Production of Microsclerotia by Species of Cylindrocladium

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ABSTRACT

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To determine factors that affect production of microsclerotia, five species of *Cylindrocladium (C. scoparium, C. crotalariae, C. floridanum, C. ilicicola,* and *C. parvum*) were studied to determine factors that affect production of microsclerotia. The most favorable temperature range for growth was 24-28 C. The C/N ratio of the substrate was most important with maximum numbers of microsclerotia formed at ratios of 40:1 and 100:1; fewer were formed at lower ratios on agar media and in sand culture. Potassium nitrate was a good nitrogen source at favorable ratios, but poorer than casein hydrolysate at unfavorable ratios. L-tyrosine as a sole source of C and N was excellent for formation of microsclerotia. Straw and sawdust also were good substrates for microsclerotia formation. Evidence suggests that the practice of mulching seed beds known to harbor *Cylindrocladium* with straw, sawdust, or other high-carbon products, may cause a significant increase in numbers of microsclerotia.

Additional key words: flotation sieving, microsclerotia, vesicle.

Several species of *Cylindrocladium* are destructive to many economic plants (6, 8, 9). Recently, *C. crotalariae* (Loos) Bell and Sobers caused extensive severe damage to peanuts in the southeastern USA (2, 7). Since 1968, *C. scoparium* Morgan has caused a severe black root rot of black walnut seedlings and has destroyed many yellow-poplar, white pine, and red pine seedlings at the West Virginia State Forest Nursery at Parsons (9).

All known species of *Cylindrocladium* form microsclerotia (MS) in most common solid and liquid media. Mature MS form within the host tissue and in organic debris in soil and they are the propagules most easily recovered from the soil (9, 11, 14). They are thought to be of primary importance for survival and dispersal and are important in recognition of the fungus in culture (4, 10, 13).

Species of *Cylindrocladium* have been separated on the basis of conidial morphology (3) and more recently on vesicle morphology (12). Because the many isolates obtained from diseased roots of various nursery seedlings exhibited a wide range in vesicle morphology, we have called all our isolates from West Virginia *C. scoparium,* pending further critical taxonomic study of the species.

The primary objective of this study was to determine some of the nutritional and environmental factors required for formation of abundant microsclerotia by species of *Cylindrocladium*. Another objective was to determine whether the number of sclerotia in the soil could be increased or decreased by additions of chemical amendments.

MATERIALS AND METHODS

Cultures of *C. scoparium* (isolates -1 and -8, indicated by superscripts following the species name) were isolated from diseased roots of yellow-poplar and black walnut nursery seedlings in West Virginia. *Cylindrocladium parvum* Anderson was also recovered from infected roots of black walnut seedlings in West Virginia. *C. floridanum* Sobers and Seymour and *C. crotalariae* were supplied by E. K. Sobers, and *C. ilicicola* (Hawley) Boedijn and Reitsma from the Centraalbureau voor Schimmelculture. *Rhizoctonia solani* Kuhn, *Papulospora* sp., and *Sclerotium rolfsii* Saccardo, which were used in some comparative experiments, were from the culture collection at West Virginia University.

The basal medium consisted of : KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; Mn^{+2}, 0.05 mg; Zn^{+2}, 0.2 mg; Fe^{+3}, 0.1 mg; thiamine, 100 μg; Difco agar, 20 g; and distilled water, 1.0 liter. The microelements were added in 1.0 ml of stock solution, 1 liter of which contained: 826 mg $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot (H_2O)_{24}$, 880 mg $ZnSO_4 \cdot 7H_2O$, and 154 mg $MnSO_4 \cdot H_2O$ with 10.0 ml of concentrated HCl to prevent the precipitation of iron. Carbon (C) and nitrogen (N) sources varied in composition and amount and will be given with the appropriate experiments in the results. Carbon-nitrogen ratios were obtained either by altering the amounts of both the C and N sources or by keeping the amount of C constant and changing the amount of N. All media were adjusted to a pH of 6.5 and autoclaved at 1.05 kg/cm^2 for 15 minutes.

Inoculum from agar cultures consisted of 3-mm diameter mycelial plugs cut from the periphery of 3- to 4-day-old colonies growing on glucose-yeast extract agar. Mycelial suspensions (8,000 propagules/ml) were prepared from fungal mats growing in liquid glucose-yeast extract and comminuted in a sterile Waring Blendor at high speed for 2 minutes.

Sand cultures (for induction of microsclerotia) were prepared by washing 1 liter of white sand and allowing it to air dry. The basal medium minus agar was thoroughly mixed into the sand and 25-cc portions were added to 250-ml Erlenmeyer flasks containing 500 mg glucose and varying amounts of KNO_3 to give the desired C/N ratios. Control cultures contained 50 mg of KNO_3 but no glucose. Flasks of media were inoculated with 1.0 ml of a mycelial suspension and incubated at 25 C for 14 days, prior to processing by a modification of the Byrd's

flotation sieving technique (5). Microsclerotia were extracted from the sand by placing a culture in a 600-ml beaker containing 250 ml of 1.5 M sucrose with 20.0 mg of NP-10 Separan (Dow Chemical Company), a soil flocculating agent. The ingredients were mixed for 20 seconds with a mechanical stirrer, and 3 minutes later the mixture was poured onto a 246-μm-mesh screen directly above a 46-μm (mesh) screen. The MS on the 46-μm (mesh) screen were washed, harvested, dried, and weighed.

Hay, sawdust, and straw cultures were prepared by grinding alfalfa hay, alfalfa straw, and red oak wood in a Wiley mill. The shredded materials were added to 250-ml Erlenmeyer flasks covered with inverted 50-ml beakers and autoclaved for 20 minutes at 1.05 kg/cm^2. About 8,000 mycelial fragments in 6.0 ml of sterile distilled water were added to each of five replicate cultures and they were incubated for 15 days in darkness at 25 C. Observations were made at low power with a stereoscopic microscope. Each experiment was repeated at least once.

Temperature and light (40 W G.E. cool-white fluorescent lamps, 1,345 lx) experiments were conducted in Freas BOD incubators adjusted to the desired temperature.

The relative abundance of MS in agar culture was estimated by use of a rating system: 0 = none, 1 = widely scattered, 2 = numerous, 3 = abundant, 4 = closely compacted.

TABLE 1. Microsclerotium formation in *Cylindrocladium scoparium* at different temperatures for 9 days on fructose-casein hydrolysate agar medium

Isolates	Microsclerotium formation rating[a]				
	12 C	20 C	24 C	28 C	32 C
C. scoparium^{-1}	0	1	3	3	0
C. scoparium^{-8}	0	2	3	3	2
C. crotalariae^{-18}	0	2	3	3	0
C. floridanum^{-10}	0	2	2	2	0
C. ilicicola^{-19}	0	2	3	3	1

[a]Each rating is based on the average value of triplicate cultures examined. Rating: 0 = none, 1 = widely scattered, 2 = numerous, 3 = abundant, 4 = closely compacted.

RESULTS

Effects of light and temperature.—Four species of *Cylindrocladium* were cultured on a fructose-casein hydrolysate (10-2 g) agar medium for 9 days in light and in darkness at a range of temperatures. Light had no apparent effect on the rate of formation or the total number of MS. The optimum temperature range for production of MS was 24-28 C (Table 1). Only *C. scoparium*$^{-8}$ grew well and produced significant numbers of MS at 32 C. The results were similar when the experiment was repeated using KNO_3 as the N source. All succeeding experiments on MS were carried out at 25 C.

Effects of carbon and nitrogen sources.—All species and isolates tested were remarkably uniform in growth and asexual sporulation on media differing only in carbon source, but growth was slower and maximum weight attained was less when KNO_3 was used as a nitrogen source than when casein hydrolysate was used. The following carbon sources were utilized well for growth: D-glucose, D-fructose, D-mannose, D-galactose, L-sorbose, D-xylose, glycerol, sucrose, raffinose, and starch.

Species and isolates of *Cylindrocladium* were remarkably similar in utilization of the N source when glucose was the C source in the medium. In general, casein hydrolysate, L-asparagine, and L-glutamic acid were utilized equally well for growth and were somewhat better than KNO_3, or ammonium sulfate.

The formation of MS occurred on a wide variety of media and was generally favored by a high concentration of C and relatively low concentration of N. Two isolates of *C. scoparium* were cultured on three agar media containing widely varying C/N ratios and amounts. The isolates were similar and produced abundant MS on the three media at the two highest C/N ratios (40:1, 100:1), and correspondingly fewer MS as the C/N ratio was lowered (Table 2). However, many MS were produced on the casein hydrolysate medium at low C/N ratios, presumably because casein hydrolysate provided some C favorable for MS production. Figure 1 shows *C. scoparium* growing on media with different C/N ratio. The MS were densely compacted at C/N ratios of 40/1 and 100/1.

TABLE 2. Microsclerotium formation in *Cylindrocladium scoparium*$^{-1}$ and *C. scoparium*$^{-8}$ when the carbon-nitrogen concentrations of three media were altered[a]

Carbon-nitrogen ratio		Microsclerotium formation rating[a]					
		Fructose-casein hydrolysate		Fructose-glutamic acid		Fructose-potassium nitrate	
C	N	*C. scoparium*	*C. scoparium*	*C. scoparium*	*C. scoparium*	*C. scoparium*	*C. scoparium*
10	0	1	1	1	1	1	1
5	1	3	3	2	2	2	2
10	1	3	3	3	3	3	2
40	1	4	4	3	4	4	4
100	1	4	4	4	4	4	4
10	4	3	3	1	1	2	2
10	8	3	3	1	1	1	1
10	12	3	3	1	1	1	1

[a]Each rating is based on the average value of three observations of triplicate cultures examined.

Several media were tested in an attempt to increase the numbers of MS and reduce the amount of mycelium. The addition of tyrosine (80 mg/liter) to a glucose-yeast extract (30 g-0.2 g/liter) medium resulted in large numbers of dark brown MS in 6-8 days with little aerial or submerged mycelium. In addition, the use of 10 g/liter of L-tyrosine to the basal medium as a substitute for both C and N source proved to be a good medium for MS production (Fig. 2). The MS are so densely compacted that they appear black. Fig. 3 shows the structure of a mature MS.

The experiment on the effects of the C/N ratio on sclerotium production was extended for comparison to *Rhizoctonia solani, Papulospora* sp., and *Sclerotium rolfsii*, using media with and without tyrosine. The response of the latter two fungi was similar to that of *Cylindrocladium;* i.e., greater numbers of sclerotia at the higher C/N ratios. However, *R. solani* formed greater numbers of sclerotia at the lower C/N ratios.

Sand culture.—Washed sand to which a complete liquid medium was added was an excellent substrate for the production of MS by *C. scoparium*$^{-1}$. Optimum MS production occurred at the higher C/N ratios (Fig. 4 and 5). A second isolate (*C. scoparium*$^{-8}$) produced similar

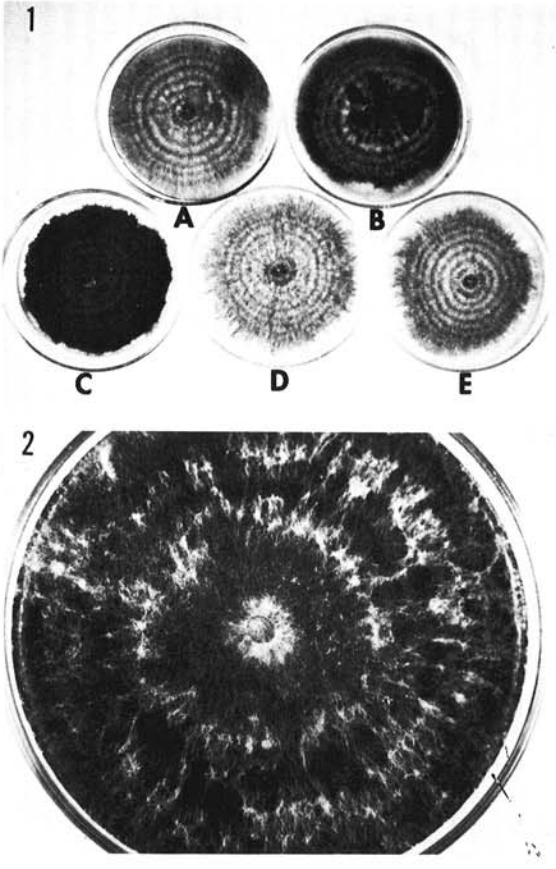

Fig. 1-(A to E) and 2. 1) Microsclerotial formation by *Cylindrocladium scoparium* isolate 8 on a fructose-potassium nitrate agar medium at different C/N. **1A)** 10:1. **1B)** 40:1. **1C)** 100:1. **1D)** 10:4. **1E)** 10:8. **2)** Densely compacted microsclerotia of *Cylindrocladium scoparium* isolate 8 on tyrosine agar medium.

Fig. 4. Microsclerotial formation by *Cylindrocladium scoparium* isolate 1 in a sand culture at a C/N of 100:1.

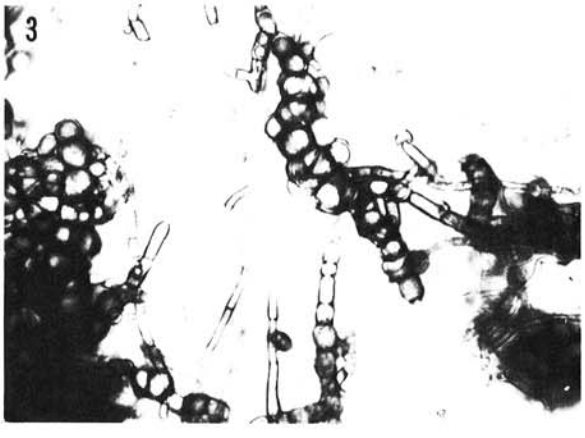

Fig. 3. A mature microsclerotium with associated hyphae of *Cylindrocladium scoparium* isolate 1.

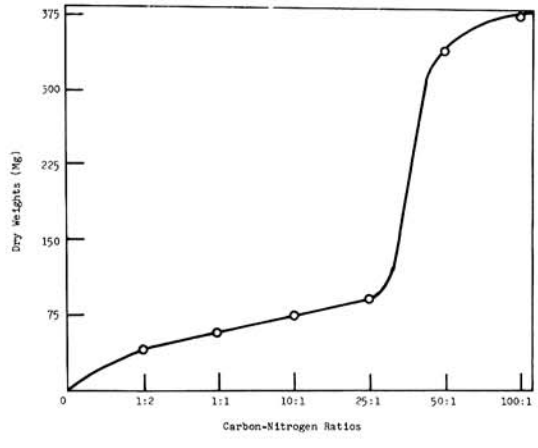

Fig. 5. Milligrams of dried microsclerotia produced by *Cylindrocladium scoparium* isolate 1 when grown in sand cultures at different carbon-nitrogen ratios.

numbers of MS, but differed in forming some MS at a C/N ratio of 1/1 after prolonged incubation.

Hay, sawdust, and straw cultures.—Alfalfa straw and sawdust are common substrates that are known to have a low N content. The vegetative growth (minimal mycelium, numerous MS) of *C. scoparium*$^{-1}$ on these natural materials was similar to that on agar media with high C/N ratios (Table 2). *Cylindrocladium scoparium*$^{-1}$ produced a thick mycelial growth and few MS on hay.

Recovery of Cylindrocladium from soil.—Hunter (9, and *unpublished*) recovered many isolates of *C. scoparium* from infected seedling roots of red pine, white pine, Virginia pine, yellow-poplar, blue spruce, and black walnut, but initially failed to recover this fungus from soil using established procedures (apple, alfalfa trapping, and soil dilution plate techniques). An effective method (9) was finally devised to demonstrate that *Cylindrocladium* MS were in the soil. We added 2 g of glucose to 25 cc of soil taken from the rhizosphere of roots of diseased black walnut seedlings. The soil cultures were incubated in the darkness for 12-14 days. Most cultures with added glucose yielded MS, whereas cultures without glucose produced none.

DISCUSSION

This study provides evidence that the C/N ratio is one of the most important factors determining the formation of MS by species of *Cylindrocladium*. As the C/N ratio increases, more MS are formed on natural and on artificial substrates. This is assumed to be the case in soil around diseased roots.

It is common practice in West Virginia nurseries to add straw or sand-sawdust mixtures to seedbeds after seeding. If viable propagules are present they could colonize this substrate and form abundant MS, which has been observed by Linderman (10) for infected azalea leaves. Aycock (1) has shown that *Cylindrocladium* can survive in infected azalea leaves for 14 months and Hodges (8) concluded that the MS are the resistant structures that make eradication of the pathogen so difficult.

Therefore, we recommend cultural practices be followed that do not increase the C/N ratio in the soil, until the factors that are responsible for the production and longevity in soil are better understood. A possible control program in nursery soils could be achieved by the integration of fertilization and fumigation procedures. Nitrogen fertilizers would lower the C/N ratio and encourage mycelial development with fewer MS, thereby making soil fumigation more effective in eradicating the fungus.

A glucose-yeast extract-tyrosine medium is recommended for laboratory production of sclerotia of most species of *Cylindrocladium* (*C. parvum* is one exception), *Sclerotium rolfsii,* and *Papulospora* sp. when large numbers are needed for study or experimentation. Light was of no significance in the induction of MS by any species of *Cylindrocladium,* but temperature was an important factor with a range of 24-28 C being optimum. In nature, the MS probably form in greatest numbers when the soil temperatures are high.

LITERATURE CITED

1. AYCOCK, R. 1973. Control and diagnosis of Cylindrocladium disease of azalea. Phytopathology 63:440 (Abstr.).
2. BELL, D. K., and E. K. SOBERS. 1966. A peg, pod, and root necrosis of peanuts caused by a species of Calonectria. Phytopathology 56:1361-1364.
3. BOEDIJN, K. B., and J. R. REITSMA. 1950. Notes on the genus Cylindrocladium (Fungi Mucedimae). Reinwardtia 1:51-60.
4. BUGBEE, W. M., and N. A. ANDERSON. 1963. Infection of spruce seedlings by Cylindrocladium scoparium. Phytopathology 53:1267-1271.
5. BYRD, D. W., JR., C. J. NUSBAUM, and K. R. BAKER. 1966. A rapid flotation technique for extracting nematodes from soil. Plant Dis. Rep. 50:954-957.
6. COX, R. S. 1954. Cylindrocladium scoparium on conifer seedlings. Delaware Agric. Exp. Stn. Tech. Bull. 301. 40 p.
7. GARREN, K. H., M. K. BRUCE, and D. M. PORTER. 1972. The Cylindrocladium crotalariae black rot of peanut in Virginia and North Carolina. J. Am. Peanut Res. Educ. Assoc. 4:67-71.
8. HODGES, C. S., JR. 1962. Diseases in southeastern forest nurseries and their control. U.S. Dep. Agric. For. Ser., Southeastern For. Exp. Stn. Pap. 142. 16 p.
9. HUNTER, B. B. 1970. Nutritional and environmental factors affecting growth, sporulation, and sclerotial formation, of species of Cylindrocladium. Ph.D. Diss. W. Va. Univ., Morgantown. 166 p.
10. LINDERMAN, R. G. 1973. Formation of microsclerotia of Cylindrocladium spp. in infected azalea leaves, flowers, and roots. Phytopathology 63:187-191.
11. MORRISON, R. H., and D. W. FRENCH. 1969. Direct isolation of Cylindrocladium and floridanum from soil. Plant Dis. Rep. 53:367-369.
12. SOBERS, E. K., and C. P. SEYMOUR. 1967. Cylindrocladium floridanum sp. n. associated with decline of peach trees in Florida. Phytopathology 57:389-393.
13. THIES, W. G., and R. F. PATTON. 1970. An evaluation of propagules of Cylindrocladium scoparium in soil by direct isolation. Phytopathology 60:599-601.
14. THIES, W. G., and R. F. PATTON. 1970. The biology of Cylindrocladium scoparium in Wisconsin forest tree nurseries. Phytopathology 60:1662-1668.