

## Physical Factors that Influence the Recovery of *Microsclerotium* Populations of *Cylindrocladium crotalariae* from Naturally Infested Soils

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### ABSTRACT

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Air-drying of soil (0.12 to 0.38% water or about -2,000 bars) resulted in no recovery of microsclerotia from soils, but rewetting soils to near field capacity for 1 to 4 wk before assay resulted in partial recovery from the deleterious effect of drying. Incubation of soil at low temperature (6 C) greatly reduced recovery of microsclerotia from soils. No microsclerotia were recovered from one soil incubated at 6 C for 1 mo. Incubation at 26 C had little or no effect on microsclerotium recovery. Tests indicated that many small

microsclerotia were present in Virginia peanut-field soils. The median 90% of microsclerotia recovered had a width in the range 25 to 103  $\mu\text{m}$ ; use of a sieve with a pore size of 25  $\mu\text{m}$  or slightly smaller is recommended for recovery of microsclerotia. Little or no interference from conidium-or ascospore-size propagules in soil was found in assays using this sieve. Soil dispersion by blending or shaking was not essential for assaying sandy peanut-field soils containing most microsclerotia free of organic matter.

*Additional key words:* *Cylindrocladium* black rot, microsclerotium survival, peanut.

Accurate estimations of the populations of plant pathogenic fungi in naturally infested soils are critical for assessment of pathogen survival and for understanding the relation of inoculum density to plant disease incidence and severity under field conditions. Accurate estimations of germinable populations or recovery of fungi from soil and plant tissue may be influenced by several field and laboratory physical factors (1, 2, 3, 6, 8, 14, 15). Inoculum of *Cylindrocladium* spp. in field soils and forest-nursery seedbeds may be subjected to seasonal and diurnal fluctuations in soil temperature and soil moisture; desiccation, for example, is believed to be lethal to the microsclerotia of *Cylindrocladium scoparium* (14). Physical factors may be important during transport or storage of soil. Refrigeration (0 to 7 C), for instance, has been employed routinely for storing soil for up to 1 mo, previous to assays of *Cylindrocladium* microsclerotia (5, 9, 10, 13) and microorganism populations (6, 15). Minimal changes in populations and soil water generally are believed to occur under refrigeration.

Effective physical separation of individual pathogen propagules on soil-dilution plates by soil dispersion methods (6, 15), and segregation of multiple propagule types (e.g., microsclerotia, conidia and ascospores for *Cylindrocladium* spp.) are important in accurate population estimates for many types of ecological (e.g., survival) studies with soilborne pathogens. Wet-sieving and selective-plateing have been employed in the isolation

of *Cylindrocladium* microsclerotia from field soils (7, 11, 14); standard dilution plating has been used to estimate total *Cylindrocladium* populations (4). Proper selection of sieve size may be critical to recovery of small microsclerotia from soil, as well as to minimizing interference from other *Cylindrocladium* propagules such as ascospores and conidia. This paper presents information on the importance of soil drying and rewetting, method of soil dispersion, temperature of soil incubation, soil-sieve size, and possible interference from ascospore- and conidium-sized propagules in estimations of microsclerotium populations of *Cylindrocladium crotalariae* (Loos) Bell and Sobers in naturally infested peanut-field soils. Only limited information presently exists on the importance of these factors. A short paper has been presented on some aspects of the work presented here (4).

### MATERIALS AND METHODS

Soils naturally infested with *C. crotalariae* were located by using aerial photography to detect active centers of *Cylindrocladium* black rot in Virginia peanut fields during the 1974 and 1975 growing seasons (12). These fields were visited on the ground and soil cores (0-15 cm in depth) were obtained from infested areas of the fields after peanut harvesting in 1974 and during the growing season prior to harvesting in 1975. Soil samples were placed in plastic bags with pinholes for gas exchange, and were stored in the laboratory at 27-29 C before use, unless otherwise indicated. Soils were mixed thoroughly by

hand for 30 min before use in experiments.

In general, the wet-sieving and dilution-plating method of Krigsvold and Griffin (7) was used to isolate *C. crotalariae* microsclerotia and similar-sized propagules from naturally infested soils. Sucrose-TBZ (thiabendazole) medium (7) was used at pH 4 in most instances. Oxgall concentration in this medium was increased from 1 to 4 g/liter because recently purchased lots of this substance were found to contain less active principle. In most experiments soil dispersion was accomplished by blending soil suspensions ( $10^{-1}$  dilution) in a Waring Blender for 15 sec at high speed. Two nested sieves with 44- $\mu$ m- and 25- $\mu$ m-square openings were used in most tests; the diagonal measurements of these square openings are 60.8  $\mu$ m and 35.5  $\mu$ m, respectively. Only the former sieve was used by us (7) and Thies and Patton (14) previously. A sieve with 150- $\mu$ m square openings was used in some experiments. A 4.8-mm sieve was used on top of all sieves to prevent loss of soil by splashing during soil washing. Soil suspensions were agitated with a magnetic stirring bar during the pipetting of soil onto dilution plates. Estimates of microsclerotium populations were based on colonies of *C. crotalariae* counted on 10 dilution-plates, prepared from each of two, 22-g soil samples, unless otherwise indicated. Colonies were counted after 5 and 7 days incubation at room temperature (25-28 C).

## RESULTS AND DISCUSSION

**Influence of soil-dispersion method on recovery of microsclerotia from naturally infested soils.**—Several microsclerotia in the same particle of organic matter (11), or those which are loosely associated, would give rise to only one colony on soil-dilution plates. This is undesirable for propagule survival studies. Blending soil suspensions (7, 14) may prevent this and has the advantage over shaking, for soil dispersion, as it requires less time. The results of two experiments (Table 1) suggested that blending (for 15 sec or 2.5 min) decreased the percentage of microsclerotia recovered on the larger

(44- $\mu$ m) sieve compared to no-blend or shake treatments, but total populations (sum of populations on both sieves) were similar in experiment A and slightly lower for blending treatments in B. Thus, use of the larger sieve only with blending (7, 14) would yield low recovery of microsclerotia from soils.

**The size of microsclerotia and microsclerotium-bearing organic matter recovered from naturally infested soils.**—Microscopic observations were made on the origin of colonies of *C. crotalariae* developing on sucrose-TBZ medium seeded with soil fractions collected on the 25- $\mu$ m sieve and on the 44- $\mu$ m sieve. Observations were made early in colony development and 30 to 40 observations were made for each of the two sieve fractions and for each of four field soils. Additional and equal numbers of observations were made for comparable soil subsamples dispersed by blending (15 sec) for two of the field soils, and for all four fields using the 150- $\mu$ m sieve with no blending of soil. For the 44- $\mu$ m-sieve fractions, 51.6 to 93.8% (mean = 70.5%) of the colonies of *C. crotalariae* from the four fields originated from microsclerotia. For the 25- $\mu$ m sieve fractions, 52.6 to 93.8% (mean = 80.0%) of the colonies of *C. crotalariae* originated from microsclerotia. The origin of the remaining colonies was unclear, because the propagules were obscured by small sand grains or soil debris. Separate tests indicated that other possible propagules of *C. crotalariae* (conidia, 4.8-7.1  $\times$  58-107  $\mu$ m, or ascospores, 6.3-7.8  $\times$  34-58  $\mu$ m) were probably not caught on the sieves nor the origin of the colonies. When conidia were added to nonsterile, *Cylindrocladium*-free, peanut-field soil ( $9 \times 10^4$  conidia/g soil) and assayed by the wet-sieving technique (25- $\mu$ m sieve), few colonies of *C. crotalariae* occurred on soil-dilution plates. In two experiments only 0.05% and 0.002% recovery of conidia was obtained, but direct plating of the soils, without sieving, yielded the expected populations.

The size of all microsclerotia observed ranged from 8.6-154.8 wide to 8.6-245.1  $\mu$ m in length (mean size = 52  $\times$  74  $\mu$ m). The median 90% of microsclerotia in all four fields had a width (least dimension) in the range 25 to 103  $\mu$ m. The results obtained for the four fields were similar, and blending had no appreciable effect on the size of microsclerotia. The mean and range of microsclerotium dimensions reported here are smaller than those recently reported by Phipps et al. (11) for *C. crotalariae* microsclerotia (mean size = 70  $\times$  103  $\mu$ m) recovered from North Carolina fields with a 38- $\mu$ m sieve. This may be due, in part, to the use by us of sieves with smaller pores than used by Phipps et al., and indicates that many small microsclerotia occur in Virginia peanut-field soils. Sieves with the smallest pores feasible should be used to obtain accurate estimates of microsclerotium populations. This also was suggested by population analyses of soils from 10 naturally infested peanut fields; six had a larger percentage of the microsclerotial population recovered on the 25- $\mu$ m sieve than on the 44- $\mu$ m sieve. Percentage of microsclerotia recovered on the 25- $\mu$ m sieve for the ten soils were 67, 77, 73, 62, 61, 35, 36, 44, 41, and 80. A 25- $\mu$ m or slightly smaller sieve may be most practical, since water does not pass readily through sieves with pores smaller than this.

Knowledge of the number of microsclerotia embedded in organic matter may be important in pathogen survival

TABLE 1. Influence of soil dispersion method on recovery of *Cylindrocladium crotalariae* microsclerotia from naturally infested soil

Experiment	Treatment	Microsclerotia/g soil <sup>a</sup> (no.)	Microsclerotia recovered on 44- $\mu$ m sieve (%)
A	Shake		
	10 min	40	40
	Blend		
	15 sec	41	32
	2.5 min	38	30
B	No blend	151	66
	Blend		
	2 sec	140	64
	15 sec	139	55

<sup>a</sup>Represents the sum of populations recovered with the 25- $\mu$ m and 44- $\mu$ m sieves.

studies in that these microsclerotia may be shielded somewhat from antagonistic microorganisms and other agents. Use of the 150- $\mu\text{m}$  sieve demonstrated that some of the microsclerotia in the test soils occurred in very large particles of organic matter (mean size =  $401 \times 871 \mu\text{m}$ ), and that a high percentage of microsclerotia recovered with this sieve occurred in organic matter (mean = 85.4% for all fields). However, only 2.6 to 8.8% as many microsclerotia were recovered from the four fields using this sieve compared to the smaller, nested sieves. In contrast to the above, only 8.4% of the microsclerotia recovered on the 25- $\mu\text{m}$  sieve, and 15.6% of the microsclerotia recovered on the 44- $\mu\text{m}$  sieve, occurred in particles of organic matter (mean size for both sieves =  $78 \times 277 \mu\text{m}$ ). These percentages appear to be in general agreement with findings of Phipps et al. (11) for North Carolina peanut-field soils, although they gave no percentages and with that of Thies and Patton (14) for *C. scoparium* in Wisconsin forest-nursery soils. Thus, the large majority of microsclerotia in these field soils occurred free or unprotected by organic matter. Blending of two test soils previous to sieving had little or no effect on the percentage of *C. crotalariae* colonies (87.5% and 86.5% for nonblended and blended soils, respectively) that originated from "free" microsclerotia (not present in organic matter). The dispersion of microsclerotia in soil by blending would not appear to be essential when assaying sandy, peanut-field soils or soil fractions that do not contain significant levels of microsclerotia in host tissues. It probably should be used for heavy soils, such as some *Cylindrocladium*-infested forest-nursery soils, that do not disperse readily.

**Influence of air-drying on microsclerotium populations recovered from naturally infested soils.**—The recovery of *C. scoparium* microsclerotia from soil was greatly decreased by air-drying soil (14), but whether this is a true loss of viability or only a temporary decrease in germinability (1) is not known. A similar effect of air-drying may exist for *C. crotalariae* microsclerotia. In separate studies conducted by researchers in our laboratory, assays of a sandy peanut-field soil at 0-13 cm depth, after a severe drought, yielded low populations of microsclerotia (mean = 0.8/g soil) compared to samples collected at 13- to 25-cm depth (mean = 5.4/g soil, J. Taylor et al., unpublished); soil samples were collected 1 wk after a drought-ending rain. When moist [near field capacity, -0.33 to -0.1 bars (Table 2)] soil samples were collected for the present study from peanut fields, mixed, and a portion air-dried in the laboratory for 24 hr, no microsclerotia were recovered from any of the nine air-dried soils examined (Fig. 1). Soils kept moist yielded high microsclerotium populations. Soil-water contents of air-dried soils ranged from 0.12 to 0.38% water, while samples kept moist ranged from 6.8 to 15.2% water. Tests indicated that the water potential for four of these air-dried soils was about -2,000 bars or somewhat lower (Table 2). Thies and Patton (14) recovered approximately 5% of the microsclerotial population after soil naturally infested with *C. scoparium* was air-dried in the laboratory for 3 mo, but gave no information on the water potential of the soils. Phipps (personal communication) found air-drying a peanut-field soil for 3 days (0.2% water content) resulted in recovery of 12% of the original population of *C.*

*crotalariae* microsclerotia. When separate air-dried (for 24 hours) soil samples were rewetted by us to the same soil water contents for continually moist soil, and incubated for 7 days, microsclerotia were recovered from eight of the nine field soils tested (Fig. 1). From 2.2 to 66.7% as many microsclerotia were recovered from these soils as from similar samples kept continually moist. Similar findings were obtained with soils obtained from other Virginia peanut fields. Thus, temporary exposure of microsclerotia to severe drought conditions may not necessarily lead to a loss of viability.

A second series of assays were performed to determine

TABLE 2. Soil water properties and contents of four field soils used to study the influence of air-drying on *Cylindrocladium crotalariae* microsclerotium populations

Field soil	Water content of:		Water content at:			
	Air-dry soil <sup>a</sup> (%)	Moist soil <sup>b</sup> (%)	-2,215 bars <sup>c</sup> (%)	-897 bars <sup>c</sup> (%)	-0.33 bars <sup>d</sup> (%)	-0.1 bars <sup>d</sup> (%)
207	0.22	6.76	0.21	0.36	5.6	8.9
217	0.12	9.28	0.25	0.50	10.2	14.7
75-51	0.36	15.20	0.30	0.48	11.4	18.3
203	0.38	9.28	0.36	0.77	10.4	16.5

<sup>a</sup>Soil was air-dried for 24 hr by spreading soil out on a laboratory bench.

<sup>b</sup>Water content for soil collected in the field.

<sup>c</sup>Determined by equilibrating soils to water vapor tensions of saturated solutions of  $\text{K}_2\text{C}_2\text{H}_3\text{O}_2$  and  $\text{Mg}(\text{NO}_3)_2$  at 25 C.

<sup>d</sup>Determined by the pressure-plate technique. Water percentages (by weight) were determined by drying soils at 105 C for 24 hr.

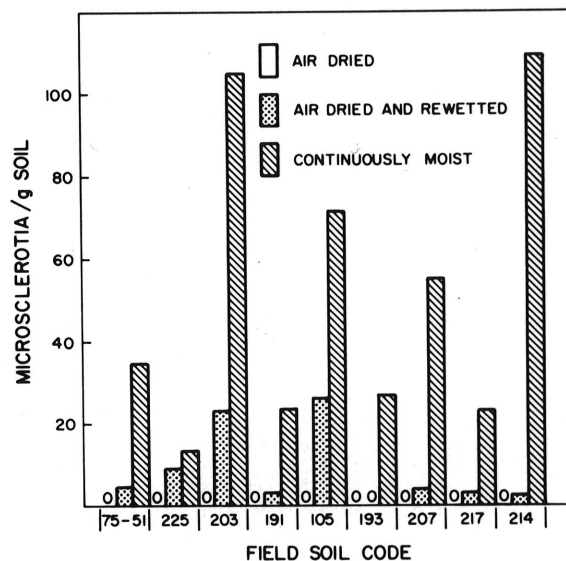


Fig. 1. Effect of air-drying and rewetting on recovery of *Cylindrocladium crotalariae* microsclerotia from nine naturally infested peanut-field soils. Soils were air-dried by spreading soil over a laboratory bench for 24 hr. No recovery of microsclerotia is indicated by a 0.

if longer periods (2-4 wk) of rewetting would result in even greater recovery from the air-drying effect (Fig. 2). Increasing the length of the wetting period possibly increased the recovery of microsclerotia from soil for only one sample (No. 217). These results indicate that maintaining the field level of soil water in samples during transport from field to laboratory may be critical to assessments of germinable microsclerotium populations for any given date in the growing season. Loss of additional water from samples already somewhat dry at the time of collection could yield erroneous germinable microsclerotium population estimates; rewetting samples in the laboratory also may change germinable populations.

**Influence of temperature of soil incubation on microsclerotium populations recovered from naturally infested soils.**—Soil samples for microbial population analyses often are stored at low temperature to minimize changes in populations before analysis. When duplicate, previously mixed soil samples from each of eight peanut fields were incubated at 6 C or 26 C for 1 mo and assayed for populations of *C. crotalariae* microsclerotia, much lower populations were recovered from the samples incubated at 6 C than at 26 C (Fig. 3). However, all populations of microsclerotia in the various field soils did not respond equally to the low-temperature treatment. For one soil (No. 188), no microsclerotia were recovered after low-temperature incubation, while for another field soil (No. 193), as high as 57% as many microsclerotia were recovered at 6 C as at 26 C. Zero-time assays for soils stored at both temperatures, and assays of samples

incubated at 26 C for 1 mo, were similar (Fig. 3). Similar results were obtained with samples from other peanut fields. Shorter periods (1-2 wk) of soil storage at 6 C also reduced microsclerotium populations. Thus, refrigeration of field-soil samples during transport from field to laboratory, or during storage in the laboratory to reduce soil-water loss (13), should be avoided. Freezing of soil resulted in no survival of the fungus. Storage at room temperature (25-28 C) appears to be best.

Although the original impetus to this research dealt with population methodology, the results reported here have ecological and physiological implications in pathogen survival. Impairment of microsclerotium germinability following a drought may affect survival, or delay or reduce disease development. Although our tests with air-dried soil were conducted indoors in the fall, probably at lower relative humidities (and, thus, water potentials) than occur generally in the field during the growing season, our unpublished field results, referred to previously, suggest that a similar phenomenon can occur in the field during the summer. Research is in progress to determine if the temperature phenomenon described above is a low-temperature-induced dormancy of microsclerotia or is a low-temperature injury with loss of viability, and also to determine the significance of the phenomenon in nature. To our knowledge our preliminary report (4) and this report are the first to

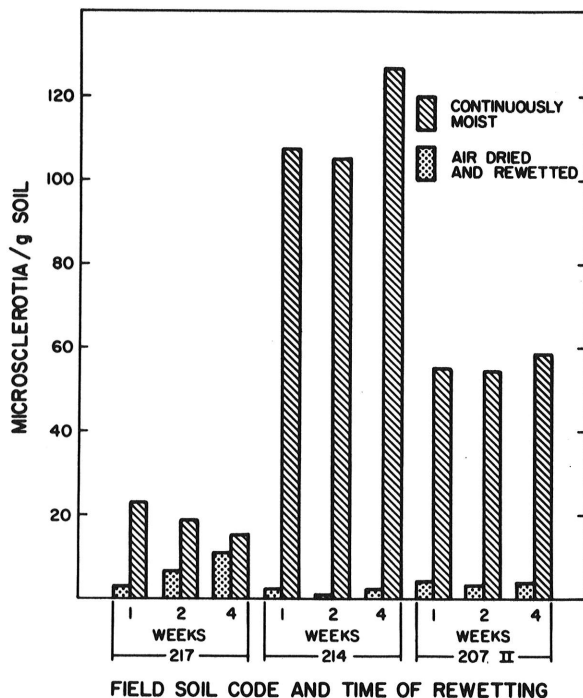


Fig. 2. Influence of length of rewetting period following air-drying on the recovery of *Cylindrocladium crotalariae* microsclerotia from three naturally infested peanut-field soils.

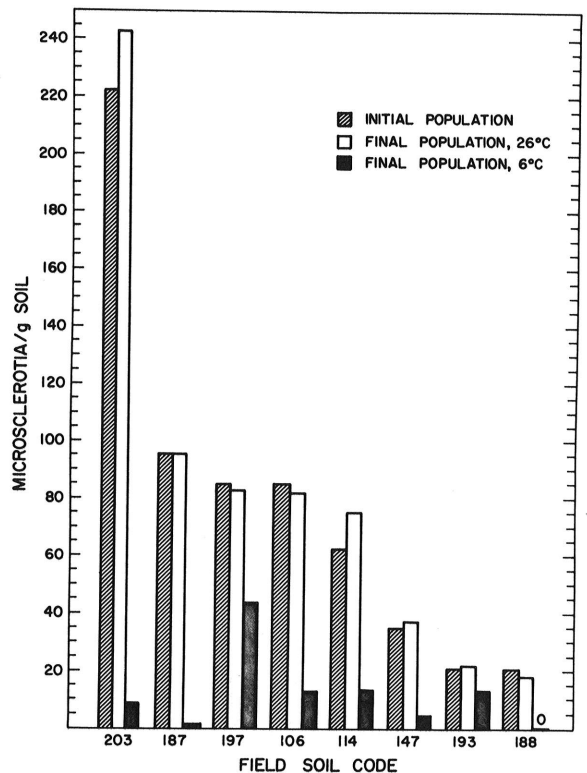


Fig. 3. Influence of incubation at 6 C and 26 C for 1 mo on the population of *Cylindrocladium crotalariae* microsclerotia recovered from eight naturally infested peanut-field soils. Initial microsclerotium populations are the average of the two subsamples incubated at the two temperatures.

describe such an effect by nonfreezing temperatures on thick-walled propagules of a soilborne fungus. Phipps (*personal communication*) has observed a similar effect of low temperature (5 C) incubation on microsclerotium populations of *C. crotalariae* in a North Carolina peanut-field soil. The effect of winter temperatures on microsclerotium germinability may critically influence the survival of *C. crotalariae* and the epidemiology of *Cylindrocladium* black rot in peanut field soils.

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