An Elutriation Method for Quantitative Isolation of Cylindrocladium crotalariae Microsclerotia from Peanut Field Soil

P. M. Phipps, M. K. Beute, and K. R. Barker

Research Associate, Associate Professor, and Professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27607.

Paper No. 4831 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh.

The use of trade names in this publication does not imply endorsement by the N.C. Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

The authors thank Lieselotte Conniff, Kathy Hayes, and D. W. Byrd, Jr. for technical assistance.

Accepted for publication 12 April 1976.

ABSTRACT

PHIPPS, P. M., M. K. BEUTE, and K. R. BARKER. 1976. An elutriation method for quantitative isolation of Cylindrocladium crotalariae microsclerotia from peanut field soil. Phytopathology 66: 1255-1259.

Plant debris and microsclerotia of *Cylindrocladium crotalariae* were elutriated from soil using a semi-automatic elutriator which was designed for separating nematodes from soil. Plant debris (larger than 425-μm) and microsclerotia-size particles (38-425 μm) in soil were collected on 425- and 38-μm sieves, respectively. Plant debris from the 425-μm sieve was blended for 2 minutes in water, then concentrated on a 38-μm sieve. Each sieve residue was treated for 1 minute in 0.25% NaClO. After rinsing, the sieve residues were suspended in water and 5-ml subsamples were pipetted into 100 ml of an isolation medium at 45 C. The medium then was mixed and poured into 10 petri dishes. The isolation medium contained glucose, 15 g; KNO_3, 0.5 g; yeast extract, 0.5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Tergitol (NPX), 1 ml; thiabendazole, 1 mg; chloramphenicol, 100 mg; chlortetracycline, 40 mg; and 20 g agar per liter of water. After 5 days of incubation, the presence of brown microsclerotia and asexual sporulation permitted recognition and counting of *C. crotalariae* colonies in assay plates. The elutriation and enumeration procedure was effective in recovery of at least 91% of laboratory-grown microsclerotia in artificially infested soil. Numbers of microsclerotia in 23 naturally-infested, peanut field soils ranged from 0.2 to 72/g soil.

Additional key words: Arachis hypogaea L., *Calonectria crotalariae* (Loos) Bell and Sobers, Cylindrocladium black rot of peanuts.

Cylindrocladium black rot (CBR) of peanuts (*Arachis hypogaea* L.), caused by *Cylindrocladium crotalariae* (Loos) Bell and Sobers (1), poses an ever-increasing threat to peanut cultivation in North Carolina and Virginia (3, 10). Microsclerotia of *C. crotalariae* are produced abundantly in the cortex of infected peanut roots and serve as effective propagules for survival of the pathogen in field soil (7, 11). The subsequent decay and disintegration of infected root tissues results in release of microsclerotia into soil where dissemination may occur by tillage and combine harvesting operations as well as water runoff. Wind-blown debris particles, large enough to carry microsclerotia, have been trapped up to 235 m downwind of operating peanut combines (11).

Recently, a new semi-automatic elutriator (NC-El) was developed for quantitative estimation of nematode populations in soil (2). Observations of the residues, collected on 38-μm sieves during elutriation of soil, revealed spores of certain fungi as well as nematodes. The current study was conducted to determine the efficacy of this machine for recovery of microsclerotia of *C. crotalariae* from soil. Both an elutriation and enumeration procedure for quantitative estimation of microsclerotia in naturally-infested soil are described.

MATERIALS AND METHODS

Collection of soil samples.—Soil samples were collected from 23 peanut fields in North Carolina where CBR had been observed since 1970. Core samples were taken with a 2-cm diameter soil-sampling tube to depths of 15-20 cm at 30-50 systematically determined locations in each field. The subsamples from each field were placed in a polyethylene bag and mixed by hand immediately after collection. All field samples were stored at room temperature (25-28 C) and assayed within 2 weeks. Prior to assay, three 25-g subsamples of each soil were dried at 105 C for 24 hours to determine moisture content.

Soil elutriation procedure.—A detailed description of the NC-El, including specifications and operation procedures for elutriation of nematodes from soil, has been reported (2). Rates of air and water flow to each soil-elutriating unit were adjusted to 40-50 cm^3/second and 80 ml/second, respectively. Plant debris larger than 425 μm was collected on 425-μm sieves (15-cm diameter) during elutriation, whereas two-fifths of the suspended particles, 38-425 μm in size, were collected on 38-μm sieves (20-cm diameter).

Enumeration of microsclerotia.—Plant debris collected on the 425-μm sieves was blended in a Waring Blendor for 2 minutes (1.5 minutes at low speed, 30 seconds at high speed) in 200 ml of water to permit quantitative estimation of microsclerotia in this fraction.

After concentrating the blended debris on a 38-μm sieve, it was washed into a 250-ml beaker and suspended in 160 ml of water by using a mechanical stirrer. This fraction was labeled the "debris fraction". The particles (38-425 μm in size) collected on the 38-μm sieve during elutriation of soil were not blended, but were suspended in water as described above and labeled the "soil fraction".

Quantitative assays for microsclerotia in each suspended fraction were made by pipetting 5-ml subsamples into 100 ml of an isolation medium at 45 C. The medium was swirled, then dispensed into 10 petri dishes (9-cm diameter) and incubated 5-8 days at room temperature under continuous fluorescent light (22 hlx). After sampling each suspended fraction, the total volume was measured to permit calculation of dilution factors.

Basal constituents of the isolation medium included glucose, 15 g; yeast extract, 0.5 g; KNO_3, 0.5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 20 g; and deionized water, 1 liter. After autoclaving, 200-ml aliquots of the medium were amended with Tergitol (NPX), 0.2 ml; thiabendazole, 0.2 mg; chloramphenicol, 20 mg; and chlortetracycline, 8 mg. Tergitol (NPX) was added directly to the medium, whereas the following amounts of the other agents were added from stock solutions: thiabendazole (16.6 mg of 60% wettable powder formulation suspended in 50 ml water), 1 ml; chloramphenicol (1 g dissolved in 50 ml 95% ethanol), 1 ml; and chlortetracycline (0.4 g dissolved in 50 ml 50% ethanol), 1 ml. Tergitol (NPX) was used to suppress growth of fungi (12) and lower the surface tension of the medium which permitted the pouring of a thin agar layer in each plate. Thiabendazole served primarily to inhibit growth by certain undesired fungi (8), and the antibacterial compounds, chloramphenicol and chlortetracycline, prevented development of bacterial colonies.

Efficiency of the elutriation and enumeration methods.—Microsclerotia of five isolates of *C. crotalariae* were produced in a liquid medium as described by Rowe et al. (11). After 8-10 weeks of incubation (in darkness to prevent perithecium formation), the mycelial mats were comminuted in a Waring Blendor for 5 minutes. Temperatures in the blender top were checked during blending to prevent exceeding 30 C. The slurry of microsclerotia and mycelium was washed on a 74-μm sieve until all mycelial fragments were removed. The microsclerotia then were suspended in water by using a mechanical stirrer and the density of the suspension was determined by pipetting 1-ml subsamples onto gridded Millipore filters (3-cm diameter). Counts of microsclerotia were made with a stereodissecting microscope. A total of six, 1-ml samples were counted and four, 1-ml samples were assayed by direct plating to confirm the accuracy of visual counts.

Peanut field soil with no history of CBR was artificially infested by pipetting 15 ml of the standardized, microsclerotial suspension into 185 g of air-dried soil. Each soil sample then was mixed until uniformly moist.

TABLE 1. Recovery of *Cylindrocladium crotalariae* from sieve residues as affected by the time of exposure to 0.25% NaClO[x]

Exposure time (minutes)	Microsclerotia per gram of soil[y]		
	Soil fraction	Debris fraction	Total
0	8.3 bc[z]	1.5 c[z]	9.8
0.5	15.3 a	1.8 bc	17.1
1	11.9 ab	3.7 ab	15.6
2	5.3 cd	3.8 a	9.1
4	2.2 d	2.0 abc	4.2

[x]Data are the average number of microsclerotia isolated from four samples of naturally-infested soil processed by the elutriation procedure.

[y]Soil fraction; particles collected on 38-μm sieves during elutriation of soil. Debris fraction; plant debris collected on 425-μm sieves and treated after blending for 2 minutes.

[z]Mean values followed by the same letter(s) are not statistically different ($P = 0.01$) by Duncan's new multiple range test.

RESULTS

Elutriation and enumeration of microsclerotia in naturally-infested soil.—In preliminary experiments, elutriation of 200-g samples of naturally-infested, field soil (loamy sand) yielded sufficient quantities of debris and particles on 38-μm sieves for quantitative assay. Visual observations of the effluent from each elutriator indicated that essentially all plant debris and small organic particles were removed from the sample after 6-8 minutes. Larger samples of soil required a longer elutriation period, and the collected fractions were too large for processing on a single sieve.

Assays of the two fractions, collected during elutriation of 200-g samples of soil for 8 minutes, yielded high numbers of undesired fungi in assay plates which prevented quantitative estimation of *C. crotalariae* propagules. Dilution assays of each fraction resulted in disappearance of *C. crotalariae* colonies prior to sufficient reduction of numbers of undesired fungal colonies.

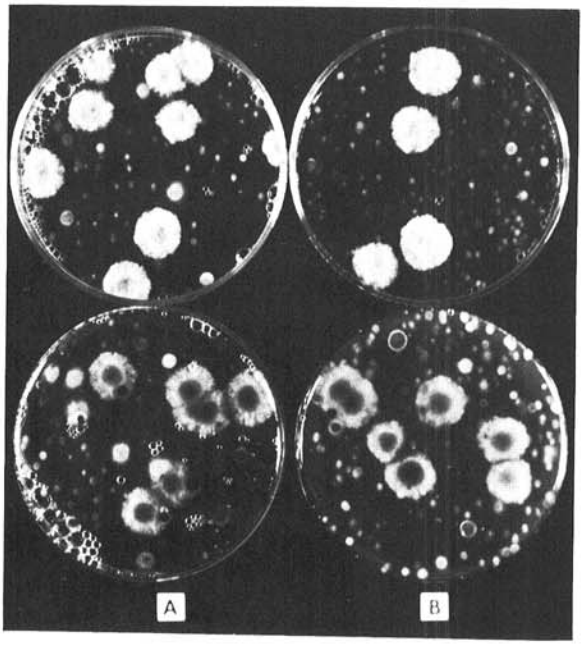

Fig. 1-(A, B). Upper and lower surfaces of assay plates exhibiting colonies of *Cylindrocladium crotalariae* after 5 days of incubation: **A)** soil fraction; **B)** debris fraction.

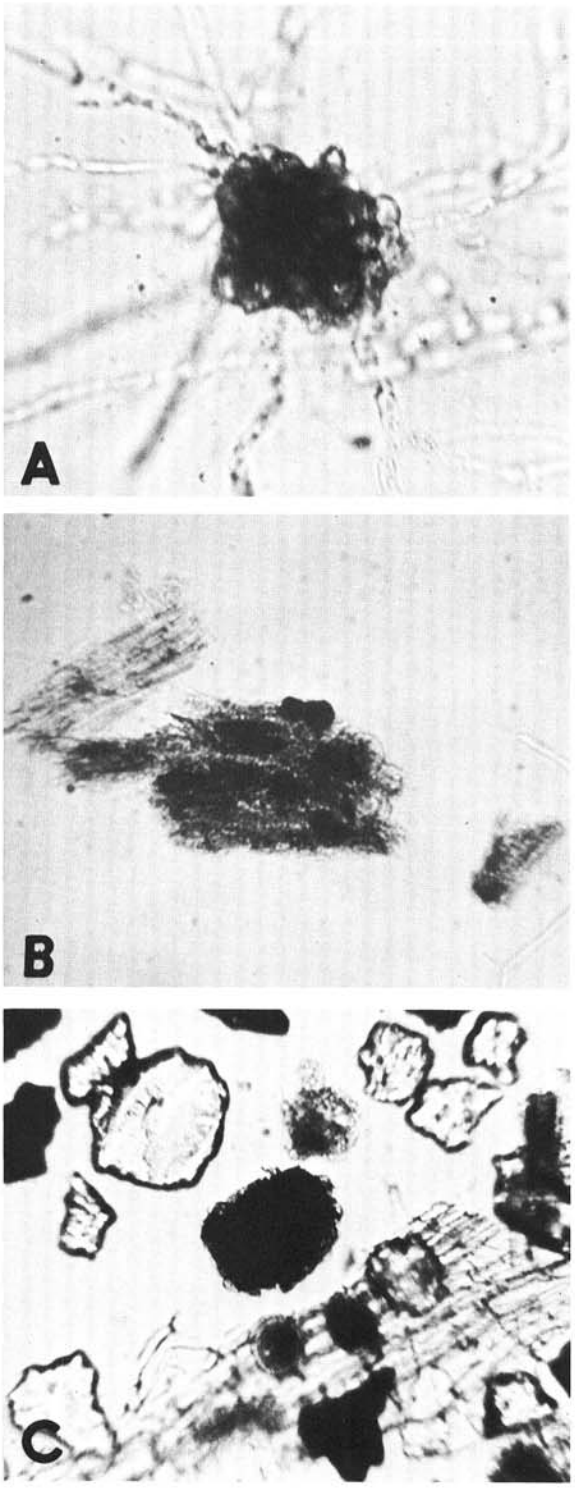

Fig. 2-(A to C). Microsclerotia of *Cylindrocladium crotalariae* isolated from naturally-infested soil: **A)** microsclerotium (65 × 78 μm) observed as colony source in assay plate of soil fraction; **B)** microsclerotia bound in particle (812 × 1,809 μm) of plant debris; **C)** microsclerotium (104 × 143 μm) from 38-μm sieve residue.

Based on these results, an attempt was made to reduce the number of unwanted fungi by treatment of each sieve residue with NaClO, as was shown effective in quantitative isolation of *Macrophomina phaseolina* sclerotia from soil (9). Each sieve residue was washed into a 600-ml beaker using a squeeze bottle containing different concentrations of NaClO in water. After swirling the residues to achieve uniform treatment, the contents were washed onto a 38-μm sieve and rinsed for 1 minute. The treatment was timed from initial contact with NaClO until the residues were washed on the 38-μm sieve.

In studies comparing 0.5 and 0.25% NaClO treatments for 2 minutes, both were effective in eliminating sufficient numbers of undesired fungi in assay plates, but the 0.25% treatment yielded greatest recovery of *C. crotalariae*. A detailed study then was made to determine the optimum exposure to 0.25% NaClO for quantitative isolation of microsclerotia from each fraction. Both the debris and soil fractions were treated 0, 0.5, 1, 2, and 4 minutes in 0.25% NaClO, then rinsed for 1 minute on a 38-μm sieve. Microsclerotia present in the soil fraction were more sensitive to NaClO exposure than ones in the debris fraction (Table 1). The 0.5- and 1-minute exposure periods resulted in greatest frequency of isolation of *C. crotalariae* from the soil fraction, whereas exposure periods up to 4 minutes had little effect on recovery from the debris fraction. Since the 1-minute exposure period was effective in reducing numbers of undesired fungi in both fractions and permitted a high frequency of isolation of *C. crotalariae,* this exposure period was adopted for use in subsequent assays. This experiment was repeated using the same soil (loamy sand) and one additional time using naturally-infested soil (fine sandy loam) from a different field. In each study, the 1-minute exposure period was most effective for reducing numbers of unwanted fungi and quantitative isolation of *C. crotalariae*.

Origin of colonies in assays of naturally-infested soil.—After 5 days of incubation, colonies of *C. crotalariae* readily were identified in assay plates by the presence of brown microsclerotia and profuse asexual sporulation (Fig. 1). More than 100 colonies of *C. crotalariae* were dissected during initial development to determine their origin. Microsclerotia either free in the agar medium or imbedded in plant debris were the only propagules found to be sources of colonies (Fig. 2-A, B). Length and width measurements of over 100 microsclerotia from plates of the soil fraction ranged

TABLE 2. Efficiency of the elutriation procedure for quantitative estimation of microsclerotia of *Cylindrocladium crotalariae* in an artificially-infested peanut field soil

Soil elutriating unit	Microsclerotia per gram of soil		Recovery (%)
	Estimated no. added	No. reisolated	
1	12.8	12.7[a]	99.3 ± 14.4[b]
2	12.8	11.6	90.6 ± 31.0
3	12.8	13.1	102.6 ± 13.6
4	12.8	12.3	96.5 ± 26.7

[a]Values are the average of three replicate samples. Each sample was elutriated for 8 minutes and the collected sieve residues were treated for 1 minute in 0.25% NaClO.

[b]Standard error of mean, $P = 0.05$.

from 39 to 325 μm (mean 102.7 μm) and from 26 to 195 μm (mean 70.0 μm), respectively. Microsclerotia in plates of the debris fraction were commonly contained in plant debris, whereas ones in the soil fraction were usually free in the medium. In several cases, more than one microsclerotium were observed in debris particles from the debris fraction and in one case, 19 separate, microsclerotia-like structures were counted in a single piece of debris.

Microsclerotia also were observed in the soil and debris fractions before plating (Fig. 2-C). Extensive microscopic observations of the collected fractions did not reveal either conidia or ascospores of *C. crotalariae*.

Efficiency of the elutriation method.—In two separate trials, a peanut field soil (fine sandy loam) was artificially infested to densities of 15.1 and 24.8 microsclerotia/g soil. Four replicate samples in each experiment were elutriated for 8 minutes, and the collected fractions were treated with 0.25% NaClO for 1 minute. A mean recovery efficiency of 91.2% (range 74.7 to 107.7 at $P = 0.05$) and 96.0% (range 63.5 to 128.5 at $P = 0.05$) were obtained in assays of the collected sieve residues from each soil, respectively.

In one additional test, three replicate samples of artifically infested soil were elutriated by each unit of the NC-El to compare their efficiency. In this experiment, a peanut field soil (sandy loam) was artificially infested to a density of 12.8 microsclerotia/g soil and assayed as described above. Each elutriator yielded a mean recovery efficiency of at least 91% (Table 2). No microsclerotia were detected in assays of the noninfested soil or the debris fraction from artificially infested soil.

Quantity of microsclerotia in soil from North Carolina peanut fields.—During February and March 1975, soil samples were collected from 23 fields in which CBR had been observed in preceding years. In 1974, these fields had been planted to either tobacco, cotton, corn, soybeans, or peanuts. Two replicate, 200-g samples from each field were assayed. Densities of microsclerotia ranged from 0.2 to 72/g soil. In each field soil, more microsclerotia were recovered from the soil fraction than the debris fraction. The lack of data on populations prior to planting crops in these fields precluded assessment of their influence on numbers of microsclerotia in soil.

DISCUSSION

Survival studies have indicated that microsclerotia of *C. crotalariae* are the primary survival structure in soil, and that conidia and ascospores are capable of only short-term survival (6). The elutriation procedure described here proved to be a rapid and efficient method for quantitative estimation of microsclerotia in naturally-infested soil. Elutriation of four 200-g samples of soil in a period of 8 minutes represents a major advantage over wet-sieving of soil by hand. Since plant debris (larger than 425 μm) and smaller, microsclerotia-size particles (38-45 μm) were collected separately during elutriation, information on the dynamics of survival of microsclerotia in soil may be obtained.

Soil from 23 naturally-infested peanut fields in North Carolina contained 0.2 to 72 microsclerotia/g soil. The dimensions of microsclerotia, observed as sources of *C. crotalariae* colonies in assay plates, were similar to those reported by Rowe et al. (11) for microsclerotia in diseased peanut roots. They reported sizes of microsclerotia ranging from 33.3 μm to 311.1 μm (mean 88.4 μm) long and 22.2 μm to 133.3 μm (mean 52.7 μm) wide. Microsclerotia elutriated from naturally-infested soil frequently were free of plant debris, which suggests they probably were released in soil after decay of associated plant debris.

Microsclerotial densities, ranging from 1 to 103/g soil, have been detected in naturally-infested soil in Virginia by a soil-blending and wet-sieving procedure (8). In one field soil, total populations of *C. crotalariae* as high as 360 propagules/g soil were detected by direct plating of blended soil suspensions (4). Assay of this soil by the wet-sieving method yielded 167 microsclerotia/g soil. The increased population of *C. crotalariae* detected by direct assay of blended soil was thought to result from the presence of propagules smaller than 25 μm, which could include: small microsclerotia and single cells of microsclerotia, ascospores, conidia, and/or mycelial fragments (G. J. Griffin, *personal communication*).

In Hawaii, 350 to 3,050 propagules of *Calonectria crotalariae* were detected in soil samples from the vicinity of diseased papaya seedlings by direct plating of soil suspensions triturated in an Omni-Mixer (5). Propagule densities of that magnitude have not been reported in peanut field soils. Direct isolation of *C. crotalariae* from blended suspensions of peanut field soil have been effective only in assay of a few field soils in which propagule densities exceeded 100/g soil (G. J. Griffin, *personal communication*).

Based on current evidence, isolation procedures that involve wet sieving of soil are an effective means for quantitative estimation of *C. crotalariae* microsclerotia in soil. The elutriation method provides for rapid, efficient estimation of microsclerotial densities currently present in peanut field soils of North Carolina. This assay procedure currently is being used to determine the effect of rotational crops and peanut cultivars on populations of *C. crotalariae* microsclerotia in soil.

LITERATURE CITED

1. 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.
2. BYRD, D. W., JR., K. R. BARKER, H. FERRIS, C. J. NUSBAUM, W. E. GRIFFIN, R. H. SMALL, and C. A. STONE. 1976. Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. J. Nematol. 8:206-212.
3. GARREN, K. H., M. K. BEUTE, and D. M. PORTER. 1972. The Cylindrocladium black rot of peanut in Virginia and North Carolina. J. Am. Peanut Res. Educ. Assoc. 4:66-71.
4. GRIFFIN, G. J. 1975. Total Cylindrocladium crotalariae population in naturally infested peanut field soil. Proc. Am. Phytopathol. Soc. 2:27 (Abstr.).
5. HWANG, S. C., and W. H. KO. 1975. A medium for enumeration and isolation of Calonectria crotalariae from soil. Phytopathology 65:1036-1037.
6. HWANG, S. C., and W. H. KO. 1976. Biology of conidia, ascospores, and microsclerotia of Calonectria crotalariae in soil. Phytopathology 66:51-54.
7. JOHNSTON, S. A., and M. K. BEUTE. 1975.

Histopathology of Cylindrocladium black rot of peanut. Phytopathology 65:649-653.
8. KRIGSVOLD, D. T., and G. J. GRIFFIN. 1975. Quantitative isolation of Cylindrocladium crotalariae microsclerotia from naturally infested peanut and soybean field soils. Plant Dis. Rep. 59:543-546.
9. PAPAVIZAS, G. C., and N. G. KLAG. 1975. Isolation and quantitative determination of Macrophomina phaseolina from soil. Phytopathology 65:182-187.
10. ROWE, R. C., M. K. BEUTE, J. C. WELLS, and J. C. WYNNE. 1974. Incidence and control of Cylindrocladium black rot of peanuts in North Carolina during 1973. Plant Dis. Rep. 58:348-352.
11. ROWE, R. C., S. A. JOHNSTON, and M. K. BEUTE. 1974. Formation and dispersal of Cylindrocladium crotalariae microsclerotia in infected peanut roots. Phytopathology 64:1294-1297.
12. THIES, W. G., and R. F. PATTON. 1970. An evaluation of propagules of Cylindrocladium scoparium in soil by direct isolation. Phytopathology 60:599-601.