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

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ABSTRACT


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; KNO₃, 0.5 g; yeast extract, 0.5 g;
KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.5 g; Tergitol (NPX), 1 ml;
thiabendazole, 1 mg; chloramphenicol, 100 mg;
chlorotetracycline, 40 mg; and 20 g agar per liter of water.
After 5 days of incubation, the presence of brown
microsclerotia and assexual 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., Calonecctia crotalariae (Loos) Bell and Sobers, Cylindrocladium black rot of

Cylindrocladium black rot (CBR) of peanuts (Arachis
hypogaea L.), caused by Cylindrocladium crotalariae
(Loos) Bell and Sobers (1), poses an ever-increasing
treat 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 combine (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 elution 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 cm²/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; KNO₃, 0.5 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 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 chlorotetraacycline, 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% w/vtare powder formulation suspended in 50 ml water), 1 ml; chloramphenicol (1 g dissolved in 50 ml 95% ethanol), 1 ml; and chlorotetraacycline (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 chlorotetraacycline, prevented development of bacterial colonies.

Efficiency of the elutriation and enumeration methods.—Microsclerotia of five isolates of C. crotaleariae were produced in a liquid medium as described by Rowe et al. (11). After 8-10 weeks of incubation (in darkness to prevent peritheium formation), the mycelial mats were comminuted in a Waring Blender 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 grided Millipore filters (3-cm diameter). Counts of microsclerotia were made with a stereomicroscope. 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.

### 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. crotaleariae propagules. Dilution assays of each fraction resulted in disappearance of C. crotaleariae 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 crotaleariae after 5 days of incubation: A) soil fraction; B) debris fraction.](image-url)

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

<table>
<thead>
<tr>
<th>Exposure time (minutes)</th>
<th>Soil fraction</th>
<th>Debris fraction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.3 a</td>
<td>1.5 be</td>
<td>9.8</td>
</tr>
<tr>
<td>0.5</td>
<td>15.3 a</td>
<td>1.8 be</td>
<td>17.1</td>
</tr>
<tr>
<td>1</td>
<td>11.9 ab</td>
<td>3.7 ab</td>
<td>15.6</td>
</tr>
<tr>
<td>2</td>
<td>5.3 cd</td>
<td>3.8 a</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>2.2 d</td>
<td>2.0 abc</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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

1Soil 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.

2Mean values followed by the same letter(s) are not statistically different (P = 0.01) by Duncan's new multiple range test.
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**

<table>
<thead>
<tr>
<th>Soil elutriating unit</th>
<th>Microsclerotia per gram of soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated no. added</td>
</tr>
<tr>
<td>1</td>
<td>12.8</td>
</tr>
<tr>
<td>2</td>
<td>12.8</td>
</tr>
<tr>
<td>3</td>
<td>12.8</td>
</tr>
<tr>
<td>4</td>
<td>12.8</td>
</tr>
</tbody>
</table>

*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.

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. 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 artificially 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 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 Omn-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 qualitative 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


