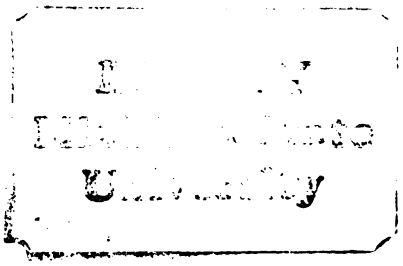

This is to certify that the

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FACTORS AFFECTING CARPOGENIC GERMINATION

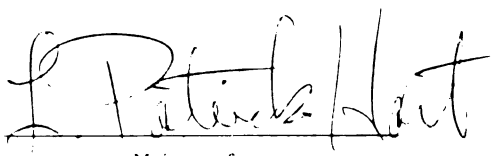
OF SCLEROTINIA SCLEROTIORUM (LIB.) DE BARY

presented by

William Lawrence Casale

has been accepted towards fulfillment
of the requirements for

M.S. degree in Botany and Plant Pathology



Major professor

Date 2/23/84

FACTORS AFFECTING CARPOGENIC GERMINATION OF *SCLEROTINIA SCLEROTIORUM* (Lib.) de Bary

By

William Lawrence Casale

A THESIS

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ABSTRACT

FACTORS AFFECTING CARPOGENIC GERMINATION OF
SCLEROTINIA SCLEROTIORUM (Lib.) de Bary

By

William Lawrence Casale

Isolate and size differences among sclerotia of Sclerotinia sclerotiorum affected carpogenic germination, whereas soil pH or splitting sclerotia in half did not. Carpogenic germination of sclerotia in PEG 8000 solutions was 100% from 0 to -4 bars osmotic potential. Germination in soil was 50% at -0.5 bars and <10% below -1 bar matric potential. Sclerotia imbibed similar amounts of water from -0.5 to -10 bars soil matric potential. Carpogenic germination was stimulated by leaching sclerotia prior to incubation, and inhibited when material diffusing from sclerotia accumulated in, or was added to, the incubation medium. More sclerotia germinated in non-sterile soil than sterile soil. These data suggest a diffusible endogenous inhibitor of carpogenic germination. Sclerotia incubated in >4 ug atrazine/g soil or a 10 uM atrazine solution formed only abnormal apothecia; percent germination was unaffected. Numerous stipes with abnormal apothecia grew from the aborted hymenia of immature apothecia soaked in 50 uM atrazine for 30 min.

To my parents

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major professor, Dr. L. Patrick Hart, for his guidance and support during the course of these studies.

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PART I: SOIL pH, SCLERCTIAL SIZE AND CONDITION AS INFLUENCES ON CARPOGENIC GERMINATION

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary [=Whetzelinia sclerotiorum (Lib.) Korf and Dumont] is an ascomycetous fungus, pathogenic to 361 species of plants in 64 families (33). Economically important hosts include plants in the families Solanaceae, Brassicaceae, Umbelliferae, Compositae, Chenopodiaceae and Leguminosae (47). Sclerotinia sclerotiorum is a discomycete of the family Sclerotiniaceae in the order Helotiales (4, 21). Libert gave the first Latin description of the present day S. sclerotiorum in 1837, naming it Peziza sclerotiorum (26). In 1884, deBary (11) changed the binomial to Sclerotinia sclerotiorum. Comprehensive histories of the taxonomy and current status of the genus have been given by Kohn (20), Purdy (31), and Willetts and Wong (47).

Infection of host plants by S. sclerotiorum occurs either by means of ascospores or mycelium arising from sclerotia or neighboring plants (47). No functional conidia are produced (microconidia have been observed, but their role is unknown). Ascospores are the primary inoculum for white mold of beans (1, 2, 7, 36, 37, 42) and lettuce drop (30), although mycelium from sclerotia has been reported to be infectious to bean (29). Ascospores are forcibly ejected from apothecia (14) and dispersed by wind as far as 25 m

(42). The fungus infects healthy plants via germinated ascospores only if external nutrients are present (2, 12, 32). Senescent and injured organs or plant litter can provide this source of nutrients (40).

Soon after infection, a light-brown watery rot develops, followed by the appearance of cottony-white mycelium and collapse of non-woody tissue (27). After several days, small aggregates of mycelium develop, darken and become dry, hard survival structures called sclerotia. A mature sclerotium consists of a darkly pigmented rind, 2-3 cells thick; just below this is a cortex of pseudoparenchymatous tissue, 2-4 cells thick, and a central medulla of loosely arranged filamentous hyphae (22). Details of the formation and composition of sclerotia have been reported (8, 9, 10, 25, 43).

Sclerotia can remain viable in field soil for 4-5 years (3, 39, 49), and under some conditions for at least 10 years (5). The primary influence on survival appears to be biological, with normally occurring soil temperatures and pH being of minor importance (3). More than 30 species of fungi and bacteria have been reported as antagonistic or parasitic to Sclerotinia species, but only a few have been studied under field conditions (6, 16, 19, 28, 44, 45).

Sclerotia of S. sclerotiorum may germinate myceliogenically (production of mycelium) and/or carpogenically (production of apothecia). Myceliogenic germination occurs when sufficient external nutrients are

present [germination has not been observed on non-amended natural soil (2, 36)]. Secondary or daughter sclerotia may be formed in soil or culture, adjacent to or at a distance from the mother sclerotia (48).

Apothecial initials evidently develop directly from medullary hyphae, usually just beneath the sclerotial rind [although microconidia are formed, they do not appear to function as spermatia] (22, 35). Saito (35) recognized four stages of apothecial initiation: 1) deeply staining areas of medullary tissue develop near the rind, and 2) become enclosed by thick-walled, darkly pigmented cells. These primordia 3) organize into clearly distinguished tissue, which 4) ruptures the rind and grows as an apothecial stipe. The stipes are positively phototropic, and differentiation of apothecial discs occurs only in the presence of light (18, 25) [for S. trifoliorum, a closely related fungus, wavelengths greater than 390 nm were ineffective (15)]. Ultrastructural (19, 20) and histochemical (22) studies of the stipe and apothecium have been done.

Several factors affecting carpogenic germination have been studied. Sclerotia grown on media containing vegetable extracts produce apothecia more frequently than those grown on potato-dextrose or synthetic media (34, 47). Providing mature sclerotia with a conditioning or after-ripening period under moist conditions (41) at high (13) or low (35) temperatures may enhance subsequent carpogenic germination. There is general agreement that 10-20 C is the optimum

temperature range for the production of apothecia (36, 47). Cycling thermoperiods are not required for carpogenic germination of S. trifoliorum (38). The effect of light on apothecial disc differentiation has already been discussed, but Letham (23) suggested that light could affect the initiation of apothecia. The relationship between moisture and carpogenic germination is important and will be discussed in PART II. Sclerotia incubated in closed tubes produced stipes but disc formation was inhibited (24). It is unclear, however, whether accumulation of inhibitory compounds or oxygen deficiency affected disc differentiation. There appear to be no reports of the influence of pH on carpogenic germination.

Preliminary studies described herein yielded information for future experiments. A simple method of producing large quantities of readily germinable sclerotia was developed. Isolates of S. sclerotiorum were screened for speed of germination and requirement for cold treatment prior to incubation, with selection of those isolates that facilitated experimentation. To insure reasonable uniformity of experimental units in future studies, the influence of sclerotial size on carpogenic germinaton was examined. Soil pH as a possible influence on germination was also investigated, as was the ability of sclerotia to alter the pH of soil in their immediate vicinity. Split and intact sclerotia were compared to determine whether cutting affected carpogenic germination.

MATERIALS AND METHODS

Production of sclerotia

Sclerotia of *S. sclerotiorum* were obtained in commercial fields in Michigan from infected dry bean plants (*Phaseolus vulgaris* L.) (Table 1). Sclerotia were surface-sterilized by first quickly dipping them in 95% ethanol to reduce surface tension, then placing them in 0.5% sodium hypochlorite (NaOCl) for 3 min followed by 2 min in sterile distilled water. Individual sclerotia were germinated myceliogenically on potato dextrose agar (PDA) and 5 mm discs were cut from the advancing colony margin after 3-4 days. Autoclaved canned green beans were arranged in a single layer in plastic petri dishes, inoculated with inverted mycelial discs, sealed with Parafilm (American Can Company, Greenwich, CT 06830) and stored at room temperature and light.

Sclerotia were harvested after one month. This insured maximum yield, as new initials formed while others were maturing. Sclerotia were separated from macerated bean tissue in a metal strainer under a strong stream of tap water. Sclerotia were air-dried for 24 h, and stored at 5 C in plastic bags.

Comparison of carpogenic germination among six isolates

Sclerotia from six isolates of *S. sclerotiorum* (A, B, D, H, M and R) were stored in plastic bags at 5 C or room temperature (22 ± 2 C) for 1, 2, 7.5 or 10.5 months.

Table 1. Source of isolates of *Sclerotinia sclerotiorum*.

Isolate	Source
A	Sclerotia collected from heavily infected dry bean plants in Bay Co., MI (8/19/81).
B	Isolated from dry bean plants infected at soil line in Bay Co., MI (7/15/81).
D	Isolated from heavily infected dry bean plants in Huron Co., MI (August, 1981).
H	Isolated from infected dry bean plants in Huron Co., MI (7/16/81).
M	Sclerotia separated from cull pile screenings from a central Michigan bean elevator in 1980.
R	Sclerotia obtained from R. Weinzerl, Department of Plant Pathology, Oregon State University, Corvallis 97331.

Additional sclerotia from one isolate (H) were stored at 5 C for two months, then -5 C for five months. Following storage, six sclerotia from each isolate were incubated on water saturated Capac sandy clay loam soil in petri dishes sealed with Parafilm and incubated at 15 C. Each treatment was replicated twice. The stipes produced were counted daily starting 19 days after the beginning of incubation. A sclerotium was considered germinated if at least one stipe had broken through the rind.

Incubation of several size classes of sclerotia

Sclerotia from the B-isolate were separated into four size classes by length and mean fresh weight (n=15) of individual sclerotia: group 1, 11-24 mm, 81.17 mg; group 2, 7-13 mm, 46.78 mg; group 3, 5-6 mm, 19.83 mg; and group 4, 2- 3 mm, 6.73 mg. Sclerotia were incubated at 15 C in water- saturated non-sterile Capac soil in Parafilm-sealed petri dishes. There were five sclerotia per dish and three dishes per size class.

Incubation of intact and split sclerotia

Twenty-five sclerotia (B-isolate) were cut in half and fifty sclerotia were left intact (large sclerotia were chosen for cutting so that the fifty sclerotia halves were similar in size to the intact sclerotia). After cutting, all sclerotia were incubated in plastic bags at 5 C for three days. Non-sterile Capac soil was adjusted to -0.5 bars matric potential (Figure 1) and 50 g placed in each of

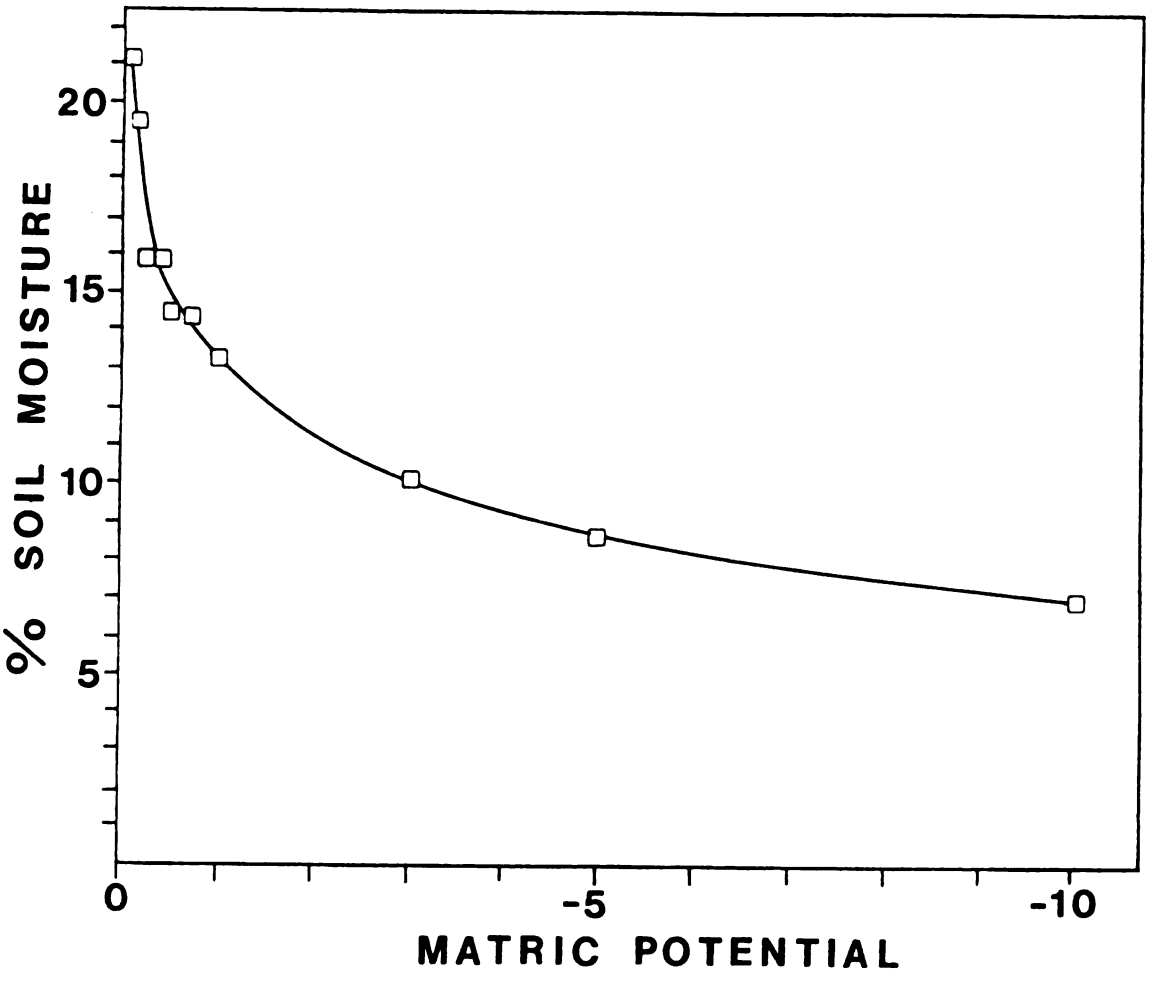

Figure 1. Soil moisture curve relating percent soil moisture to matric potential for Capac sandy clay loam soil.

20 petri dishes. Five intact sclerotia or sclerotia halves were lightly pushed into the soil in each dish. The dishes were sealed with Parafilm and stored at 15 C.

Incubation of sclerotia at various soil pH

Fifty milliliters of distilled water was added to 250 g of Capac soil (pH 5.6) in each of five beakers. The soil pH was adjusted with hydrochloric acid or potassium hydroxide and allowed to equilibrate for 84.5 h. Five B-isolate sclerotia were placed on the surface of 50 g of equilibrated soil in each of four petri dishes at each pH value (pH 4.82, 5.28, 6.11, 6.43 and 7.04). The dishes were sealed with Parafilm and the sclerotia incubated at 15 C. The number of germinated sclerotia and the number of stipes produced were counted at regular intervals. After 33 days, the pH of soil immediately beneath each sclerotium and the pH of soil in each dish as distant as possible from any sclerotium was determined with an electronic pH meter .

RESULTS

Production of sclerotia

White cottony mycelium grew throughout inoculated bean tissue after one week to produce sclerotial initials, tufts of white mycelium, 1-5 mm in length. Clear droplets developed on the surface of the initials two to three days later; these gradually disappeared as the sclerotia matured

and darkened. Sclerotia, dry and black, were apparently mature after another 4-7 days. Sclerotia were irregular in shape and ranged from <1 to >20 mm in length, resembling sclerotia produced on infected plants. Larger sclerotia arose from the fusion of several neighboring initials. Sclerotia grown on PDA were generally smaller and more regular in shape (circular/concave-convex).

Germination rates of six isolates

With the exception of the R-isolate, all isolates had 83-100% carpogenic germination after seven months incubation in soil at 15 C. There were differences in the speed of germination among isolates (Table 2). Isolate-A sclerotia conditioned at 5 and 23 C prior to incubation had 50% germination after 22 and 37 days, respectively. Thus, a conditioning period at 5 C accelerated, but was not required for carpogenic germination. Isolate-D behaved similarly. Fifty percent of H-, B- and M-isolate sclerotia had germinated by 7 months, but not before 46 days, at both 5 and 23 C. Only two of the 24 R-isolate sclerotia germinated after seven months.

The speed of carpogenic germination of H-isolate sclerotia conditioned at 5 or 23 C for 1, 2, or 7.5 months was similar, with the most rapid germination by sclerotia conditioned at 5 C for 10.5 months (50% by 22 days), and those conditioned at 5 C for two months then -5 C for 5 months (50% by 21 days) (Table 3). Thus, the effect on carpogenic germination of 5 C conditioning was apparently no

Table 2. Number of carpogenically germinated sclerotia per 12 sclerotia for six isolates of Sclerotinia sclerotiorum with two conditioning temperatures.

Isolate	Conditioning[a] temperature (C)	Incubation time (days)[a] 19	22	26	29	37	42	210
A	5	1	6	8	8	8	8	10
	23	0	1	2	5	6	6	12
D	5	0	1	3	7	8	12	12
	23	0	0	2	4	4	11	11
H	5	0	1	1	2	2	2	10
	23	0	1	2	2	4	4	12
B	5	0	0	0	0	2	3	12
	23	0	0	0	0	0	0	12
M	5	0	0	0	0	0	0	12
	23	0	0	0	0	0	0	12
R	5	0	0	0	0	0	0	0
	23	0	0	0	0	0	0	2

[a] Sclerotia were conditioned in sealed plastic bags for 7.5 months at 5 or 23 C prior to incubation.

[b] Sclerotia were incubated at 15 C in water-saturated soil contained in Parafilm-sealed petri dishes.

Table 3. Number of carpogenically germinated sclerotia per 12 sclerotia of Sclerotinia sclerotiorum (H-isolate) with 5 conditioning periods and 3 conditioning temperatures.

Conditioning[a] period (months)	Conditioning temperature (C)	Incubation time (days)[b] 19	21	22	26	37	210
1.0	5	0	1	1	2	9	12
	23	0	0	0	0	4	11
2.0	5	0	0	0	2	10	12
	23	0	0	1	2	6	6
7.5	5	0	0	1	1	2	10
	23	0	0	1	2	4	12
10.5	5	0	5	9	12	12	12
2.0 at 5, then 5.0 at -5		3	10	12	12	12	12

[a] Sclerotia were conditioned in plastic bags prior to incubation.

[b] Sclerotia were incubated at 15 C in water-saturated soil contained in Parafilm-sealed petri dishes.

different than 23 C conditioning for 7.5 months or less, although conditioning for 10.5 months or at -5 C accelerated germination.

Effect of splitting sclerotia and sclerotial size on carpogenic germination

The number of carpogenically germinated sclerotia was independent of size up to 21 days incubation, but dependent on size after 22 days (Figure 2). Germination at 21 and 22 days was statistically analyzed for independence of sclerotial size using chi-square ($P=0.05$) testing of 4 x 2 (size class x germination) contingency tables. Essentially 100% of the largest sclerotia (size classes 1 and 2), 50% of size class 3, and 30% of size class 4 had germinated after 37 days. The mean number of stipes per germinated sclerotium after 23 days was 3.6, 2.9, 1.1 and 1.1 for size classes 1, 2, 3 and 4, respectively. In future experiments, sclerotia as nearly the same size as possible were selected.

Germination of intact and split sclerotia was compared (Table 4). When the number of germinated sclerotia after 24 and 46 days was analyzed using 2 x 2 (condition of sclerotia x germination) contingency tables and chi-square ($P=0.05$), germination was independent of the condition (intact or split) of the sclerotia . The mean number of stipes produced per germinated sclerotium was similar for intact and split sclerotia, but could not be statistically tested.

The cut surfaces of the split sclerotia, initially white, turned off-white to black (similar to rind tissue)

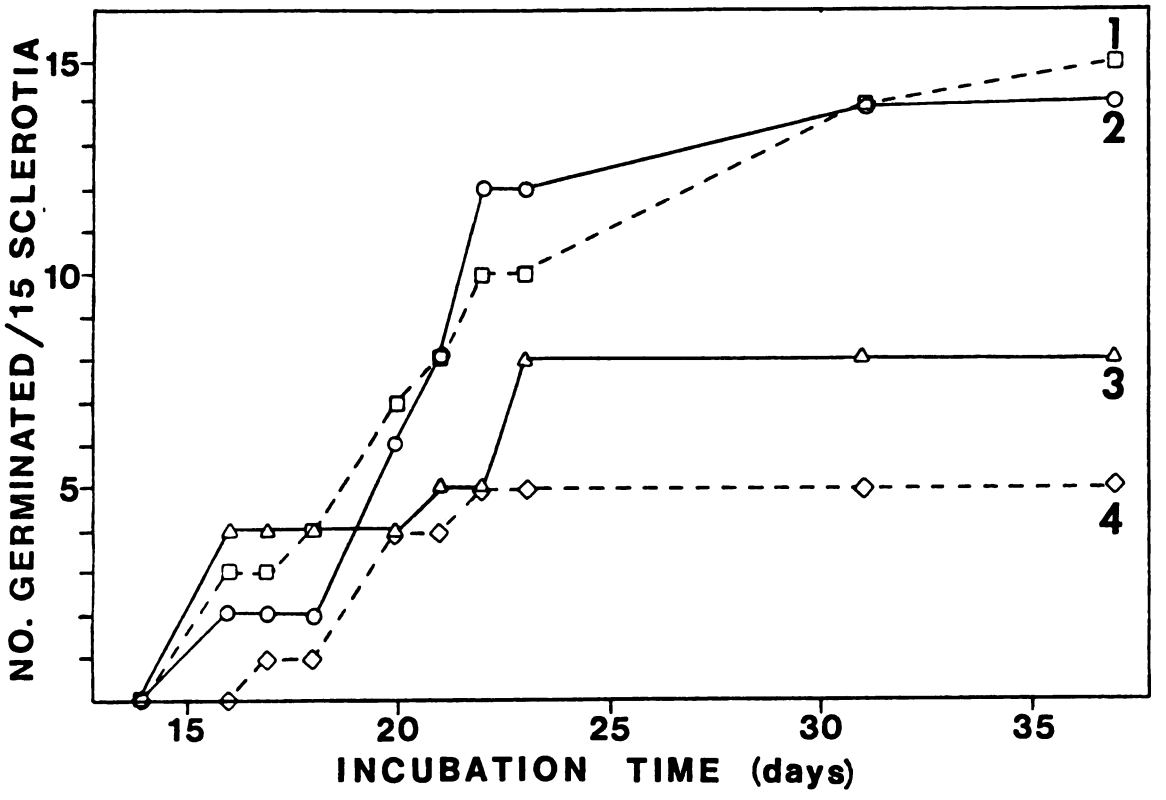

Figure 2. Comparison of carpogenic germination among four size classes (1 = 81 mg, to 4 = 7 mg) of sclerotia of Sclerotinia sclerotiorum incubated at 15 C in water-saturated soil.

Table 4. Carpogenic germination of split (half) and intact (whole) sclerotia of *Sclerotinia sclerotiorum* incubated in soil at -0.5 bars matric potential.

	No. germinated/50		No. stipes/ germinated sclerotium	
Sclerotia	24 days	46 days	24 days	46 days
Whole	23[a]	46[a]	1.35[b]	1.54[b]
Half	14	44	1.07	1.39

[a] Values in a column were not significantly different, based on chi-square ($P=0.05$).

[b] Differences could not be statistically tested.

after 24 days of incubation.

Influence of soil pH on carpogenic germination

After 84.5 h equilibration, the soil pH's used were 4.82, 5.28, 6.11, 6.43 and 7.04. After incubation of sclerotia for 33 days, the soil immediately beneath sclerotia in treatments two to five was significantly more acidic than the surrounding soil (Table 5).

The number of sclerotia that germinated after 24 days was tested using a 5 x 2 (soil pH x germination) contingency table and chi-square ($P=0.05$): germination was independent of soil pH for the range of pH examined (Table 5). Contingency tables could not be used to analyze germination data at 19 and 33 days due to the low number of germinated sclerotia at 19 days and ungerminated sclerotia at 33 days.

DISCUSSION

Sclerotia collected from infected plants or plant debris in the field were difficult to free of bacterial and fungal contaminants. Sclerotia grown on green beans in petri dishes and harvested by washing under tap water were easily surface sterilized in sodium hypochlorite. The green bean substrate may provide sclerotia with nutrients similar to those obtained from infected bean plants in the field.

There was a large variance in the time required for germination among sclerotia of the same isolate grown and

Table 5. Number of carpogenically germinated sclerotia per 20 sclerotia of *Sclerotinia sclerotiorum* incubated at various soil pH's.

	Initial soil pH				
Incubation time	4.8	5.3	6.1	6.4	7.0
19 days	0[x]	1	5	4	1
24 days	15	10	14	8	11
33 days	18	20	18	20	20
	Final soil pH[y]				
Beneath sclerotia	4.2a[z]	4.2a	5.1c	5.7d	6.2e
Surrounding soil	4.5ab	4.9 bc	6.0 de	6.6 f	7.0 g

[x] Differences in the numbers of sclerotia germinated at 24 days were not significant, based on chi-square ($P=0.05$). Differences at 19 and 33 days could not be statistically tested.

[y] Final soil pH was measured after 33 days immediately beneath each sclerotium, and also at a point as distant as possible from any sclerotium (surrounding soil).

[z] Differences between final pH values with a common letter are not significant according to Tukey's test ($P=0.05$).

Analysis of variance for final soil pH

Source	df	Mean square	F
Treatment	9	5.1290	131 **
Error	40	0.0391	

** Differences among treatments are significant at $P=0.01$.

harvested at the same time. This may be due to natural variation among individual sclerotia or to sensitivity to slight differences in environmental factors. The variance may, however, result from the method of sclerotial production, since new sclerotia are being initiated and formed throughout the 30 day incubation on green bean tissue and sclerotia are harvested at the same time. This results in a difference of several weeks maturation time among the sclerotia. Although sclerotia appear to be morphologically mature, differences in physiological maturity may occur among sclerotia in a petri dish. Variance may be reduced by harvesting the sclerotia as soon as the first sclerotia apparently mature. This would sacrifice yield in favor of reducing variability.

Cutting sclerotia in half had no effect on the percent germination (Table 4). This finding was supported by Saito (35), who studied the initiation and development of apothecia from small cubes of sclerotial medullary tissue. Variability in experiments due to differences among individual sclerotia may, therefore, be reduced by applying different treatments to alternate halves of the same sclerotium. There were slightly more stipes produced per germinated intact sclerotium than from half sclerotia, but this difference could not be statistically tested due to the experimental design and limitations of contigency tables. The regeneration of the dark pigmented layer in cut sclerotia has been reported (35).

The size of sclerotia was positively correlated with the percent germination after 20 days incubation, but not before 20 days. The largest sclerotia produced the most stipes, with fewer stipes formed as sclerotial size decreased. Large sclerotia contain greater amounts of medullary tissue from which apothecial initials may arise (therefore, potentially more stipes) and more reserves to support apothecial development than smaller sclerotia. The results indicate that selecting sclerotia of similar size increases uniformity of germination and stipe production.

Conditioning at 5 C was not required for carpogenic germination of the isolates tested (Tables 2, 3). The conditioning period is not well defined, and little or nothing is known of the processes associated with it. Saito (35) believes it is the time of formation of stipe initials within the medullary tissue. There were considerable differences among isolates in the incubation time prior to germination (specifically, stipe emergence). Variation among isolates with respect to conditioning and germination rate may have resulted in some of the disagreement found in the literature.

Carpogenic germination of sclerotia was unaffected by differences in the soil reaction between pH 4.82 and 7.04. Since the unadjusted pH of the Capac soil used in future experiments (pH 5.6) was within this range, no attempt was made to alter its pH. The pH of the soil immediately below the sclerotia decreased. This change was particularly

pronounced in the more alkaline soils. Culture filtrates and cellular extracts of S. sclerotiorum contain high concentrations of organic acids (46), and sclerotia have been shown to leak amino acids (17), which may account for the observed drop in soil pH. The lowering of soil pH in the immediate sclerotial environment may have resulted in the apparent independence of carpogenic germination and soil pH. A lower soil pH might also inhibit bacteria in the vicinity of the sclerotia, thereby reducing rotting and enhancing survival. The change of pH in the immediate sclerotial environment must, therefore, be considered when studying the effect of pH on S. sclerotiorum.

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PART II: THE INFLUENCE OF WATER POTENTIAL ON CARPOGENIC GERMINATION

INTRODUCTION

Diseases caused by Sclerotinia sclerotiorum are most severe under wet conditions (20, 10). Cultural practices and modifications of plant architecture that reduce moisture in the soil or canopy have had some success in reducing disease incidence and severity (4, 6, 10, 24, 26, 28, 29).

Wet conditions might affect inoculum production, infection of the host plant and lesion development. S. sclerotiorum does not infect unless there is a film of water on the plant surface, although growth of the fungus from ascospores, mycelial disks or sclerotia is not adversely affected by osmotic potentals of -40 bars and higher (9). Lesion expansion on bean leaves was stimulated by lowering osmotic potential from -1 to -28 bars, and was not reduced until -64 bars, but expansion of established lesions was stopped when leaf surfaces were allowed to dry.

There have been few reports on the relationship between water potential (Ψ_w) and carpogenic germination. Bedi (3) reported that sclerotia did not germinate carpogenically at 100% relative humidity, but required free water. Morrall (21, 22) compared carpogenic germination of sclerotia incubated in soil of various percentages of moisture. The water status of such soils is unclear since actual water potentials were not determined. However, sclerotia

germinated at soil moisture contents of 31% of field capacity or greater.

Several studies have examined the effect of specific water potentials on carpogenic germination of S. sclerotiorum. Sclerotia incubated in sucrose, KCl or a salts mixture osmoticum produced mature apothecia at 0 bars osmotic potential, but only a few stipe initials developed at -6 bars (no intermediate osmotic potentials were tested) (9). Apothecial formation was completely inhibited below -6 bars. Sclerotia floated on polyethylene glycol (PEG) 4000 solutions germinated from 0 to -6 bars osmotic potential, but not lower (21).

Duniway et al (7) determined matric potential optima of -240 mbars and from -80 to -160 mbars for two isolates of S. sclerotiorum incubated in a ceramic plate soil moisture extractor. There was a sharp decrease in the number of apothecia produced below -80 and -40 mbars, respectively, for the two isolates.

Soil matric potential can be manipulated by placing a semi-permeable membrane between soil and a solution of PEG (23, 31, 32, 33). Morrall (22) incubated sclerotia in soil adjusted to specific matric potentials by immersing soil contained in dialysis bags in PEG 20,000 solutions. Sclerotia germinated carpogenically between 0 and -7.5 bars, but not at lower matric potentials. Sclerotia at -4 bars soil matric potential imbibed over 90% of the maximum amount of water imbibed after subsequent soaking in pure water; at

-10 bars, sclerotia imbibed 85% of the maximum (21). Germinated sclerotia were incubated similarly in soil adjusted with PEG 20,000 solutions after their stipes were removed: 75-100% regerminated from -1 to -10 bars, and at -15 bars, 50% regerminated (21). Apothecial production can evidently be sustained at soil matric potentials lower than those which initiate germination.

PEG has been used to impose water stress on plants by decreasing the osmotic potential of the rooting medium (13, 14, 16, 18). Roots were found to have a low permeability to PEG 1000-20,000, unless they were damaged mechanically (17). Lawlor (17) reported that the amounts of inorganic contaminants in commercial PEG was unlikely to cause damage in plants. PEG was selected for use as an osmoticum in the present study to reduce uptake of the osmoticum by sclerotia.

The objectives of this study were to determine the range of water potentials at which carpogenic germination of S. sclerotiorum can occur, and to compare carpogenic germination of sclerotia held at constant osmotic and soil matric potentials.

MATERIALS AND METHODS

Water imbibition by sclerotia

Water imbibition by sclerotia of isolates R and M was compared. Isolate-R sclerotia were kept at either 5 C or room temperature (22 ± 2 C) for 32 days prior to the experiment. Isolate-M sclerotia were more brittle than laboratory produced sclerotia and although they readily germinated to produce mycelia, less than 1% germinated carpogenically.

Subsamples from each group were weighed and dried at 105 C for 24 h, then reweighed to determine the initial percent moisture. Sclerotia from each group (700 mg, replicated 4 times) were placed in petri dishes containing 30 ml of distilled water. The sclerotia in each dish were removed at regular intervals, quickly blotted dry, weighed and immediately returned to fresh distilled water.

The relationship between soil matric potential and percent soil moisture was determined with a 15 bar ceramic plate soil moisture extractor (Soil Moisture Equipment Co., Santa Barbara, CA) (8) for Capac sandy clay loam collected from the Michigan State University Botany and Plant Pathology Field Laboratory, East Lansing . The amount of water imbibed by sclerotia in soil adjusted to matric potentials from -0.5 to -10.0 bars was examined. Capac soil was adjusted to specific matric potentials by drying saturated soil to appropriate moisture levels calculated

from the soil moisture curve (Figure 1). Soil was thoroughly mixed at regular intervals to insure uniform soil moisture. Changes in the soil matric potential due to condensation of water on the lid were minimized by placing sufficient soil in each petri dish so that the soil was in contact with the lid. Five sclerotia were weighed and completely buried in the soil in each of four replicate dishes at each matric potential. Dishes were sealed with Parafilm, placed in plastic bags and incubated at 15 C. The sclerotia were removed from the soil, quickly brushed to remove any adhering soil and immediately weighed.

Incubation of sclerotia at various osmotic potentials

Distilled water was adjusted to specific osmotic potentials (Ψ_s) with polyethylene glycol (PEG) 8000 (M.W. 6000-7500; Union Carbide Corporation). The concentration of PEG 8000 used to obtain osmotic potentials of -0.5, -1.0, -2.25, -3.0, -4.5, -6.5 and -8.8 bars in the first experiment was determined from a formula by Michel and Kaufman (19):

$$\Psi_s = -(1.18 \times 10^{-2})C - (1.18 \times 10^{-4})C^2 + (2.67 \times 10^{-4})CT + (8.39 \times 10^{-7})C^2T \qquad (1)$$

where C is the concentration of PEG 8000 in g/kg water, and T is the temperature in degrees C. A standard deviation of 0.28, determined empirically, was reported.

A revised formula (12) was used to obtain Ψ_s of 0, -0.5, -1.0, -2.0, -4.0, -6.0, -8.0, -10.0, -15.0 and -20.0 bars in

the second experiment:

$$\Psi_s = 1.29[PEG]^2T - 140[PEG]^2 - 4[PEG] \qquad (2)$$

where [PEG] is the concentration of PEG 8000 in g/g water, and T is the temperature in degrees C.

In the first experiment, 50 g of Capac soil, sieved through a 2 mm mesh screen and dried at 105 C for 24 h, was placed in each of 35 plastic petri dishes (90 x 15 mm), and 20 ml of PEG 8000 solution added to each petri dish, resulting in a thin layer of solution above the soil. Sclerotia (H-isolate) were pre-soaked in distilled water for 7 h (Figure 3) to reduce changes in osmotic potential of the PEG 8000 due to the imbibition of water by the sclerotia. Five sclerotia were gently pressed into the soil in each dish so that they were just covered by the osmoticum. Five replicate dishes were used at each osmotic potential.

In the second experiment, 24 g of washed and ignited sand (Mallinkrodt, Inc., Paris, Kentucky 40361) was used in place of the soil, and 10 ml of PEG 8000 solution plus 80 ug/ml each of penicillin-G, streptomycin sulfate and neomycin sulfate was added to each petri dish. Five sclerotia (A-isolate), pre-soaked for 7 h, were placed on the surface of the sand with the upper sclerotial surface exposed to air in each of four replicate petri dishes at each osmotic potential. The dishes in both experiments were sealed with Parafilm, weighed and incubated at 15 C.

After 30 days, the dishes were reweighed to determine evaporative loss and the number of germinated sclerotia recorded. Sclerotia were considered germinated if at least one stipe had broken through the rind.

Incubation of sclerotia at various soil matric potentials

Capac soil was adjusted to matric potentials of -0.5, -1.0, -1.5, -2.0, -3.0, -5.0 and -7.0 bars as described previously. Fifty grams were placed in each of 35, 90 x 15 mm petri dishes. Five replicate dishes were included at each soil matric potential, and five sclerotia were buried in each dish. The sclerotia in Parafilm-sealed dishes were incubated at 15 C.

RESULTS

Water imbibition by sclerotia

The soil moisture curve of percent soil moisture as a function of soil matric potential is shown in Figure 1.

The rate of water imbibition by sclerotia in distilled water was similar for the three groups of sclerotia tested (Figure 3). Because of differences in the initial percent moisture content among the three groups, weight increase (water imbibition) was calculated as a percent of the initial dry weight (weight of sclerotia after 24 h at 105 C). Sclerotia of the R-isolate stored at 5 C, R-isolate stored at room temperature and of the M-isolate lost 3.2 ±

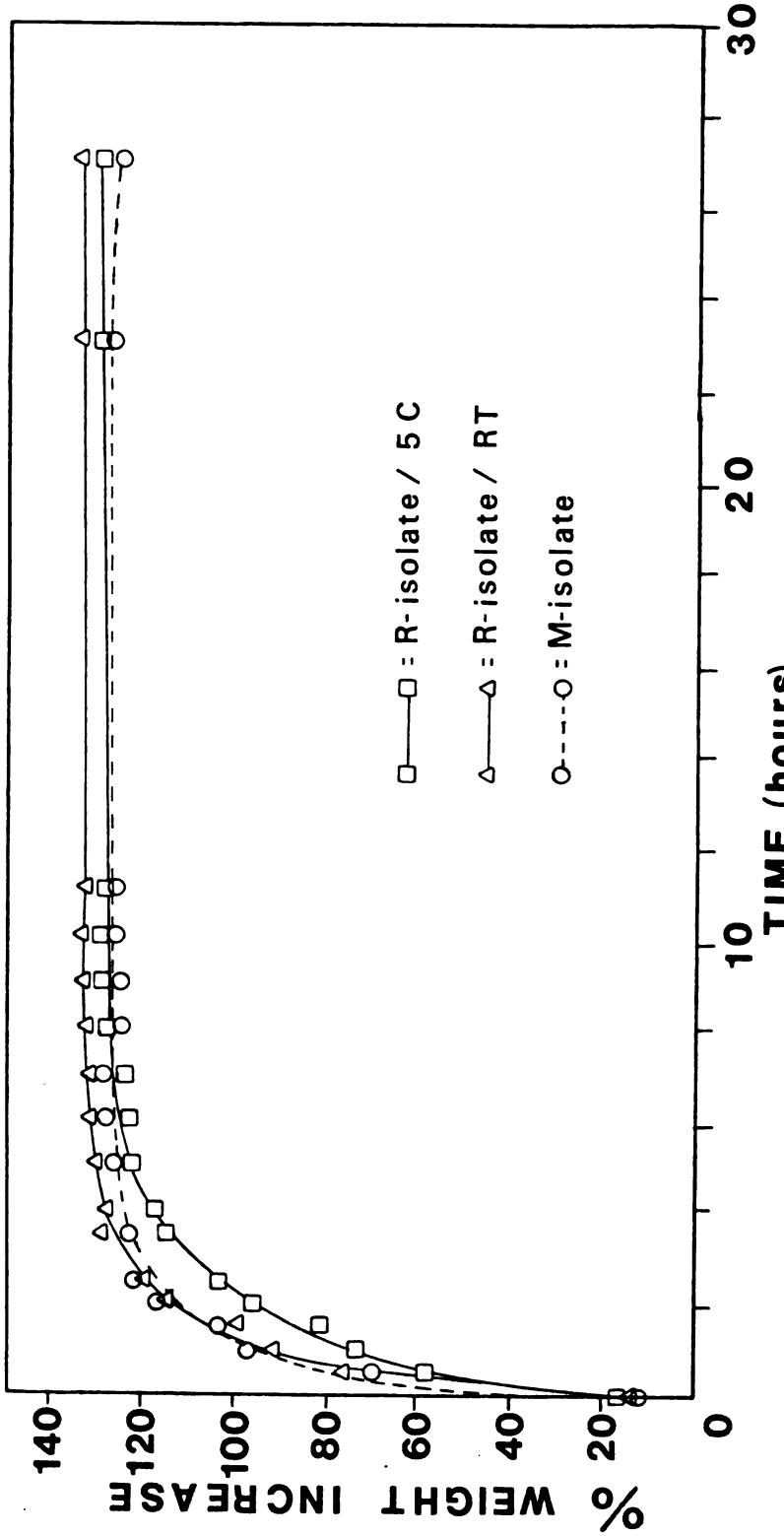

Figure 3. Rate of water imbibition (weight increase expressed as a percentage of the initial dry weight) by sclerotia of Sclerotinia sclerotiorum incubated in distilled water. Laboratory-grown R-isolate sclerotia were kept at 5 C or room temperature (RT) for 32 days prior to the experiment; M-isolate sclerotia were collected from bean elevator screenings. Four 700 mg replicates from each of the samples of sclerotia were included. Standard deviations of the means were between 0.1 and 3.6 percent.

1.32%, 5.0 ± 1.49%, and 14.5 ± 1.44% (mean of four replicates the standard deviation of the mean) of their initial dry weight after 118 h, respectively. Consequently, the actual percent moisture during the course of the experiment was slightly higher than indicated. At 118 h the weight of sclerotia was no greater than it had been at 27 h.

Similar amounts of water were imbibed in 48 h by sclerotia held at matric potentials from -0.5 to -10 bars (Figure 4). Weight increase was measured as a percentage of the initial fresh weight. In the previous experiment, R-isolate sclerotia stored at 5 C had an increase of 97% of the initial fresh weight after 48 h in distilled water (Ψ_w=0 bars).

Influence of water potential on carpogenic germination

In the first experiment, the number of germinated sclerotia in PEG 8000 solutions was highest at -1.0 bar, and approximately 30% germinated at -8.8 bars (Figure 5). Sclerotia rotted at the higher osmotic potentials, which affected the germination. In the second experiment, antibiotics were included; germination was 100% between 0 and -4.0 bars, and nearly 30% at -8.0 bars (Figure 5); there was no germination at -15.0 or -20.0 bars. Evaporative water loss during the experiments, which would have altered osmotic potential, was negligable.

In soil, 50% of the sclerotia germinated at -0.5 bars, and less than 10% germinated at matric potentials below -1 bar (Figure 4).

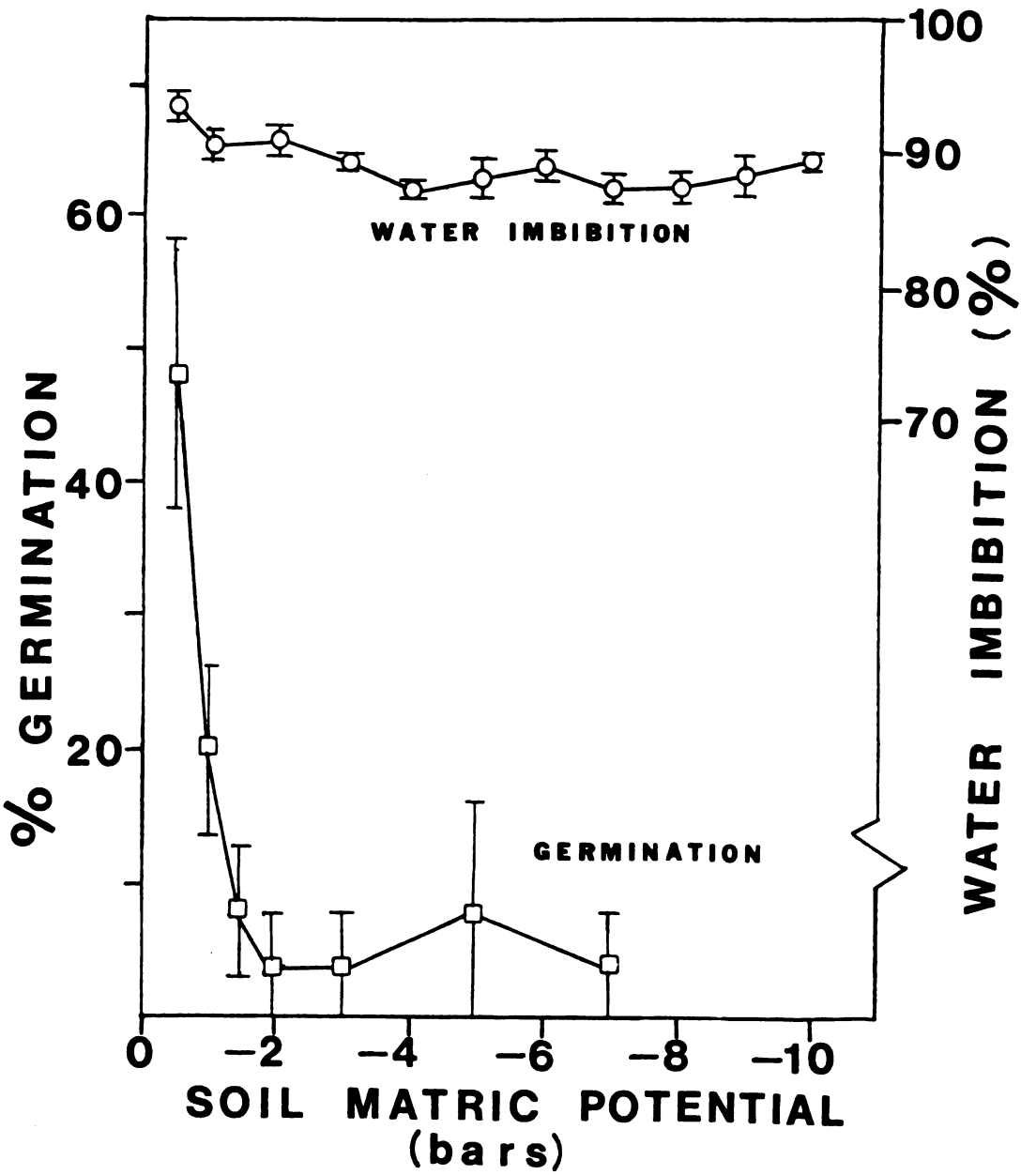

Figure 4. Effect of soil matric potential on carpogenic germination of sclerotia of Sclerotinia sclerotiorum (H-isolate) incubated for 30 days at 15 C, and on the amount of water imbibed by sclerotia after 48 h expressed as a percent of the initial fresh weight.

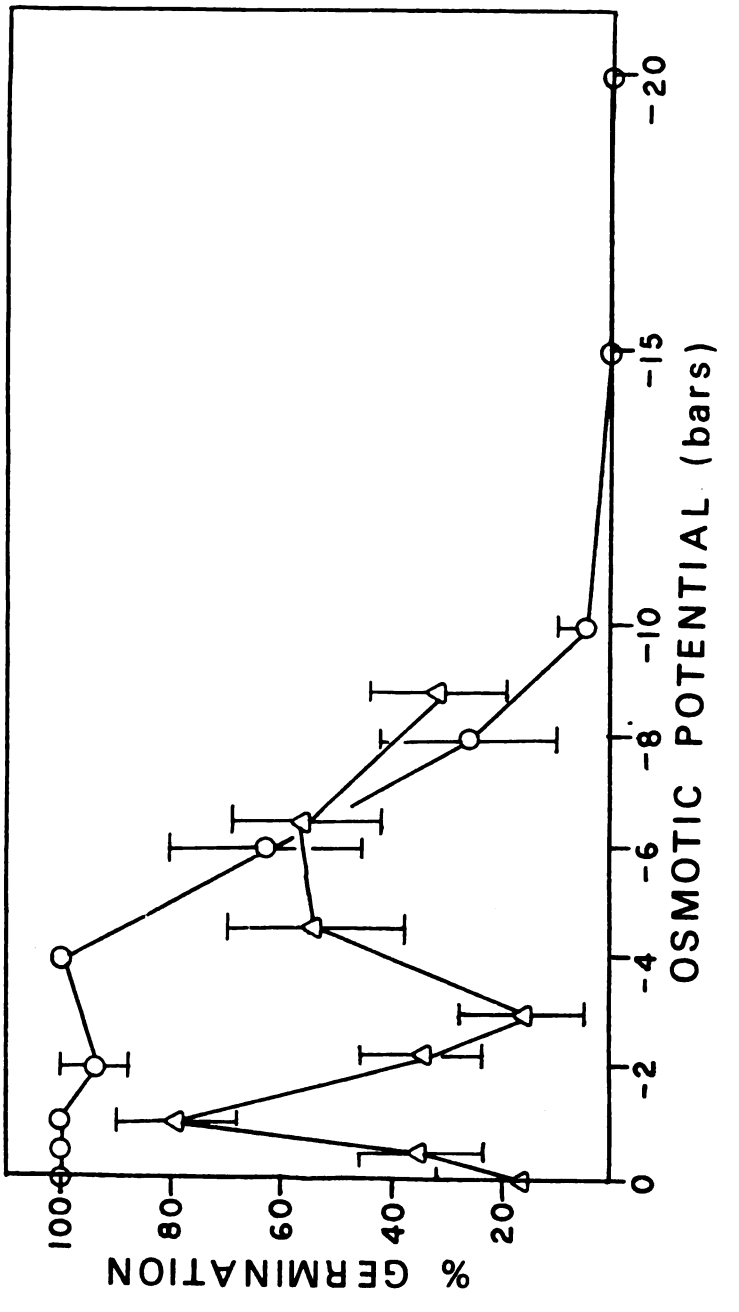

Figure 5. Effect of osmotic potential on carpogenic germination of unrotted sclerotia of Sclerotinia sclerotiorum in PEG 8000 solutions after 30 days at 15 C. (O) = A-isolate; (Δ) = H-isolate. Vertical bars equal twice the standard deviation of the mean.

DISCUSSION

The rate of water imbibition and dry weight lost upon soaking was similar for sclerotia (R-isolate) conditioned at 5 C or room temperature (Figure 3), suggesting that the 5 C treatment did not increase either the amount of soluble compounds able to diffuse out of sclerotia or the permeability of the membranes to such compounds. The M-isolate sclerotia gained weight at a rate similar to the R-isolate sclerotia. There was, however, three times the initial dry weight lost from M-isolate sclerotia compared to the R-isolate. Their final percent moisture was therefore higher than the R-isolate. Huang (11) reported greater leakage of amino acids from what he referred to as abnormal sclerotia: those having a severely fractured rind and a brown medullary region. The M-isolate sclerotia used in the present experiment had tan to grey medullae and were more brittle than the laboratory produced R-isolate sclerotia which had white medullae. The condition of the M-isolate sclerotia may have been due to the method of their storage at a bean elevator.

Since the maximum amount of water was imbibed by sclerotia after 7 h incubation in distilled water (Figure 3), a soaking period of 7 h was used for subsequent experiments to obtain a maximum amount of water imbibition with a limited amount of leakage of solutes, particularly in the water potential experiments, where imbibition of water

by sclerotia would have lowered the water potential of the incubation medium.

The different patterns of germination exhibited by sclerotia incubated in soil adjusted to specific matric potentials (Figure 4) and by sclerotia incubated in PEG 8000 solutions (Figure 5) of similar water potentials indicate that all properties of a system for maintaining specific water potentials must be considered. Aside from water potential, ion effects and characteristics such as diffusibility of active compounds within the system may have significant effects on the behavior of the organism under study. Phytophthora cinnamomi had reduced growth rate and increased susceptibility to water stress on matric-controlled as compared to osmotic-controlled agar media, and it was suggested that this response reflected differences between the two systems in solute transport (27). Phytophthora cinnamomi and Alternaria tenuis were less tolerent of soil matric potentials than of equivalent osmotic potentials of agar media controlled osmotically with KCl or sucrose (2). Growth of Fusarium roseum f. sp. cerealis was stimulated as the osmotic potential was lowered over the range, -1.5 to -8.2 bars; growth was not stimulated when soil matric potential was lowered over the same range (5).

The hypothesis that carpogenic germination of S. sclerotiorum was stimulated by the loss of inhibitory compounds through diffusion was supported by sclerotial

germination occurring at much lower solute potentials than soil matric potentials. The amount of water imbibed by sclerotia, however, decreased only slightly at the lower soil matric potentials (Figure 4), suggesting that the dry weight lost consisted, in part, of compounds which inhibited carpogenic germination. Although water imbibition was only slightly affected, the volume of soil water present at lower matric potentials may have been insufficient for the diffusion of inhibitory compounds away from sclerotia. Polyethylene glycol 8000 solutions provided a medium through which inhibitory compounds could diffuse, even at relatively low osmotic potentials. "Regermination" of sclerotia of S. sclerotiorum at -15 bars (21) indicates that sclerotia can imbibe enough water to support germination at this low water potential, provided germination has been initiated.

Morrall (22) reported 28% germination of sclerotia of S. sclerotiorum at a soil matric potential of -7.3 bars, while in the present study only 4% of sclerotia germinated at -7 bars matric potential (Figure 4). The higher germination rate reported by Morrall may have been due to saturating soil in dialysis bags prior to immersion in PEG 20,000 solutions of appropriate osmotic potentials. Three days were required for equilibration and the process was repeated during the experiment due to microbial decomposition of the bags. Loss of inhibitory compounds may have occurred during exposure to very high water potentials for several days during the incubation period. Sclerotia in the present study

germinated at osmotic potentials lower than those reported by Grogan and Abawi (9) or Morrall (21). This may be due to different types or volumes of osmotica used, or to fungal isolate differences.

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PART III: EVIDENCE FOR A DIFFUSIBLE ENDOGENOUS INHIBITOR OF CARPOGENIC GERMINATION

INTRODUCTION

Results of experiments reported in PART II suggest that carpogenic germination of Sclerotinia sclerotiorum is stimulated by diffusion of some compound(s) out of and away from sclerotia (see PART II, DISCUSSION). The composition of sclerotia (8, 9, 16, 17, 18) and amino acids leaked from sclerotia (7) have been examined, but there have been no reports of endogenous compounds influencing germination in this manner.

The present study was undertaken to determine: a) whether leaching sclerotia stimulated germination, and b) if incubating previously leached sclerotia in sclerotial leachate inhibited germination.

MATERIALS AND METHODS

Electrolyte leakage by surface-sterilized sclerotia

Loss of electrolytes was used to determine if changes in permeability of sclerotial membranes resulted from surface sterilization. Sclerotia were rinsed under running deionized water for 30 sec to remove surface material, air-dried for 2 h, then divided into six subsamples (approximately 730 mg fresh weight each) and weighed.

"Surface-sterilized" sclerotia were placed in a metal sieve and rinsed with 10 ml 95% ethanol, then 50 ml deionized water. Rinsed sclerotia were placed in 40 ml 0.5% NaOCl for 3 min, 40 ml sterile deionized water for 2 min, blotted dry and transfered to 15 ml fresh deionized water. Conductivity was measured with an electronic conductivity meter (Markson Science Inc., Del Mar, CA 92014) immediately and at 30 min intervals for 2.5 h. "Non-surface-sterilized" sclerotia were treated similarly, but deionized water was substituted for 95% ethanol and 0.5% NaOCl. Three replicates were included.

The experiment was repeated with sclerotia air-dried for 60 min after blotting and before soaking in 15 ml deionized water; conductivity was measured for three hours. In a third experiment, conductivity was measured over 43 h, and 100 ug/ml each of penicillin-G, streptomycin sulfate and neomycin sulfate was added to eliminate bacterial growth.

Incubation of sclerotia in soil, soil filtrate or water

Material diffusing from sclerotia was removed from, and a carbon source added to, several incubation media to determine the effect on carpogenic germination.

Sclerotia were incubated in sterile or non-sterile soil or soil filtrate, each with or without glucose amendment. Sterile soil was obtained by placing Capac sandy clay loam in glass beakers covered with aluminum foil and autoclaving at 121 C for 30 min, 3 times. Five surface-sterilized sclerotia (H-isolate) were placed on 50 g of sterile or non-

sterile soil in each of five replicate 90 x 15 mm plastic petri dishes and 20 ml sterile distilled water or sterile distilled water plus 1% glucose was added to each dish. The dishes were sealed with parafilm and incubated at 15 C.

To remove contaminants from sand to be used in other treatments, the sand was rinsed well with water and placed in an enamel bucket with enough hydrochloric acid (37%) added to just cover the sand. After 24 h of frequent mixing, the acid was poured off. The sand was then stirred under running distilled water until the pH of the runoff water was equal to the pH of the distilled water.

Soil filtrate was obtained by filtering five parts Capac soil mixed with two parts distilled water (w/v) through #1 Whatman paper in a Buchner funnel. Twenty milliliters of sterile or non-sterile soil filtrate or soil filtrate plus 1% glucose was added to each of several petri dishes containing 50 g acid washed, autoclaved sand. Five sclerotia were placed in each petri dish, and there were five replicates per treatment. The dishes were sealed and incubated as described previously.

Sclerotia were also incubated in water which was changed at regular intervals or not changed. Five sclerotia, 50 mg acid washed sand, and 20 ml sterile distilled water (with or without 1% glucose amendment) were added to each dish. Sclerotia in half of the dishes were kept in the same solution during the entire incubation period, and the remaining sclerotia were aseptically removed every 24-48 h

and replaced in fresh sterile distilled water or sterile distilled water plus 1% glucose. Changing of the solutions prevented the accumulation of any compounds that leaked from sclerotia. Five replicate dishes were incubated as described above.

Recovery of sclerotial leachate

Two grams of sclerotia (H-isolate) was rinsed with deionized water and placed in 75 ml sterile deionized water (two replicates). The solution was collected and replaced with fresh sterile deionized water each hour for the first 12 h, then every 12 h for five days. The samples were concentrated separately in a rotary exaporator at 45 C, dried under nitrogen and stored over calcium chloride, in vacuo, for 48 h.

Assay of sclerotial leachate for total carbohydrate

Because of similarities in the effects of glucose and sclerotial leachate in the incubation medium, sclerotial leachate was assayed for the presence of carbohydrate.

Three grams (fresh weight) of sclerotia (H-isolate) in each of three 150 ml flasks was rinsed by shaking in 50 ml sterile deionized water for 15 sec; the water was poured off and replaced with 50 ml fresh sterile deionized water. At 1, 3, 7, 12, 24, 48 and 72 h, the solution was poured off, collected, frozen at -5 C until needed, and replaced with fresh sterile deionized water. After thawing, each leachate sample was filtered, reduced to dryness in a rotary

evaporator at 45 C and resuspended in 40 ml deionized water (the 6-7 h leachate sample had to be diluted 4 times for the assay).

The leachate was assayed for total carbohydrate by the phenol method (4, 5). The absorbancy at 488 nm of the samples was measured on a Varian Series 634 spectrophotometer (Varian Aerograph, 2700 Mitchell Drive, Walnut Creek, CA 94598). Deionized water (for reagent blank) and glucose standards (25, 50, 75 and 100 ug/ml) were prepared and absorbancy measured in the same manner as the leachate samples. Samples were replicated three times.

Gas chromatography of sclerotial leachate

Alditol acetate derivatives of the sugars and polyols in the sclerotial leachate were prepared from the samples collected above. Aliquots containing 20 ug leachate were dried in 1 ml reaction vials under nitrogen. Twenty-five milliliters of freshly made sodium borohydride (20 mg/ml in 3 M ammonium hydroxide) was added to each vial and incubated for 1 h at 23 C. Glacial acetic acid (1-3 drops) was then added until bubbling ceased. After the addition of 100 ul methanol, the solution in each vial was carefully mixed on a vortex mixer and dried under nitrogen. This step was repeated. To each vial was then added 100 ul methanol:water (1:1); the solutions were mixed and dried under nitrogen. Another 100 ul methanol was added to each vial, the solutions mixed and dried. This last step was repeated twice. After the final drying, 50 ul acetic

anhydride was added to each vial and the vials were heated at 121 C for 1 h.

Two-microliter samples of derivatized leachate and standards were injected into a 6 ft x 2 mm glass column packed with 3% SP-2340 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823) in a Varian Aerograph Series 1200 gas chromatograph. A detector temperature of 300 C and inlet temperature of 230 C were used with a column temperature programmed from 180-275 C at 4 C/min. Nitrogen carrier gas was adjusted to a flow rate of 25 ml/min.

Effect of sclerotial leachate on carpogenic germination

Crude leachate was obtained by soaking sclerotia in distilled water for 82 h and filtering the resulting yellow solution through #1 Whatman paper. Sclerotia that had been leached for 13 days under running tap water were incubated on 25 g of washed and ignited sand in a 90 x 15 mm petri dish containing 10 ml of 58, 580 or 5800 ug leachate/ ml aqueous solution. Five sclerotia in each of 20 replicate dishes were incubated at 15 C.

Germinated sclerotia with short stipes were incubated in leachate solutions to determine if crude leachate had an effect on continued stipe elongation and continued stipe production in addition to its effect on initiation of carpogenic germination. Sclerotia (H-isolate) were germinated on moist soil, in the dark, at 15 C. Most sclerotia had germinated after 48 days and produced several stipes each. The number of stipes on germinated sclerotia

was counted and the sclerotia placed on a 3 mm bed of washed and ignited sand in six petri dishes, four sclerotia per dish. Ten milliliters of sclerotial leachate was added to each dish at the rate of 0, 0.29 or 2.9 mg leachate/ml. Included in all treatments were 100 ug/ml each of penicillin-G, streptomycin sulfate and neomycin sulfate. Sclerotia in 2 replicate sets of dishes were incubated at 15 C.

Removal of diffusible material from sclerotia and its effect on carpogenic germination

Sclerotia were leached on a layer of glass wool in a glass funnel under running tap water. Other sclerotia were spread in the bottoms of glass petri dishes and maintained at 100% relative humidity (RH) by positioning them above deionized water in sealed Mason jars. Paper towels lining the walls of the jars extended into the water to increase the surface area of the water. A third group of sclerotia was exposed to ambient relative humidity (=air-dry).

Sclerotia (H-isolate) were treated as above for 30 days. Leached, 100% RH and air-dry sclerotia were placed on a 3 mm bed of washed and ignited sand moistened with 6 ml distilled water in 100 x 15 mm plastic petri dishes, 10 sclerotia per dish, and 4 replicate dishes per treatment. The dishes were sealed with Parafilm and incubated at 15 C. The experiment was repeated using sclerotia (A and D-isolate) which were leached or held at 100% RH for 23 days prior to incubation on moist sand, with nine replicates per treatment.

RESULTS

Electrolyte leakage by surface-sterilized sclerotia

Surface-sterilized sclerotia leaked significantly more electrolytes in the first 30 min than non-surface-sterilized sclerotia (Figure 6). After the first 30 min, however, the differences were not significant. Results were similar when sclerotia were air-dried for 60 min prior to incubation and when conductivity was measured for 43 h.

Carpogenic germination of sclerotia incubated in soil, soil filtrate or water

Seventy-two percent of sclerotia incubated in non-sterile soil germinated carpogenically, while of the sclerotia incubated in non-sterile soil + glucose and sterile soil, only 12 and 16% germinated, respectively (Table 6). Four and 32% of the sclerotia rotted in the non-sterile soil and non-sterile + glucose, respectively. Of all the soil filtrate treatments, only the non-sterile soil filtrate treatment had any germination (8%) after 50 days, although 8% of the sclerotia rotted.

Fifty-two percent of the sclerotia which were incubated in regularly changed sterile distilled water germinated carpogenically. There was no carpogenic germination in the regularly changed sterile distilled water + glucose or either of the unchanged sterile distilled water treatments.

Some sclerotia in all treatments except sterile soil,

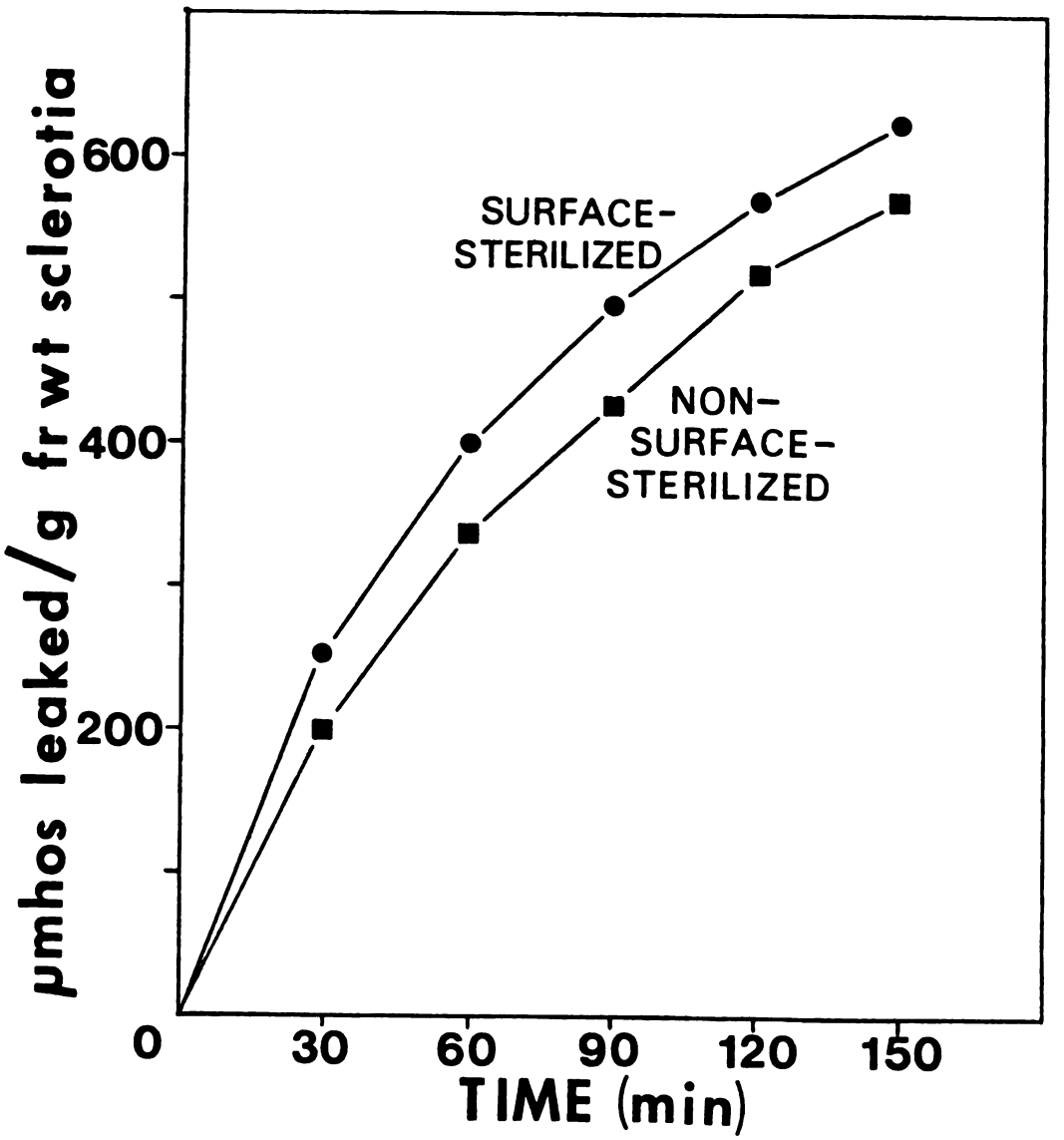


Figure 6. Electrolyte leakage from surface-sterilized and non-surface-sterilized sclerotia of Sclerotinia sclerotiorum. Surface-sterilized sclerotia were rinsed briefly in 95% ethanol, placed in 0.5% NaOCl for 3 min, then placed in deionized water for 2 min. Non-surface-sterilized sclerotia were treated similarly to surface-sterilized sclerotia, but with deionized water replacing 95% ethanol and 0.5% NaOCl. After treatment, each sample (400-750 mg) of sclerotia (3 replicates) was immediately incubated in 15 ml deionized water at 23 C.

Table 6. Carpogenic germination of sclerotia of Sclerotinia sclerotiorum after 50 days incubation at 15 C in sterile or non-sterile soil or soil filtrate, or in distilled water which was changed regularly or not changed. One percent glucose was added to a similar set of treatments.

Treatment[y]	Percent germination[x] - 1% glucose	+ 1% glucose
Soil + H_2O:		
Sterile	16 cd[z]	36 bc
Non-sterile	72 a	12 cd
Sand + soil filtrate:		
Sterile	0 d	0 d
Non-sterile	8 cd	0 d
Sand + sterile H_2O:		
Changed	52 ab	0 d
Not changed	0 d	0 d

[x] Means of 5 replicates.

[y] Five sclerotia were incubated on 50 g soil or sand plus 20 ml distilled water or soil filtrate in each petri dish (90 x 15 mm).

[z] Differences between entries with a common letter are not significantly different according to Tukey's test ($P=0.05$).

Analysis of variance

Source	df	Mean square	F
Treatment	11	2930.36	12.38 **
Error	48	236.67	

** Differences among treatments are significant at $P=0.01$.

non-sterile soil and regularly changed sterile distilled water germinated myceliogenically.

Recovery and assay of sclerotial leachate

There was 46 mg dry material/g dry wt sclerotia recovered from sclerotia soaked in sterile deionized water for 5 days; most of this material (35 mg/g dry wt) was recovered in the first 12 h, with 43 mg/g dry wt recovered in the initial 72 h (Figure 7).

For the total carbohydrate assay, the concentration of carbohydrates was expressed in ug glucose equivalents (ug glc eq), where

$$\text{no. ug glc eq/g sclerotia} = \text{(no. glucosyl units)} \times \frac{\text{volume of leachate (ml)}}{\text{mass of sclerotia (g)}} \quad (3)$$

where 1 glucosyl unit equals the absorbancy of 1 ug D-glucose/ml prepared by the phenol method.

The total detectable carbohydrate recovered in the first 12 h was 69% of that recovered in the first 72 h (4031 ug glc eq/g sclerotia) (Table 7). Therefore, detectable carbohydrate accounted for 8 and 9% of the total mass of leachate recovered after 12 and 72 h, respectively. These values are approximate since the phenol method will detect pentoses, hexoses, heptoses and amino sugars.

Of the carbohydrates detected by gas chromatography, glucose and mannose (or their polyol forms) were found in greatest abundance. Lesser concentrations of

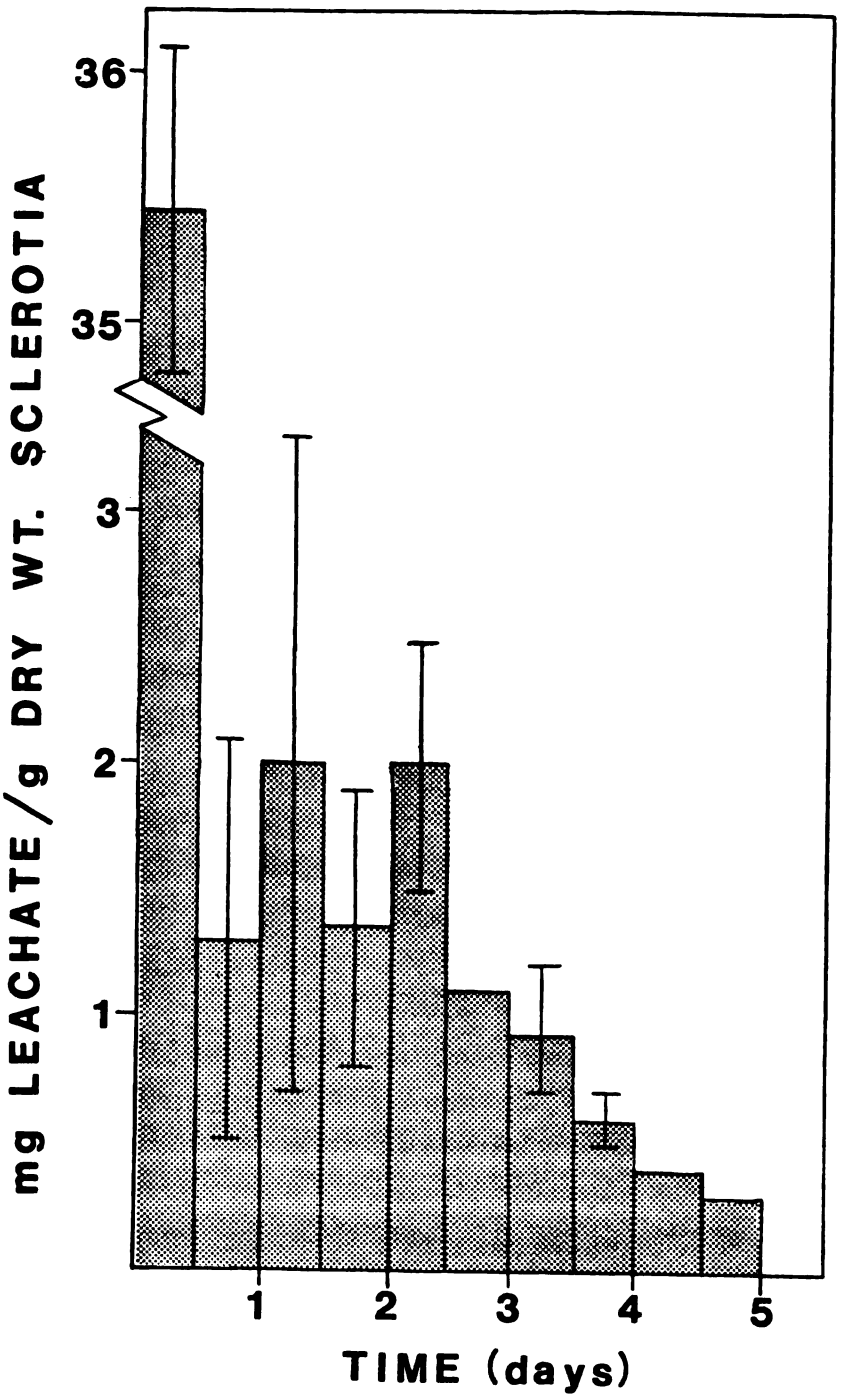


Figure 7. Recovery of leachate at 12 h intervals from sclerotia of Sclerotinia sclerotiorum incubated in sterile deionized water for five days. Vertical lines equal twice the standard deviation of the mean. Bars without vertical lines were not replicated.

Table 7. Total carbohydrates (detectable by phenol method) in sclerotial leachate samples[a] of Sclerotinia sclerotiorum collected over 72 h.

Sampling time (h)	ug glucose equivalents/ g sclerotia	ug glucose equivalents/ g sclerotia/ hour
0 - 1	342 ± 35.8[b]	342 ± 35.8
1 - 3	484 ± 10.3	242 ± 5.3
3 - 7	1233 ± 17.8	309 ± 19.1
7 - 12	726 ± 14.4	145 ± 3.2
12 - 24	611 ± 14.3	51 ± 4.1
24 - 48	473 ± 5.9	20 ± 0.3
48 - 72	162 ± 5.6	7 ± 0.3

[a] Leachate was obtained by soaking each of 3 replicate 2.7 g samples of sclerotia in 50 ml sterile deionized water. The leachate solutions were collected and replaced with fresh sterile deionized water at 1, 3, 7, 12, 24, 48 and 72 h.

[b] Means of 3 replicates ± the standard deviation of the mean.

glyceraldehyde, deoxyribose, arabinose, galactose and glucosamine (or their polyol forms) were also detected.

Germination of sclerotia incubated in sclerotial leachate

Leached sclerotia were incubated in 0, 58, 580 or 5800 ug leachate/ml. The lowest concentration used (58 ug/ml) was about 16% of that recovered in a previous experiment after 5 days (Figure 7), based on the mass of sclerotia incubated in the leachate solution. Germination in 580 ug leachate/ml was lower than that in controls, and only 1% germinated in 5800 ug leachate/ml after 35 days (Tables 8a, b). Increasing amounts of mycelium were produced in the leachate solutions as the concentration of leachate increased. Secondary sclerotia developed from sclerotia that germinated myceliogenically in the 5800 ug leachate/ml solution.

In a similar experiment, germinated sclerotia (H-isolate) with short stipes, but no apothecia, were incubated for 21 days in 0, 0.29 or 2.9 mg leachate/ml. Significantly fewer new stipes were produced after 14 days in 0.29 mg leachate/ml than in either water controls or 2.9 mg leachate/ml treatments (Table 9). By 21 days, however, differences were not significant. Stipes formed prior to incubation continued to elongate in crude leachate solutions as well as in controls.

Table 8a. Carpogenic germination of sclerotia of Sclerotinia sclerotiorum incubated in leachate solutions. The test sclerotia were leached under running tap water for 13 days prior to incubation. Leachate for the treatment was collected during 72 h from other sclerotia.

Leachate concentration	Percent of sclerotia germinating carpogenically after:[x]		
(ug/ ml)	14 days	23 days	34 days
0	18 a[y]	55 a	83 a
58	11 ab	39 ab	79 ab
580	0 b	21 bc	71 b
5800	0 b	0 c	1 c

[x] Means of 4 replicates. Each replicate consisted of 5 dishes, each dish containing 5 sclerotia, 25 g sand and 10 ml leachate solution.

[y] Per column, entries with a common letter are not significantly different according to Duncan's Multiple Range test ($P=0.05$).

Table 8b. Analyses of variances for carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* incubated in leachate solutions from Table 8.

Analysis of variance for 14 day data

Source	df	Mean square	F
Treatment	3	313.0	6.75 *
Error	12	47.67	

Analysis of variance for 23 day data

Source	df	Mean square	F
Treatment	3	2241.0	10.49 **
Error	12	213.67	

Analysis of variance for 34 day data

Source	df	Mean square	F
Treatment	3	5977.3	194.91 **
Source	12	30.67	

* Differences among treatments are significant at $P=0.05$.

** Differences among treatments are significant at $P=0.01$.

Table 9. New stipe production by sclerotia of Sclerotinia sclerotiorum incubated in sclerotial leachate solutions. The sclerotia were first germinated in soil.

Leachate concentration	No. new stipes/ dish after:[x]	
(mg/ ml)	14 days	21 days
0.0	44.0 b[y]	75.0 a
0.29	24.5 c	77.0 a
2.9	71.0 a	108.0 a

[x] Means based on 2 replicates, each consisting of 4 germinated sclerotia, 25 g sand and 10 ml sclerotial leachate solution in a 90 x 15 mm petri dish.

[y] Per column, entries with a common letter are not significantly different according to Duncan's Multiple Range test ($P=0.01$).

Analysis of variance for 14 day data

Source	df	Mean square	F
Treatment	2	1090.5	107.26 **
Error	3	10.17	

Analysis of variance for 21 day data

Source	df	Mean square	F
Treatment	2	684.7	<1 n.s.
Error	3	880.0	

** Differences among treatments are significant at $P=0.01$.

n.s. Differences among treatments are not significant.

Germination of leached and unleached sclerotia

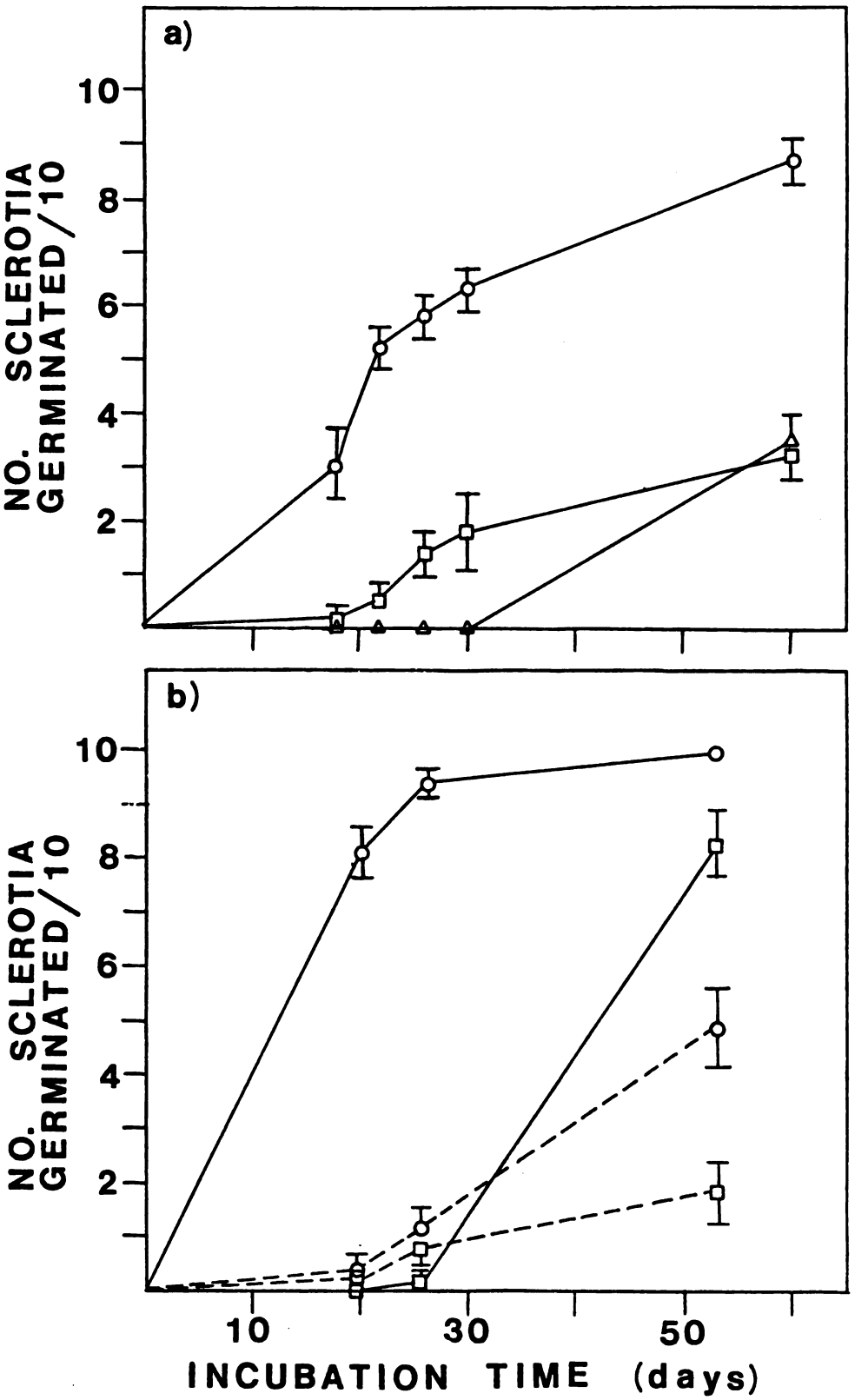
Leaching sclerotia under running tap water prior to incubation on moist sand enhanced carpogenic germination of all three isolates of S. sclerotiorum (Figure 8). After 20 days, 5, 40 amd 80 percent of the leached sclerotia of isolates D, H and A, respectively, had germinated, whereas less than 5% of each isolate had germinated when held at 100% RH or ambient RH prior to incubation. Forty-nine, 87 and 100 percent of the leached D-, H- and A-isolate sclerotia, respectively, had germinated after 60 days. Of the sclerotia held at 100% RH prior to incubation, only isolate A had greater than 50% germination .

DISCUSSION

It was hypothesized in PART II that the leakage of inhibitory compounds from sclerotia stimulated carpogenic germination. When material diffusing from sclerotia was removed from the incubation medium, either directly or via possible microbial sinks in non-sterile soil, carpogenic germination was higher than if this material was allowed to accumulate (Table 6). The addition of glucose also suppressed carpogenic germination in all treatments. Although glucose, as well as other sugars and/or polyols, was detected in sclerotial leachate, inhibition by other leachate components cannot be ruled out. Phenol-detectable

Figure 8. Effect of leaching on carpogenic germination of sclerotia of Sclerotinia sclerotiorum incubated on moist sand. (a), H-isolate sclerotia were leached under running tap water (○), held at 100% relative humidity (□), or held at ambient relative humidity (△) for 30 days prior to incubation. (b), A-isolate (——) or D-isolate (----) were leached (○) or held at 100% relative humidity (□) for 23 days prior to incubation. Vertical lines represent twice the standard deviation of the mean.

Figure 8.


a)
b)
NO. SCLEROTIA GERMINATED/10
10
8
6
4
2
10
30
50
INCUBATION TIME (days)

carbohydrate comprised only 9% of the total leachate (Table 7; Figure 7), although large amounts of trehalose and mannitol were found in ground and extracted sclerotia, with lesser amounts of arabitol, glycerol, glucose, galactose and traces of fructose, galactitol and arabinose (8, 17). Huang (7) detected seven amino acids in sclerotial leachate.

Carpogenic germination of sclerotia was almost completely inhibited by incubation in soil filtrate (Table 6). An exchange of ions between the soil solution and the colloidal fraction or organic matter in the soil may have removed inhibitory compounds from or altered the osmotic potential of the soil solution during incubation, accounting for the near absence of germination in soil filtrate.

The addition of leachate to the incubation solution inhibited carpogenic germination of sclerotia previously leached to remove readily diffusible compounds (Table 8a). It is unlikely that the inhibition of carpogenic germination was due to the lowering of osmotic potential by the leachate. Although the precise composition of the sclerotial leachate is unknown, limits can be set on the probable osmotic potential of the leachate solutions by the formula,

$$\Pi = icRT \qquad (4)$$

where Π is the osmotic pressure in bars, i is the average number of particles per molecule, c is the concentration of the solute in moles/liter, R is the gas constant (0.0832 bar

liter^{-1} mol^{-1} K^{-1}), and T is the absolute temperature in degrees K (15).

An upper limit can be set by assuming the leachate to be composed entirely of hexose, in which case the osmotic potential of the 5800 ug leachate/ml solution would be -0.77 bars. As an extreme lower limit, a 5800 ug NaCl/ml solution would have an osmotic potential of -4.76 bars. The actual osmotic potential of the 5800 ug/ml solution would lie between these limits, which is higher than the lowest osmotic potential at which carpogenic germination occurred (Figure 5).

Surface sterilization of sclerotia was necessary to prevent microbial degradation of sclerotial leachate. The effect of surface sterilization on permeability of sclerotial membranes was, therefore, of concern. Although surface-sterilized sclerotia initially leaked a greater amount of electrolytes than non-surface-sterilized sclerotia, there was no further difference after the first 30 min (Figure 6). This likely reflected changes in membrane permeability of only the outer layer of tissue from which solutes would diffuse most rapidly. Since stipe initiation occurs in medullary tissue (14), it was assumed that surface sterilization would not unduly affect the response of sclerotia to compounds inhibitory to carpogenic germination.

Sclerotial leachate did not appear to inhibit continued stipe production and elongation by previously germinated

sclerotia, suggesting that leachate may affect only the formation of apothecial initials and not subsequent events.

Leaching sclerotia prior to incubation (Figure 8) may enhance carpogenic germination by removing a specific inhibitory compound(s) or by depleting nutrient reserves. The presence of inhibitory compounds is suggested by inhibition of carpogenic germination of sclerotia in sclerotial leachate. Its also possible that sufficient nutrient reserves (possibly glucose) in sclerotia act as a suppressor to carpogenic germination. Sclerotia contain sufficient nutrients to support mycelial germination, since this event occurred when sclerotia were incubated in leachate, and unleached sclerotia sometimes germinate myceliogenicallywhen incubated in small volumes ofwater.

Suppression of germination of nutrient-independent fungal propagules by nutrient depletion has been reported (2, 3, 6, 11, 12). Germination was suppressed by incubation of such propagules on a bed of sand leached with water or phosphate buffer (6), or by incubation with bacteria or actinomycetes (10). Conidia rendered nutrient-dependent by leaching, germinated when incubated with spore exudate (2).

These reports concerned hyphal germination of fungal propagules, whereas two modes of germination of sclerotia of S. sclerotiorum are possible. Myceliogenic germination and mycelial growth are suppressed by an environment of low nutrient status (13), while carpogenic germination appears to be stimulated by an environment low in nutrients and by

depletion of endogenous compounds by leaching or utilization of such compounds by soil microorganisms (Table 6, 8; Figure 8). (Soil microorganisms might also affect the permeability of sclerotial membranes to endogenous inhibitory compounds.) This mechanism could enhance survival by preventing myceliogenic germination when exogenous nutrients are scarce or there is significant competition for existing nutrients. Under these conditions, ascospore production would permit dispersal of the fungus to environments possibly more conducive to growth of the organism. With leakage of endogenous material being greatest under wet conditions, the necessary moisture for ascospore germination and host invasion is likely to be present during conditions stimulatory to apothecia production. When exogenous nutrients are present and competition is low, sclerotia could germinate myceliogenically and spread through the soil to infect living plants or increase the number of propagules in the form of secondary sclerotia.

Sclerotia of S. sclerotiorum have been shown to interact with their environment by influencing the soil pH (Table 5), leaking carbohydrates and other compounds that might be utilized by other soil microorganisms (Table 7; Figure 7), and by the influence of leaching and microbial action on their germination (Table 6; Figure 8). The "dormancy" of sclerotia in the soil is not, therefore, a time of inactivity.

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PART IV: EFFECT OF HERBICIDES ON CARPOGENIC GERMINATION AND APOTHECIAL DEVELOPMENT

PART IV examines the specific effects by atrazine and other herbicides having similar modes of action in plants, on carpogenic germination and apothecial development .

MATERIALS AND METHODS

Mycelial growth on herbicide-amended agar media

Aatrex [atrazine] (Ciba-Geigy), Princep [simazine] (Ciba-Geigy), Lexone [metribuzin] (Mobay) and Karmex [diuron: 3-(3,4-dichlorophenyl)-1,1-dimethylurea] (Dupont) were diluted to 5, 50 and 500 ug a.i./ml in 1% Bacto-agar (Difco):water, autoclaved at 121 C for 20 min, poured into petri dishes and allowed to cool. A 5 mm diameter mycelial disc of S. sclerotiorum was placed in the center of each plate with the mycelium in contact with the surface of the medium. Four replicates at each herbicide concentration and 1% Bacto-agar control were placed under fluorescent lights at 23 C. Colony diameters were determined after 3 days by taking the mean of two measurments at right angles to each other.

Effect of herbicide-amended soil on carpogenic germination

Stock solutions (50 mg a.i./ml distilled water) of Aatrex, Princep and Lexone were diluted with distilled water to 25, 5.0 and 2.5 mg a.i./ml. Ten milliliters of herbicide solution or distilled water control was added to 50 g oven-dried Capac sandy clay loam soil in each 90 x 15 mm petri

dish. Ten sclerotia were placed on the soil surface in each dish (2 replicates) and incubated in plastic bags in the dark at 15 C. The dishes were placed under fluorescent light (2800 lux, 14 h photoperiod) at 15 C after 35 days incubation. Eighteen days after exposure to light (53 days after beginning incubation) the sclerotia in one replicate were washed under running distilled water for 1 min and placed in a petri dish with 20 ml distilled water (replaced with fresh distilled water after 24, 48 and 84 h) to determine whether the herbicides had residual effects on apothecia development. All sclerotia were incubated under fluorescent lights at 15 C. A sclerotium was considered carpogenically germinated if at least one stipe had broken through the rind.

A similar experiment was performed using lower concentrations of herbicides and substituting Karmex for Princep (because of its similarity to atrazine, simazine [Princep] was excluded from several experiments). Stock solutions (2.5 mg a.i./ml distilled water) of Aatrex, Lexone and Karmex were diluted stepwise to 500, 100, 20 and 4 ug a.i./ml. Ten milliliters of herbicide solution or distilled water control was added to 50 g oven-dried soil in each petri dish. Ten sclerotia were placed on the surface of the herbicide-amended soil (four replicates) in petri dishes, placed in plastic bags and incubated in the dark at 15 C. After 28 days, the dishes were sealed with Parafilm, two

replicates placed under fluorescent light and two replicates left in the dark at 15 C.

Effect of analytical-grade herbicides on carpogenic germination

To confirm that carpogenic germination and apothecial development were being affected by the active herbicide and not another compound in the formulations, sclerotia were incubated in solutions of analytical-grade atrazine, metribuzin and diuron. Atrazine was insoluble and diuron only slightly soluble in water, but both were soluble in methanol:water. Sclerotia were incubated in 0, 1, 2, 3, 4 and 5% methanol:water to determine a non-toxic methanol concentration.

Atrazine (5mM in methanol) was slowly diluted 100 times with distilled water. The resulting 50 uM atrazine in 1% methanol solution was diluted with 1% methanol to 50, 10, and 2 uM atrazine. Ten sclerotia were placed on 25 g washed and ignited sand with 10 ml atrazine solution or 1% methanol control (two replicates) in each of eight 90 x 15 mm petri dishes. The dishes were sealed with Parafilm and incubated under fluorescent light (2800 lux, 14 h photoperiod) at 15 C.

In another experiment, sclerotia were incubated on 25 g washed and ignited sand in 90 x 15 mm petri dishes containing 10 ml of 0, 2, 4, 6, 8 or 10 uM atrazine, or 10, 50 or 100 uM metribuzin or diuron in 1% methanol. The

sclerotia were incubated in the dark at 15 C for 65 days, then placed under fluorescent lights at 15 C.

Effect of atrazine on apothecial disc development

Carpogenically germinated sclerotia bearing stipes without apothecia, and with the apothecial disc beginning to expand were soaked in either 1% methanol or 50 uM atrazine in 1% methanol for 30 min. Sclerotia were then rinsed in distilled water, placed on 10 g washed and ignited sand in each of several 60 x 15 mm petri dishes containing 4 ml distilled water (four sclerotia per dish) and incubated under fluorescent light at 15 C.

RESULTS

Mycelial growth on herbicide-amended agar media

Colony diameters of S. sclerotiorum in agar containing atrazine, simazine and metribuzin treatments at 5 and 50 ug/ml, and diuron at 5 ug/ml were not significantly different than the control, but mycelial growth was significantly slower at higher concentrations (Table 10).

Effect of herbicide-amended soil on carpogenic germination

In the first experiment, there was 90-100% carpogenic germination of sclerotia incubated in soil amended with atrazine, simazine or in unamended soil controls (Table 11). None of the sclerotia incubated in the metribuzin-amended

Table 10. Growth of Sclerotinia sclerotiorum on herbicide amended 1% Bacto agar media after three days. Media were inoculated with 5 mm diameter mycelial discs.

Herbicide	Concentration (ug/ ml)	Colony diamter (mm)[x]
Atrazine	5	50.6 ab[y]
	50	46.0 bc
	500	15.6 e
Simazine	5	50.8 ab
	50	51.9 a
	500	40.5 cd
Metribuzin	5	51.3 ab
	50	46.8 ab
	500	7.1 f
Diuron	5	51.6 a
	50	37.8 d
	500	0.0 g
Control	---	49.9 ab

[x] Mean of four replicates.

[y] Entries with a common letter are not significantly different according to Tukey's test ($P=0.05$).

Analysis of variance

Source	df	Mean square	F
Treatment	12	1352.975	271.81 **
Error	39	4.978	

** Differences among treatments are significant at $P=0.01$.

Table 11. Effect of herbicide-amended soil[x] on carpogenic germination and apothecial development of Sclerotinia sclerotiorum. Sclerotia were removed from herbicide-amended soil after 53 days, and placed in distilled water.

		In herbicide-amended soil 53 days				In herbicide amended soil 53 days, then distilled water 29 days		
			No./ 20 sclerotia				No./ 10 sclerotia	
Herbicide	Concentration (mg a.i./ g soil)	% Germination	Stipes	Apothecia	Secondary sclerotia	% Germination	Stipes	Apothecia
Control	---	95	41 b[y]	0	0	---	---	---
Atrazine	0.5	100	58 ab	0	0	100	64	20
	10.0	100	81 a	0	0	100	83	8
Simazine	0.5	90	52 ab	0	0	ND[z]	ND	ND
	10.0	100	43 b	0	0	100	41	10
Metribuzin	0.5	0	0 c	0	21	0	0	0
	10.0	0	0 c	0	6	10	3	0

[x] Ten sclerotia were incubated in each 90 x 15 mm petri dish containing 50 g soil and 10 ml herbicide solution.

[y] Differences between entries with a common letter are not significant according to Tukey's test ($P=0.05$).

[z] ND = not determined.

soils germinated carpogenically, but at 0.5 and 1.0 mg/g soil several germinated with mycelium that grew across the soil surface to produce secondary sclerotia. Although many stipes were produced by sclerotia in the atrazine and simazine treatments, mature apothecia developed only in the unamended control after 53 days. Sclerotia removed from the atrazine and simazine amended soil after 53 days, produced only abnormally formed apothecia (Figure 9C).

In the second experiment, all of the stipes produced by sclerotia incubated in 20-500 ug atrazine/g soil were darkened, abnormally formed and produced no apothecia when exposed to light for 18 days (Table 12; Figure 9B). Nearly all stipes produced by sclerotia in >0.8 ug atrazine/g soil were abnormal, and only abnormal apothecia developed. There was no carpogenic germination of sclerotia in 500 ug metribuzin/g soil, and normal stipes but no apothecia were produced by sclerotia in 100 ug metribuzin/g soil. Sclerotia in all other treatments produced normal stipes and apothecia (Table 12; Figure 9A).

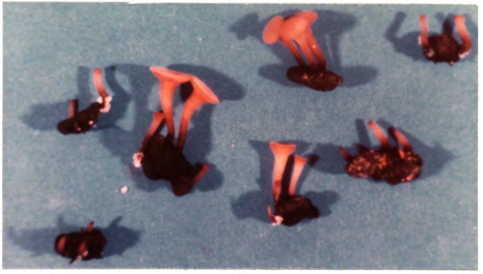
Effect of analytical-grade herbicides on carpogenic germination

Sclerotia incubated in 1% methanol:water for 17 days germinated at a slightly lower rate (75 ± 5%) than those incubated in water (95 ± 5%), but produced normal apothecia. Rates of carpogenic germination for sclerotia incubated in 0, 2, 10 and 50 uM atrazine in 1% methanol:water were not significantly different after 17 days (Table 13). Stipes

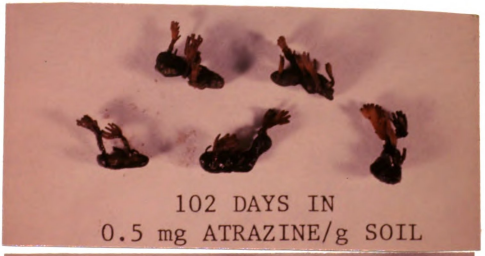
Figure 9. (A): Normal stipes and apothecia produced by sclerotia of Sclerotinia sclerotiorum incubated in water saturated soil at 15 C, under fluorescent light. (B): Abnormally formed, multiple-branched stipes of sclerotia incubated in atrazine amended soil at 15 C, under fluorescent light. (C): Abnormal apothecia produced by sclerotia incubated as in (B) for 53 days, then placed in distilled water at 15 C, under fluorescent light.

Figure 9.

A)



B)


102 DAYS IN
0.5 mg ATRAZINE/g SOIL

C)

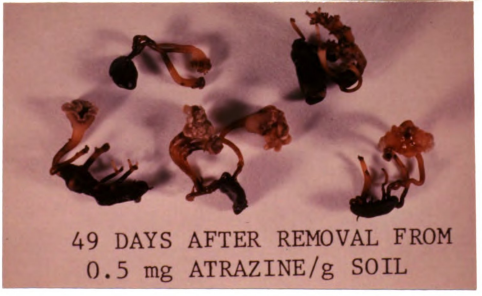

49 DAYS AFTER REMOVAL FROM
0.5 mg ATRAZINE/g SOIL

Table 12. Effect of herbicide amended soil on carpogenic germination and apothecial development of Sclerotinia sclerotiorum. Sclerotia were incubated for 28 days in the dark, then 18 days under flourescent light, at 15 C.

Herbicide[x]	Conc. (ug a.i./ g soil	Percent germination of unrotted sclerotia[y]	Total no./ 20 sclerotia: Stipes Normal	Stipes Abnormal	Apothecia Normal	Apothecia Abnormal
Control	---	50 abc[z]	16	0	1	0
Atrazine	0.8	35 abcde	9	0	2	0
	4.0	65 a	2	19	0	4
	20.0	5 de	0	1	0	0
	100.0	55 ab	0	18	0	0
	500.0	22 bcde	0	6	0	0
Metribuzin	0.8	40 abcd	11	0	6	1
	4.0	50 abc	18	0	13	0
	20.0	48 abc	17	0	1	0
	100.0	16 cde	4	0	0	0
	500.0	0 e	0	0	0	0
Diuron	0.8	17 bcde	4	0	3	0
	4.0	20 bcde	4	0	3	0
	20.0	45 abc	12	0	3	0
	100.0	28 bcde	10	0	3	0
	500.0	20 bcde	5	0	2	0

[x] Ten sclerotia were incubated in each 90 x 15 mm petri dish containing 50 g soil and 10 ml herbicide solution.

[y] Means of two replicates.

[z] Differences between entries with a common letter are not significant according to Duncan's Multiple Range test ($P=0.05$).

Analysis of variance for % germination

Source	df	Mean square	F
Treatment	15	920.33	3.49 *
Error	16	263.44	

* Differences among treatments are significant at $P=0.05$.

Table 13. Effect of atrazine on carpogenic germination and apothecial development of Sclerotinia sclerotiorum. Ten sclerotia were incubated in each petri dish containing 10 ml atrazine in 1% methanol:water, under fluorescent light at 15 C, for 17 days.

Atrazine concentration (uM)	Percent germination	Total no./ 20 sclerotia			
		Stipes		Apothecia	
		Normal	Abnormal[a]	Normal	Abnormal[b]
0	75[c]	41	0	33	0
2	70	44	0	21	7
10	70	0	64	0	26
50	95	0	118	0	4

[a] Abnormal stipes were darkened and misshapen (Figure 10B).

[b] Abnormal apothecia were malformed and sterile (Figures 10C, 11).

[c] Mean of 2 replicates.

Analysis of variance for percent germination

Source	df	Mean square	F
Treatment	3	283.33	1.03 n.s.
Error	4	275.00	

n.s. Differences among treatments are not significant at $P=0.05$.

produced by sclerotia in 1% methanol controls and 2 uM atrazine were light colored and shaped normally (Figure 10A); stipes of sclerotia in 10 and 50 uM atrazine were darkened, and abnormally shaped (Figure 10B). While all of the apothecia produced by sclerotia in the 1% methanol control were normal, 25, 100 and 100% of apothecia produced by sclerotia in 2, 10 and 50 uM atrazine, respectively, were malformed and sterile (Table 13; Figures 10, 11).

Results were similar when sclerotia were incubated in 2, 4, 6, 8 and 10 uM atrazine (nearly all apothecia produced in >4 uM atrazine were abnormal), while sclerotia in 10, 50 and 100 uM metribuzin or diuron produced all normal apothecia (data not shown).

Effect of atrazine on apothecial disc development

The hymenium of apothecia soaked in 50 uM atrazine for 30 min darkened after 2 days, although stipes appeared normal. Numerous stipes grew from the hymenial surface of these apothecia after 10 days, and each developed a malformed apothecium (Figure 12A, B). Stipes soaked in atrazine appeared normal after 2 days, but developed highly distorted apothecia by 10 days (Figure 12C). Sclerotia soaked in 1% methanol control developed normal apothecia (Figure 12A).

Figure 10. (A): Normal stipe (top) and apothecium produced by sclerotium of Sclerotinia sclerotiorum incubated in 1% methanol:water at 15 C, under fluorescent light. (B): Abnormal stipes produced by sclerotium incubated in 10 uM atrazine in 1% methanol:water at 15 C, under fluorescent light. (C): Abnormal apothecia produced by sclerotium incubated as in (B).

Figure 10.

A)

B)

C)

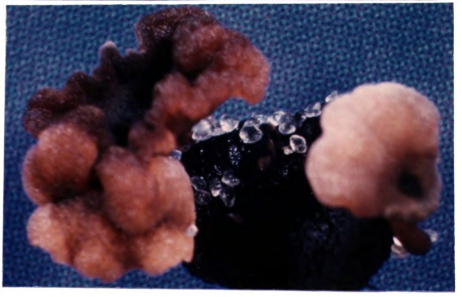
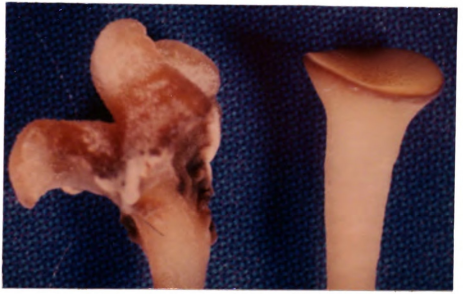


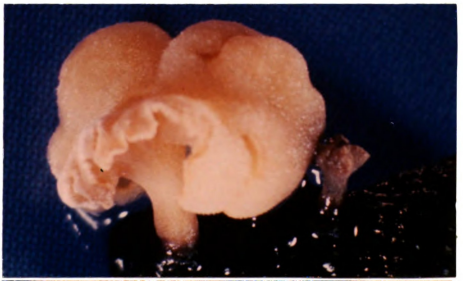
Figure 11. (A): Normal (right) and abnormal apothecia produced by sclerotia of Sclerotinia sclerotiorum incubated in 0 and 10 uM atrazine, respectively, in 1% methanol:water at 15 C, under fluorescent light. (B) and (C): Distorted and unexpanded apothecia of sclerotia incubated as in (A).

Figure 11.

A)



B)



C)

Figure 12. (A): Normally (left) and abnormally developed apothecia of Sclerotinia sclerotiorum soaked in 0 and 50 uM atrazine, respectively, in 1% methanol:water for 30 min prior to incubation in distilled water at 15 C, under fluorescent light, for 10 days. (B): Darkened hymenia of apothecia shown in (A), with numerous stipes growing from their surface; early stage (left) and later stage with development of malformed apothecia. (C): Extensive branching and aborted apothecia produced by stipe soaked in 50 uM atrazine in 1% methanol:water as in (A).

Figure 12.

A)

B)

C)

DISCUSSION

Studies on the effect of herbicides on soil-borne plant pathogens have been reviewed by Kaiser et al (11) and Katan and Eshel (12). Atrazine stimulated growth (17, 18, 20, 24), respiration (6, 23) or glucose catabolism (26) of some fungi at low concentrations (about 10 ug/ml), but was often inhibitory at higher concentrations (20, 21, 22, 23). Some fungi have been found to utilize simazine as a nutrient (13).

None of the herbicides tested in the present study stimulated mycelial growth on agar. Metribuzin, a triazine with an asymmetric ring, had a different effect on germination of sclerotia than did atrazine or simazine (symmetric triazines). Metribuzin prevented carpogenic germination, but there was considerable mycelial growth across the soil and secondary sclerotia were produced. It does not seem likely that metribuzin directly stimulated mycelial growth, since mycelial growth was not stimulated on metribuzin-amended agar. It is possible that metribuzin acted indirectly through suppression of antagonistic or competitive microorganisms, or by the microbial breakdown of metribuzin into compounds that could be metabolized by S. sclerotiorum. Diuron (a substituted urea) inhibited mycelial growth and carpogenic germination.

Based on my definition of carpogenic germination as a single stipe breaking through the rind, atrazine and

simazine did not affect carpogenic germination. The stipes produced, however, by sclerotia incubated in atrazine- or simazine-amended soil were distorted, highly branched, and apothecial discs were difficult to distinquish or were absent (Figure 9B). Removal of sclerotia from the atrazine- and simazine-amended soils or incubation in low concentrations of atrazine in 1% methanol diminished the effect of the herbicides and differentiation of apothecial discs occurred, but these discs were morphologically abnormal and sterile (Figures 9C, 10, 11).

Direct application of atrazine to apothecia resulted in abortion of the hymenium and the growth of numerous stipes from the hymenium; stipes soaked in atrazine branched repeatedly and developed aborted apothecia (Figure 12).

All of the herbicides used in this study inhibit photosynthetic electron transport in plants (1), probably by affecting a quinone step. Atrazine and simazine were the only compounds that differentially allowed carpogenic germination while inhibiting normal apothecial development. Apothecial disc expansion is light dependent, and atrazine may affect the photosensing mechanism. This could not be the only site of action, however, since stipes grown in the dark were deformed. Carlile (3) suggested that the activity of respiratory enzymes may be indirectly affected by light. If apothecial disc expansion is associated with increased respiration (and the energy required for cell division and growth would undoubtably require it), the possibility that

atrazine is inhibiting electron-transport would help explain the affect on normal development.

Branching of stipes after decapitation (9, 14), and abnormal apothecia produced spontaneously during 15-20 C incubation (14), or elicited by dichloropropene dichloropropane (7, 16), uranium nitrate (2), and cadmium salts (4, 5) have been reported previously. The abortion of the hymenium with subsequent production of numerous stipes from the hymenial surface after application of atrazine has not been previously reported.

This study indicates that triazine herbicides are potentially useful to study the mechanism of apothecial disc differentiation, and possibly as a means of reducing apothecial development of S. sclerotiorum in the field.

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