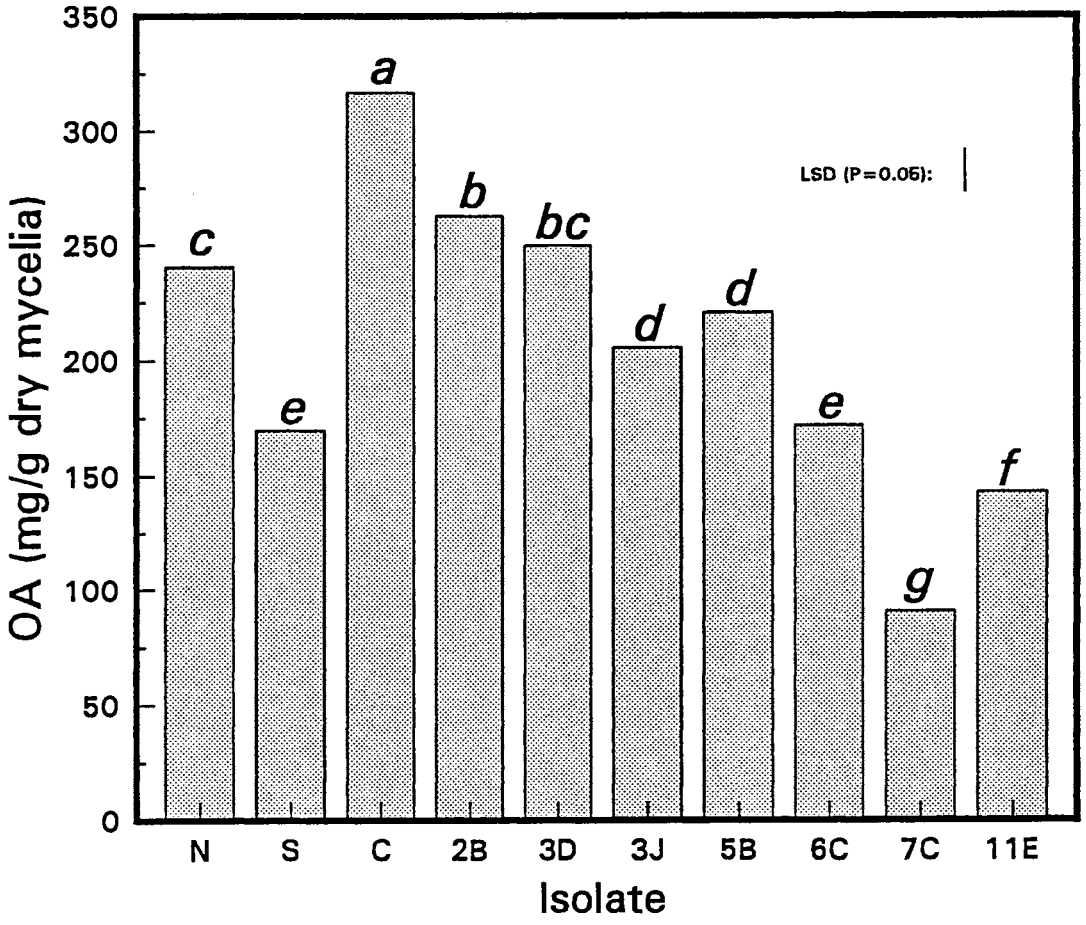

Figure 2. Oxalic acid (OA) production by 10 isolates of *S. minor* in potato dextrose broth. The vertical line above the bars indicates the $LSD_{0.05}$ ($LSD_{0.05}$=16.6). The data represent the means of two tests. Data in bars with the same letters do not differ significantly (P=0.05) as determined by LSD test.

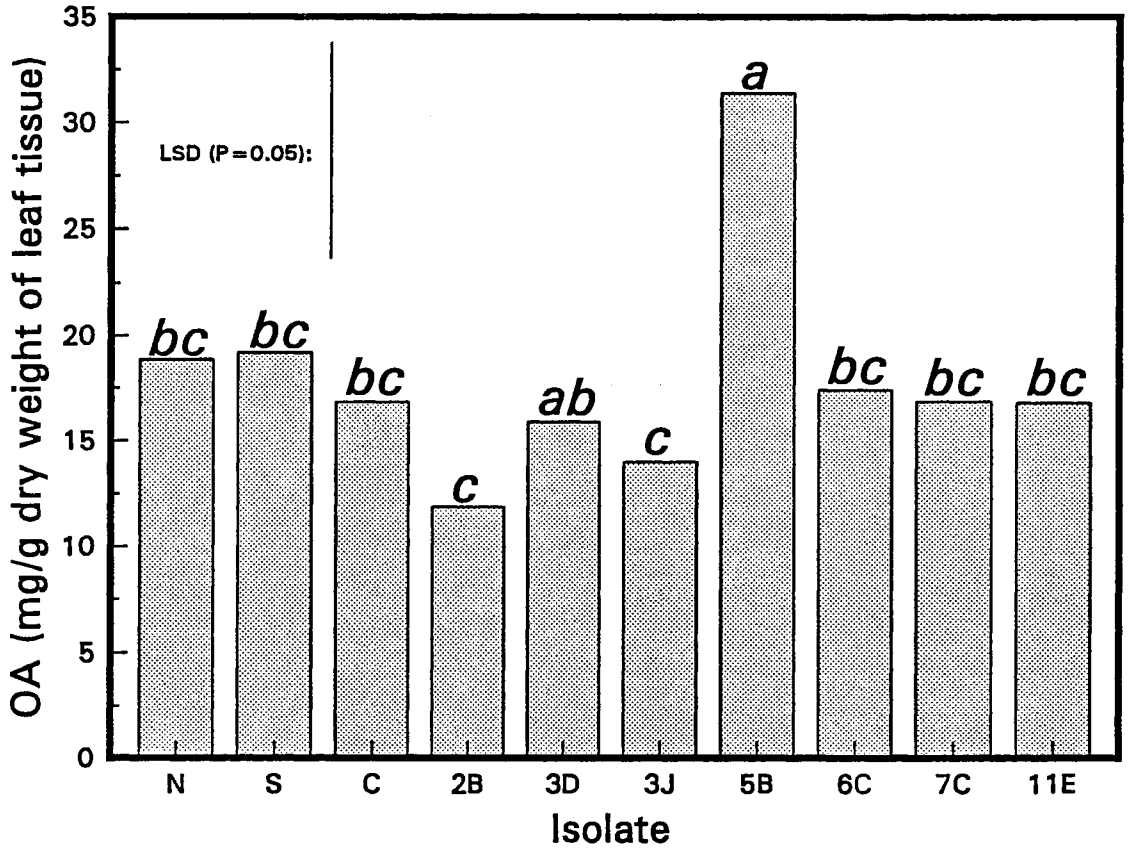

Figure 3. Oxalic acid (OA) production by 10 isolates of *S. minor* on detached Romaine lettuce leaves. The vertical line above the bars indicates the $LSD_{0.05}$ ($LSD_{0.05}$=10.5). Data represent the means of two tests. Data in bars with the same letters do not differ significantly (P=0.05) as determined by LSD test.

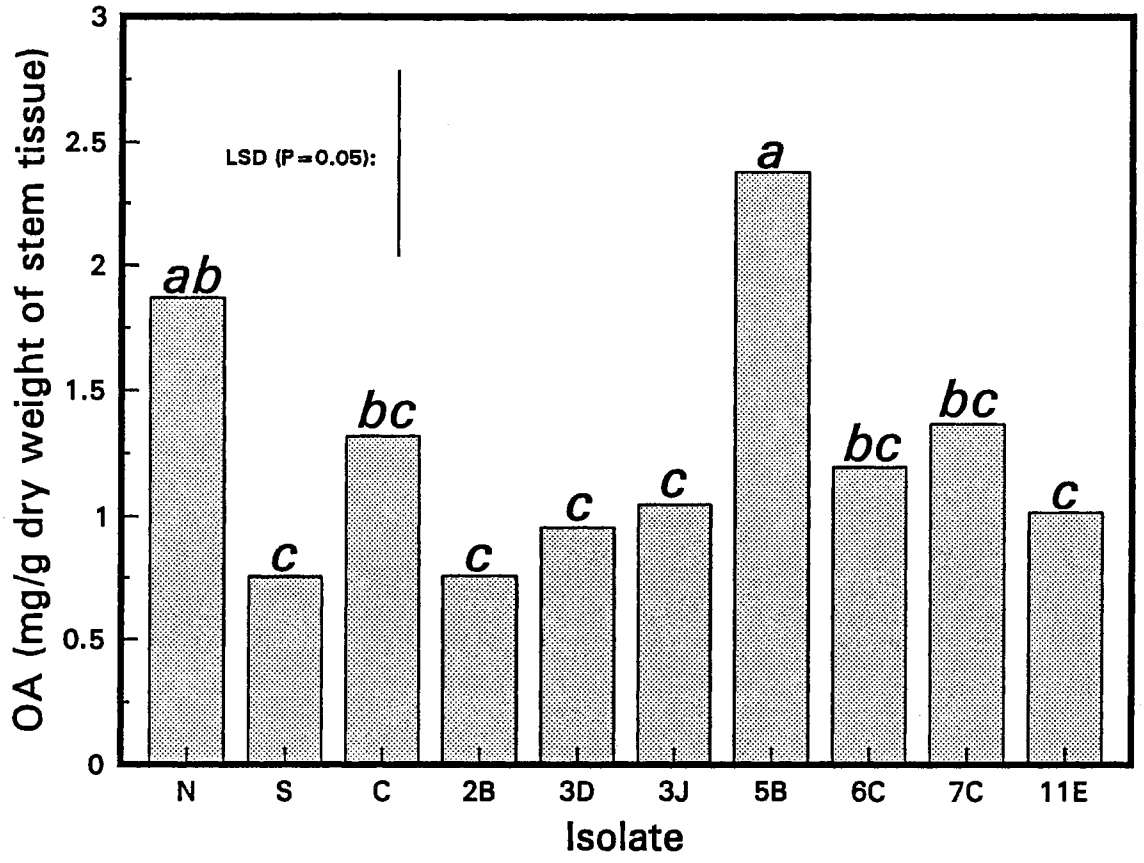

Figure 4. Oxalic acid (OA) production by 10 isolates of *S. minor* on peanut cv. Okrun stems. The vertical line above the bars indicates the $LSD_{0.05}$ ($LSD_{0.05}$=0.7). Data represent the means of two tests. Data in bars with the same letters do not differ significantly (P=0.05) as determined by LSD test.

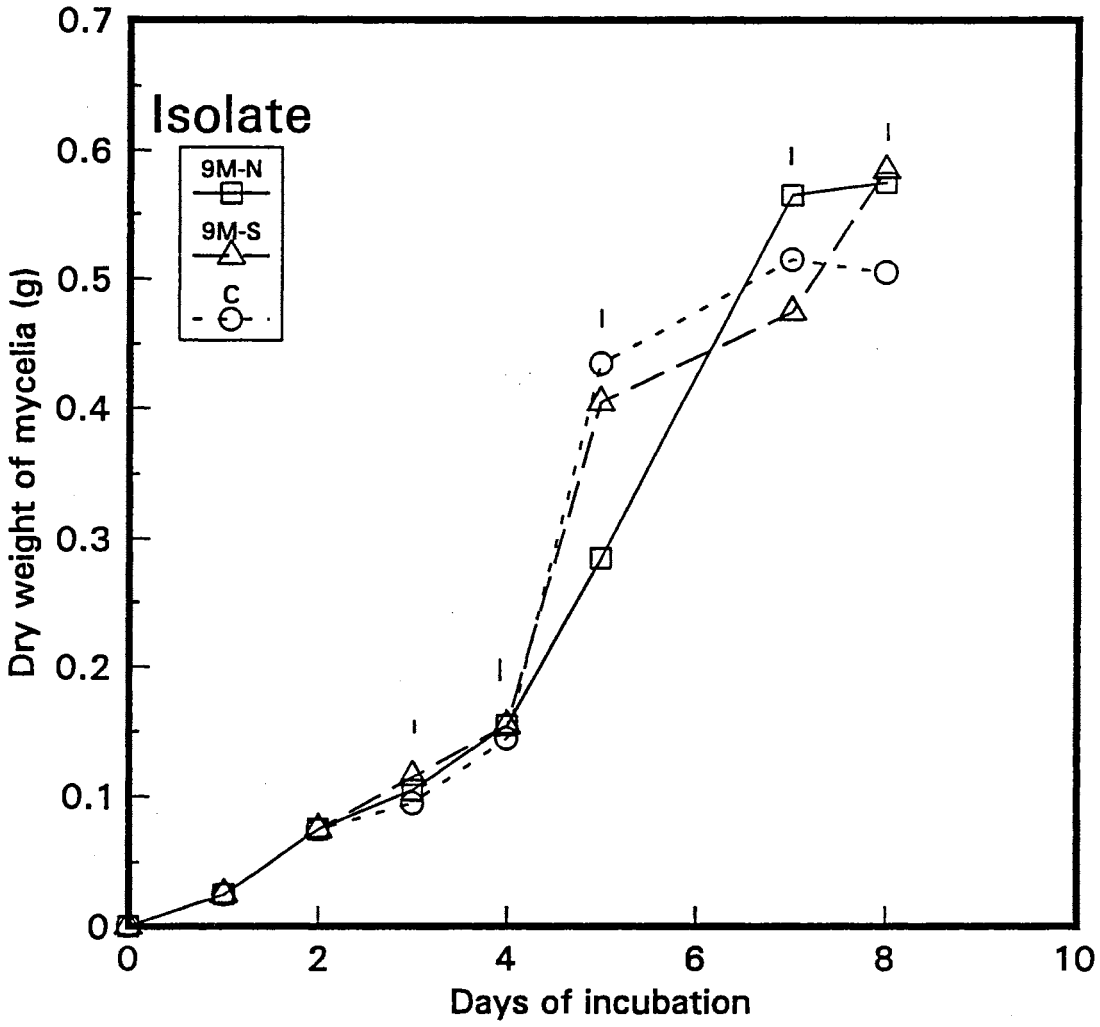

Figure 5. Growth of three isolates of *S. minor* in potato dextrose broth. Vertical lines above the data points represent the $LSD_{0.05}$ values of the data at different days of incubation. The $LSD_{0.05}$ values are 0, 0, 0, 0.009, 0.016, 0.01, 0.011, and 0.011 for dry weight of mycelia after 0, 1, 2, 3, 4, 5, 7, and 8 days of incubation, respectively.

CHAPTER IV

FORMATION OF SCLEROTIA OF *SCLEROTINIA MINOR* IN MIXED CULTURES

ABSTRACT

A subisolate of *Sclerotinia minor* (9M-N) which does not form sclerotia, was grown in potato dextrose broth together with isolate C, which does form sclerotia. Sclerotia formed were collected after 14 days of incubation on a rotary shaker at 200 rpm at 22±1 C. Ninety-seven percent of the sclerotia produced mycelial growth on potato dextrose agar (PDA) with white sectors that were void of sclerotia. The two isolates in paired cultures on PDA exhibited some incompatibility where mycelial growth was scant in the middle of the plates. No sclerotia formed in the 9M-N section of the plates after two weeks of incubation at 22±1 C, while sclerotia were formed in the C section of the plate. Almost all the sclerotia formed by isolate C that were adjacent to the barrier line between the two isolates formed sectors void of sclerotia when cultured. Fifty-seven percent of sclerotia within 0.5 cm of the barrier line also formed sectors void of sclerotia. None of the sclerotia formed more than 0.5 cm from the barrier line produced sectors. Tests were repeated using sclerotial forming subisolate 9M-S instead of C; similar results were obtained. These results suggest that during sclerotial formation, pseudoparenchymatous tissue of isolates C

and 9M-S can entrap mycelia of 9M-N. The results also suggest that mycelium of 9M-N is able to survive in the sclerotia of isolate C or 9M-S. However, transfer of a nonsclerotial forming factor did not occur through anastomosis because sclerotia of isolate C and 9M-S formed away from 9M-N did not produce sectors. These results were confirmed by curing tests using cycloheximide-amended PDA. No evidence of involvement of extrachromosomal particles with this trait was found. Sclerotia which could form 9M-N sectors were able to produce ascospores which would grow to form colonies similar to that of 9M-N without sclerotia. No extrachromosomal genetic materials were detected from isolates C, 9M-S, or 9M-N by direct nucleic acid extraction.

INTRODUCTION

Sclerotinia minor Jagger is an ascomycete which causes diseases on a broad array of host plants of economical importance worldwide (7, 11, 16). It causes disease on peanut in diverse regions of the United States (11, 13, 16). Although *S. minor* forms apothecia when conditions are favorable, the sexual stage of this pathogen is not important in the disease cycle on peanut (16). *S. minor* characteristically forms numerous small (0.5-2 mm dia) black sclerotia, similar in size, over the entire surface of mature colonies (7, 18).

Sclerotia of an isolate of *S. minor* (9M) produced white sectors when grown on potato dextrose agar (PDA). Subisolate 9M-N was originated from white sectors of isolate 9M through subcultures of mycelial fragments on PDA (8). Subisolate 9M-

N does not form sclerotia, does not cause disease on peanut and is less virulent on detached Romaine lettuce leaves (8). Because sclerotia of *S. minor* usually do not sector on plates, the nature of the nonsclerotial forming sectors of 9M was questioned. At least three hypothesis could explain the observed sectoring. The first is natural mutation. Secondly, extrachromosomal nucleic acids such as plasmids or double stranded ribonucleic acids (dsRNAs) may be involved. Several fungal plant pathogens including *Sclerotinia sclerotiorum* have been reported to contain mycoviruses, virus-like particles, or dsRNAs that are associated with reduced pathogenecity and/or reduced growth rate and reproductive fitness (2, 3, 4, 12, 15). These extrachromosomal particles move among compatible isolates through anastomosis and spread cytoplasmically (3, 15). Thirdly, physical entrapment of the nonsclerotial forming mycelium into sclerotia could be involved.

The curing test using cycloheximide has been commonly used to detect extrachromosomal particles, especially dsRNAs (2, 14). When cycloheximide is added to culture media, fungi with extrachromosomal particles sector because the speed of replication of chromosomes and extrachromosomal particles is different under the influence of cycloheximide. Thus cultures with or without extrachromosomal particles can be recovered from these plates by observing sectors with different morphology.

Pairing tests are commonly used to detect transmission of dsRNAs between fungal isolates through anastomosis (2). When two isolates of fungus with difference in certain trait are paired on media, they will grow toward each other and anastomosis

will occur between isolates if they are compatible. If transmission of extrachromosomal particles related to certain trait occurs between the two isolates of fungi, mycelium of one isolate showing the trait of the other isolate can be retrieved far away from the merging line between the two isolates.

This study reports on the use of several experimental approaches, that included curing test, pairing test, mixed culture test, culture of single ascospores from different kinds of sclerotia, and direct nucleic acid extraction, for providing information to elucidate the biological basis for the nonsclerotial forming subisolate 9M-N. A brief report of this research was presented earlier (9).

MATERIALS AND METHODS

Isolates of *S. minor*. Three isolates of *Sclerotinia minor* (C, 9M-S and 9M-N) were used in this study. Isolate C is routinely used for greenhouse inoculation of peanut (10). Subisolates 9M-N and 9M-S were from sectors of a field isolate 9M (8). Subisolate 9M-N does not form sclerotia, and is not pathogenic on peanut. Isolates C and 9M-S both form sclerotia, and are pathogenic on peanut (8). The isolates were maintained on potato dextrose agar (PDA).

Curing test. Mycelial agar plugs (0.6 cm dia) taken from the edges of 2-day-old cultures of isolates C, 9M-S or 9M-N were transferred to PDA amended with 13.5 μg/ml of cycloheximide. For each isolate, fifty plates were used. After a week, the center plug with the original inoculum was transferred to a new petri plate containing PDA amended with cycloheximide to enlarge the sectors if any formed.

Cultures of C, 9M-S and 9M-N on PDA were carried out as controls.

Extraction of nucleic acids from fungal mycelia. A mycelial agar plug (0.6 cm dia) from the edge of a two-day-old culture of *S. minor* was inoculated in 100 ml PDB. After one week of incubation at 22±1 C on a rotary shaker at 200 rpm, mycelial mats were collected by vacuum filtration through Whatman No. 3 filter paper. The mycelia were then lyophilized. For each isolate of *S. minor*, 0.2 g of lyophilized mycelia was used to extract nucleic acids according to the methods of Biel and Parrish (1). Lyophilized mycelia were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was then transferred to a 30-ml centrifuge tube and suspended in 5 ml of lysis buffer consisting of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 2% sodium dodecyl sulfate (SDS). Proteinase K (Gibco BRL, MD) was added to the mixture to a final concentration of 250 ng/μl, and the mixture was incubated at 37 C for 1 hr. Five ml of TE-saturated (TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) phenol:chloroform (1:1) was then added to the lysate and mixed thoroughly. This mixture was centrifuged at 15,300 g for 30 minutes and the aqueous supernatant was collected. The aqueous phase was centrifuged for 20 min at 15,000 g at 4 C to remove the polysaccharides. The supernatant was later layered on 5.0 ml column of Sephadex G-50 and eluted with TE buffer. Fractions of 0.5 ml were collected in microcentrifuge tubes. The fifth and the sixth fraction tubes were used for later precipitation according to the nucleic acid concentrations determined by a spectrophotometer at 260 and 280 nm. Nucleic acids were precipitated at room temperature using a mixture of 1M NaAC and isopropanol

(1:10; V/V). After centrifugation at 10,000 g, the pellet was washed with 70% ethanol and dried in a Speed Vac SC100 vacuum dryer (Savant Instruments, Inc., NY). The pellet was later resuspended in 200 μl of distilled water and run on 1% agarose gel. Enzymatic digestion of the samples was conducted with DNase I at 10 μg/ml in 50 mM Tris-HCL, 10 mM $MgCl_2$, and 1 mg/ml BSA, pH 8.0 at 37 C for 60 min. Samples were also digested with RNase at 25 μg/ml in 0.03 M or 0.3 M NaCl at 37 C for 60 min. Nucleic acids that were hydrolysed in the 0.03 M NaCl RNase treatment, but not in the 0.3 M NaCl RNase treatment were considered to be dsRNAs. Mycelia of isolates of *Helminthosporium victoriae* which contained dsRNAs were used as positive control in the extraction procedure.

Mixed liquid culture test. The mixed culture test was carried out to detect the low frequency of transmission of viruses through anastomosis between fungal isolates, in case the number of extrachromosomal particles existing in fungus was too low to detect in curing test and direct nucleic acid extraction. In this test, two isolates, a sclerotial forming isolate and 9M-N, are grown together in the liquid culture. If there is transmission of extrachromosomal particles related to sclerotial formation between isolates, a low frequency of sclerotia with white sectors from a population of sclerotia formed in the liquid culture should be detected as a result of the movement of the extrachromosomal nucleic acids.

Fresh mycelial agar plugs (0.6 cm dia) of isolates C and 9M-N from the edge of 2-day-old cultures were inoculated together in 100 ml potato dextrose broth (PDB). The cultures were incubated on a rotary shaker (200 rpm) at 22$\pm$1 C. After 7 days,

the flasks with their contents were set aside for four weeks to allow sclerotia to form in the liquid culture. The sclerotia that formed were retrieved from the surface of the culture and placed in a desiccator containing anhydrous $CaSO_4$ to allow drying for 10 days. The dried sclerotia were surface sterilized for 10 sec in 70% ethanol and for two minutes in an aqueous solution containing 1% sodium hypochlorite, and 50 of them were plated on PDA. After 7 days at 22±1 C, the central mycelial plug with the germinated sclerotia was transferred to a new PDA plate to enlarge the sectors if any formed. The number of sclerotia showing 9M-N sectors was recorded, and the percentage of viable sclerotia showing 9M-N sectors was calculated. This test was also performed with 9M-N and 9M-S cultured together. Each test was conducted twice and there were six replicates (flasks) in each test. From every replicate, 50 sclerotia were retrieved and plated. Liquid cultures of each of isolates 9M-S, C or 9M-N were carried out as controls.

Pairing test. Mycelial agar plugs (0.6 cm dia) of 9M-N and C were paired on a 15-cm petri plate containing PDA. After 7 days of incubation at 22±1 C, sclerotia were formed by the C colony but not by the 9M-N colony. Sclerotia formed by isolate C were collected from four areas of the colony at different distances from the barrier line between 9M-N and C (Figure 1). The four areas were: adjacent to the 9M-N colony, within 0.5 cm from the barrier line, 2 cm further and the farthest (>2.5 cm from the barrier line). Collected sclerotia were surface sterilized and plated as described in the mixed liquid culture test. The number of sclerotia from the different regions showing 9M-N sectors was recorded, and the percentage of viable

sclerotia showing 9M-N sectors was calculated. Pairing of 9M-S and 9M-N was also carried out. The test was repeated twice. This paring test was also repeated twice using cultures from single ascospore cultures originated from sclerotia produced in paired cultures of isolate 9M-N and C or 9M-S.

Ascospore cultures. To culture the ascospores, apothecia of *S. minor* isolates were induced using methods similar to that of Hawthorne (5). To produce sclerotia of C and 9M-S, a wholewheat agar medium (WWA) containing 2% agar and 5% Pillsbury's whole wheat flour in 15-cm petri plates was inoculated with 0.6 cm dia mycelial plugs of C or 9M-S. To produce sclerotia of C or 9M-S containing 9M-N, WWA in 15-cm petri plates was inoculated with 9M-S and 9M-N or C and 9M-N in a paired fashion. After five weeks of incubation in the dark at 15 C for 8 h and at 10 C for 16 h, sclerotia formed by C or 9M-S were collected. Sclerotia formed by the C or 9M-S colony on the pairing plates next to the 9M-N colony were also collected. Almost all of these sclerotia were able to form 9M-N sectors as shown in the results of the pairing test. These sclerotia were designated as 9M-N+C and 9M-N+9M-S. The sclerotia were air dried for 3 to 5 days and screened on a 20-mesh (0.84 mm opening) sieve. Those that passed through were discarded because stipes formed by them very rarely develop into apothecia (5). The retained sclerotia were surface sterilized as described in the mixed culture test and placed in 15-cm petri plates each containing 20 ml autoclaved tap water. The sclerotia were then incubated for 8 weeks in the dark at a daily regime of 15 C for 8 h and at 10 C for 16 h. After 3 weeks, sclerotia that formed stipes were transferred to another 15-cm petri plate containing

20 ml of sterilized water. Mature apothecia were produced by illuminating the stipes for 8 h daily at 20 C for 14-21 days. Surface of the mature apothecia were smeared on PDA in 9-cm petri plates to transfer the ascospores. After 12 hours of incubation at 22±1 C, each germinating single ascospore was transferred to a 9-cm petri plate containing PDA with the help of a dissecting scope. The morphology of the cultures arising from the single ascospore cultures was then compared with that of 9M-S, C, and 9M-N.

Some of the data were analyzed with analysis of variance using SAS (SAS Institute, Cary, NC) and the figures were done on Freelance (Version 4.0, Lotus Development Corporation, Redwood City, CA). Fisher's protected least significant difference (LSD) test was used for mean separation. Only significant (P=0.05) data are discussed unless stated otherwise.

RESULTS AND DISCUSSION

Curing test. Neither isolate C or 9M-S formed sectors void of sclerotia on PDA containing 13.5 μg/ml cycloheximide. Isolate 9M-N did not form sclerotia on PDA containing cycloheximide. None of the isolates lost or gained traits related to sclerotial formation when cultured on cycloheximide-amended PDA. This suggests that sclerotial formation in *S. minor* is a genomic trait.

Nucleic acids from *S. minor* isolates. No extrachromosomal nucleic acids were detected in mycelia of isolates C, 9M-S and 9M-N as determined from agarose gel electrophoresis (Figure 2). Double stranded RNAs were always detected in

Helminthosporium victoriae isolates 26 and 190 (Figure 2). By using DNase, RNase at 0.03 M NaCl, and RNase at 0.3 M NaCl treatments, it was demonstrated that the high molecular weight bands shown on the total nucleic acids gel were total DNA, and the low molecular weight smeared bands were single stranded RNAs. No dsRNA was detected in mycelia from the three *S. minor* isolates. This further confirmed that there were no extrachromosomal nucleic acids in the isolates of *S. minor* being tested. The presence or the absence of dsRNAs has been found to be associated with pathogenicity or virulence of several plant pathogenic fungi, including *Endothia parasitica* and *Sclerotinia sclerotiorum* (2, 15). These dsRNAs were easily detected by direct nucleic acids extraction. In this study, if there is any extrachromosomal nucleic acids, they must exist at such a very low concentration that they were difficult to detect.

Mixed liquid culture test. Almost all the viable sclerotia retrieved from the mixed cultures of C and 9M-N, or 9M-S and 9M-N, produced white sectors void of sclerotia (Table 1). None of the sclerotia from cultures of C or 9M-S alone produced sectors void of sclerotia. Isolate 9M-N did not form sclerotia in liquid culture. The possibility of superficial contamination of sclerotia with 9M-N mycelia was excluded as the surface sterilization procedures were able to kill all 9M-N mycelia in control experiments. This test also excluded the possibility of transmission of extrachromosomal nucleic acids related to sclerotial formation between 9M-N and C, or between 9M-N and 9M-S. If such transmission had occurred through anastomosis, the frequency of detecting sclerotia which produced sectors void of sclerotia should be

very low. Instead, the results indicate that it is more likely that sclerotia of C or 9M-S entrap mycelium of isolate 9M-N during their formation. The closer the two isolates, the higher the probability of C or 9M-S entrapping 9M-N mycelia in their sclerotia.

Pairing test. Isolates 9M-N and C, or 9M-N and 9M-S, did not overgrow each other, but exhibited some incompatibility where mycelial growth was scant between the two colonies. Almost all viable sclerotia of isolate C or 9M-S adjacent to the 9M-N colony developed nonsclerotial forming sectors (Table 2). Fifty-seven percent of viable sclerotia formed within 0.5 cm to the barrier line by C showed 9M-N sectors (Table 2). Forty-one percent of viable sclerotia formed within 0.5 cm to the barrier line by 9M-S showed 9M-N sectors (Table 2). None of the sclerotia formed more than 0.5 cm from the barrier line produced sectors (Table 2). Similar results were obtained when the test was repeated using cultures of C, 9M-S and 9M-N originated from single ascospore cultures. These results confirmed those of the mixed culture test. Transfer of a nonsclerotial forming factor did not appear to have occurred through anastomosis because sclerotia formed away from the 9M-N mycelial growth did not produce sectors. Transfer of a sclerotial forming factor did not occur either as no sclerotia formed in 9M-N colonies. Rather, more sclerotia of C or 9M-S are able to entrap mycelia of 9M-N when they are closer to the colony of 9M-N. These results suggest that during sclerotial formation, pseudoparenchymatous tissue of isolate C or 9M-S can entrap mycelia of 9M-N during their development where 9M-N survives in sclerotia and forms sectors during subsequent germination.

Culture of ascospores from different kinds of sclerotia. Sclerotia of isolate C produced stipes after 21 days of incubation under the conditions stated previously. Sclerotia of 9M-S, 9M-N+C and 9M-N+9M-S produced stipes 3-4 more days later. Isolate C was the most efficient producer of apothecia (Table 3). Ascospores from sclerotia of isolate C or 9M-S produced colonies similar to their parents which formed sclerotia evenly over the PDA plates (Table 4). Ascospores from sclerotia of 9M-N+C or 9M-N+9M-S produced two kinds of colonies, one similar to 9M-N where no sclerotia were formed, the other similar to isolate C or 9M-S which produced sclerotia. Among these ascospores cultured, more showed colony morphology similar to 9M-N (Table 4). These results suggest that sclerotia of 9M-N+9M-S and 9M-N+C contain mycelia of both 9M-N and 9M-S or C. This confirmed that during sclerotial formation, pseudoparenchymatous mycelial tissue of isolate C or 9M-S can entrap mycelia of 9M-N. Nonsclerotial forming mycelia of 9M-N are able to survive in the sclerotia of C or 9M-S. Mycelia of 9M-N in sclerotia of C or 9M-S are able to produce ascospores through the apothecia formed by the sclerotia.

Formation of sclerotia by different fungi has been studied exclusively by microscopic observations (7, 17, 18, 19). Development of sclerotia of *Sclerotinia* spp. belongs to the 'terminal' type, where the sclerotial initials are formed by the prolific and often dichotomous branching of the tip of a hypha or the tips of several (17). Mycelium is highly septate producing short-celled branches, many of which fuse together forming a compact knot of hyphae from which a sclerotium develops. It is not known whether a sclerotia of *S. minor* is a genetically homogenous entity.

From the results of this study, sclerotia of *S. minor* are not genetically homogenous as sclerotia of isolate C or 9M-S are able to entrap mycelia of subisolate 9M-N into their sclerotia. Subisolate 9M-N may have arisen from a natural mutant occurred in the field and later survived in sclerotia of isolate 9M.

The possibility of occurrence of heterokaryotic mycelia or sclerotia was ruled out by the results of the pairing test. If heterokaryosis occurs, it must have originated from anastomosis of the two isolates, 9M-N and C or 9M-S. Since anastomosis does not always happen between meeting mycelia, the possibility of finding sclerotia with 9M-N mycelia close to the 9M-N colony should not be too high. Also we should be able to find sclerotia away from 9M-N which would be able to form 9M-N sectors, and/or we should be able to detect sclerotia formation on the 9M-N colony. But the pairing test showed that almost all the sclerotia close to 9M-N colony showed nonsclerotial forming sectors. But no sclerotia far away from 9M-N were able to sector. No sclerotia formed on 9M-N colony. This rather indicates that physical entrapping of 9M-N mycelia into sclerotia is what happened when the two isolates are close to each other. The way how the sclerotia in this study sectored also ruled out the possibility of occurring of heterokaryosis during the formation of these sclerotia. If heterokaryosis had happened, cultures of these sclerotia should form sectors as the mycelia grow out. Then different sectoring positions should be observed. But these sclerotia always sector at the center of the colony where the sclerotia were plated. This indicates that heterokaryosis was not involved in the formation of sclerotia which were able to form white sectors.

Since subisolate 9M-N is not pathogenic on peanut, is vegetatively competitive, and is able to survive in sclerotia of other pathogenic isolates, it may be useful as a genetic control agent in the field to infuse the sclerotia of pathogenic isolates. The idea is very similar to the use of sterile male insects such as tsetse flies and mosquitoes to infuse the population of healthy mosquitoes, thus controlling the insect populations (6).

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Table 1. Sectoring from germinating sclerotia of *S. minor* retrieved from mixed cultures in potato dextrose broth (PDB).

Origin of Sclerotia[1]	No. Germinated (A)[2]	No. Sectoring (B)[3]	B/A (% Sectoring)[4]
C	24	0	0 a
9M-S	30	0	0 a
9M-N+C	34	33	97 b
9M-N+S	28	27	95 b
$LSD_{0.05}$			3.3

1. Sclerotia of C or 9M-S were produced in liquid culture of C or 9M-S in PDB. Sclerotia of 9M-N+C or 9M-N+S were produced from mixed liquid culture of 9M-N and C, or 9M-N and 9M-S in PDB.
2. Average number of sclerotia germinated out of 50 plated. The number is the average of two tests. There were six replicates in each test. Each replicate had 50 sclerotia that were retrieved from one flask of mixed cultures in PDB.
3. The number of sclerotia showing 9M-N sectors. The number is the average of two tests. Each test had six replicates.
4. Percentage of viable sclerotia showing 9M-N sectors. Means in the column followed by the same letter do not differ significantly ($P<0.05$) according to LSD means analysis. Effect of treatment was determined by an F test on a one-way ANOVA.

Table 2. Sectoring from germinating sclerotia of *S. minor* in paired culture test on potato dextrose agar (PDA).

Pairing[1]	Area[2]	No. Plated[3]	No. Germinated (A)[4]	No. Sectoring (B)[5]	B/A (%)[6]
C & C	1	15	15	0	0
	2	16	16	0	0
	3	15	15	0	0
	4	15	15	0	0
S & S	1	15	15	0	0
	2	15	15	0	0
	3	15	15	0	0
	4	15	15	0	0
C & N	1	25	25	24	96
	2	51	51	29	57
	3	116	115	0	0
	4	112	112	0	0
S & N	1	76	76	73	96
	2	71	71	29	41
	3	77	76	0	0
	4	66	65	0	0

1. Isolate N and S refer to subisolates 9M-N and 9M-S respectively. C & C or S & S was pairing of C and C, or S and S on PDA. C & N or S & N was pairing of isolate C and 9M-N, or pairing of subisolates 9M-S and 9M-N on PDA.
2. Area 1 refers to the sclerotia formed on the barrier line between two paired colonies. Area 2 refers to sclerotia formed within 0.5 cm to the barrier line. Area 3 refers to the sclerotia 2 cm further away from the barrier line and area 4 refers to the sclerotia formed the farthest (Figure 1).
3. The number of sclerotia plated on PDA. For pairing of C & C and S & S, only a portion of the sclerotia formed on the plates were plated. For pairing of N & C and N & S, all the sclerotia formed on the plates were plated.
4. The number of sclerotia germinated from the sclerotia plated on PDA.
5. The number of sclerotia showing 9M-N sectors.
6. Percentage of viable sclerotia showing 9M-N sectors.

Table 3. Apothecial formation in *S. minor* by different types of sclerotia.

Sclerotia Type[1]	% Sclerotia Producing Apothecia[2]
C	57 a
9M-S	10 b
9M-N+C	30 ab
9M-N+9M-S	15 b
$LSD_{0.05}$	38

1. Sclerotia of C or 9M-S were collected from wholewheat agar (WWA) inoculated with C or 9M-S. Sclerotia of 9M-N+C or 9M-N+9M-S were the sclerotia formed in the paired cultures of 9M-N and C or 9M-N and 9M-S on WWA that were next to the 9M-N colony.
2. Percentage of sclerotia formed apothecia. The number was the average of two replicates. Means in the column followed by the same letter do not differ significantly ($P < 0.05$) according to LSD means analysis. Effect of sclerotial type was determined by an *F* test on a one-way ANOVA.

Table 4. Culture of single ascospores from apothecia of different types of sclerotia of *S. minor*.

Sclerotia Type[1]	No. Ascospores cultured (A)[2]	No. Colonies like 9M-N (B)[3]	B/A (%)[4]
C	58	0	0
9M-S	74	0	0
9M-N+C	147	141	96
9M-N+9M-S	65	61	94

1. The sclerotia type. Sclerotia of C or 9M-S were collected from wholewheat agar (WWA) inoculated with C or 9M-S. Sclerotia of 9M-N+C or 9M-N+9M-S were the sclerotia formed in the paired cultures of 9M-N and C or 9M-N and 9M-S on WWA that were next to the 9M-N colony.
2. Total number of single ascospore cultures achieved.
3. Number of single ascospore cultures which showed colony morphology similar to that of 9M-N which did not form sclerotia.
4. Percentage of single ascospore cultures which showed colony morphology similar to that of 9M-N

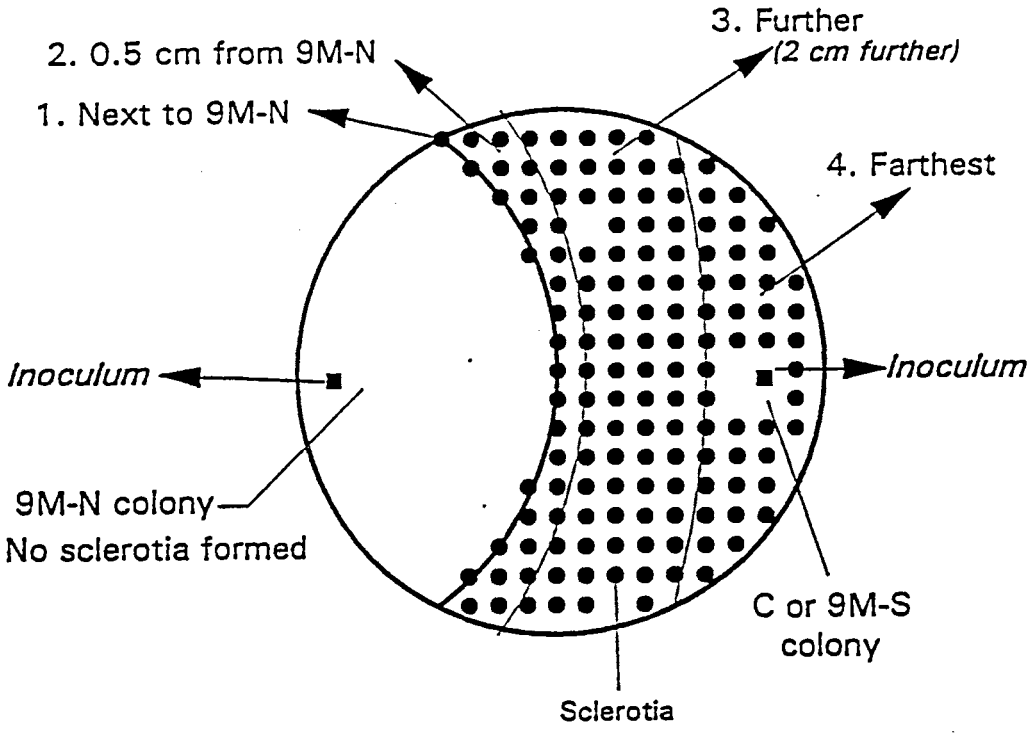

Figure 1. Schematic drawing of the paired culture tests for isolates of *S. minor* (Table 2).

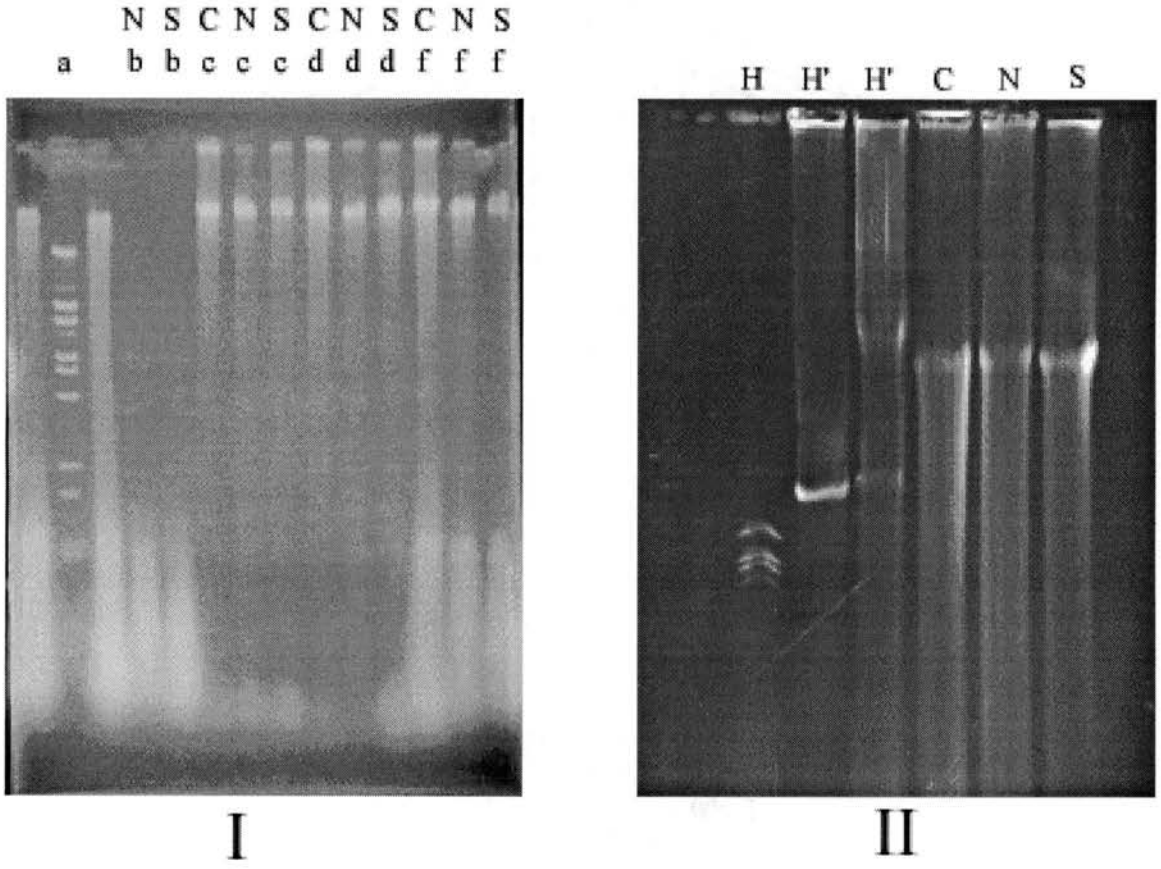

Figure 2. Gel electrophoresis of total nucleic acids extracted from isolates of *S. minor*. I and II are two electrophoresis gel photos. N, S, C, H' and H represent the isolates of fungi used in the extraction. N is the 9M-N subisolate, S is the 9M-S subisolate, and C is the C isolate of *S. minor*. H' is isolate 26, and H is isolate 190 of *Helminthosporium victoriae* which were used as dsRNA positive controls for the extraction procedures. 'a' is the λ-DNA molecular weight standard. 'b' represents the DNase treatment of total nucleic acids. 'c' represents RNase treatment of total nucleic acids at 0.03 M NaCl. 'd' represents RNase treatment of total nucleic acids at 0.3 M NaCl. 'f' represents the total nucleic acids of different isolates of *S. minor*.

SUMMARY

Several soil-inhabiting pathogens cause diseases that affect peanut health and productivity. In Oklahoma, the most damaging ones include *Sclerotinia minor*, *Sclerotium rolfsii*, *Verticillium dahliae* and *Pythium* spp. These pathogens all have broad host range, including crops and weeds, and they are able to survive in soil or on plant debris as durable survival structures such as sclerotia, microsclerotia or thick-walled oospores, which makes them very difficult to control in fields where they have become established. Few chemicals are available for control of these diseases, especially sclerotinia blight, and problems of chemical toxicity, environmental contamination, and pesticide residues in food, are an increasing concern. Therefore, alternative control methods, especially integrated pest management (IPM) systems which allow farmers to manage diseases using several approaches with minimum chemical input are of interest to researchers. Combination of chemical control, biological control, culture practices (including crop rotation, use of cover crops and organic amendments), and using resistant peanut cultivars are being researched in Oklahoma to determine their potential to economically manage these soilborne diseases. The results of this research would be of value in formulating an IPM system to manage these soilborne diseases in Oklahoma, especially sclerotinia blight.

The first two chapters of this dissertation report that rapeseed meal (RSM) and

rape greens (RG) have the potential to be used as organic amendments to the soil to reduce the pathogenic activity of some of the soilborne pathogens, as the volatiles released from the breakdown of glucosinolates in RSM or RG in soil amended with RSM or RG were able to reduce growth and sclerotial or microsclerotial formation of *S. minor*, *S. rolfsii* and *V. dahliae*. In addition, viability of sclerotia of *S. minor* and *S. rolfsii* were reduced in soil amended with RSM or RG. This study will contribute to future research of incorporating RSM and/or RG in the field to reduce the sclerotial population of *S. minor* and *S. rolfsii*, and ultimately to economically manage these diseases.

The later two chapters of this dissertation report the characterizations of isolates of *S. minor* from Oklahoma. This study will aid in the understanding of the *S. minor* pathogen, and may contribute to formulation or devising of new strategies for management of sclerotinia blight. A nonsclerotial forming subisolate of *S. minor* (9M-N), obtained from white sectors of a field isolate (9M), was used to demonstrate that oxalic acid is not the sole pathogenicity factor in *S. minor* on either peanut or lettuce. Tests in which isolates of *S. minor* grew in mixed cultures suggest that isolates of *S. minor* can entrap mycelia of other isolate into their sclerotia. Thus long term application of isolate 9M-N, which is not pathogenic on peanut and does not form sclerotia, has the potential as a biocontrol agent to infuse the population of pathogenic isolates of *S. minor*, and ultimately reduce the sclerotial population in field soil.

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