

Assessment of Microsclerotia of *Verticillium albo-atrum* in Field Soils

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ABSTRACT

A substrate consisting of Czapek's agar, less sugar, plus 200 $\mu\text{g/ml}$ streptomycin sulfate and covered with a layer of cellophane film, trapped *Verticillium albo-atrum* from soils. The presence of the fungus in 32 soils was qualitatively determined by spreading 0.5-g samples of soils on this substrate and counting groups of microsclerotia that developed on the cellulose film after 6 days at 26 C. Groups of microsclerotia were observed without interference from other microorganisms after soil was washed from the surface of the substrate. About 90% of all the groups of microsclerotia detected in soils collected from June to September 1971 were found to arise from microsclerotia free in the soil. Nine of ten

cultures of the fungus isolated from this substrate were pathogenic to cultivar Acala S J-1 cotton seedlings. The procedure was made quantitative by the wet-sieving of 15-g soil samples, followed by the culturing of residues of soils arrested by 37-, 53-, and 74- μ sieves, upon which about 90% of free microsclerotia were arrested. The procedure was quantitative at an inoculum density of 0.13 microsclerotia/g soil. The inoculum density in 33 soil collections, ranging from sandy loams to clay loams in texture, varied from 0.03 to about 50 microsclerotia/g soil.

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The microsclerotium is the persistent soil-borne infective propagule of *Verticillium albo-atrum* Reinke & Berthe. Microsclerotia of the fungus can germinate repeatedly (3), and may produce short-lived conidia in soil (3, 11). Neither a threshold number of microsclerotia required to induce minimal disease nor the inoculum density required to induce an epidemic in the field appears to be known. However, Wilhelm (13) showed the importance of inoculum density when he reported on the reduced infection index of a soil deprived of a susceptible host for different lengths of time. A summary of the literature relating to the importance of inoculum of this fungus to disease development and of the historical problems limiting quantitative determination of infective propagules was made recently by Powelson (11).

We report here qualitative and quantitative procedures for assessing viable microsclerotia of *V. albo-atrum* in field soils which should be helpful in the development of an understanding of the epidemiology of the fungus. Our procedures are based upon mechanical separation of microsclerotia from soils by wet-sieving, together with isolation of the fungus on a suitable substrate. The soil-sieving system has been useful in separating other sclerotia-producing fungi (6, 7, 12) as well as chlamydospores of *Phytophthora cinnamomi* (8) from soils.

MATERIALS, METHODS, AND RESULTS.—*Qualitative determination of viable propagules of V. albo-atrum in soil.*—Soil samples were collected between young cotton plants in fields of Merced, Madera, Kings, and Tulare Counties, California, on 7 to 10 June 1971. Samples were taken at 0-6 inches depth from three rows/field at 3 to 4 places/100 feet of planted row. The subsamples of each row were bulked to make three replicates which were homogenized by hand mixing. Soil, air-dried for 2 to 3 weeks, was sieved, and particles passing a 2-mm

sieve but resting on a 1-mm sieve were used for assays of propagules of the fungus. Three 0.5-g samples from each of the three replicates were spread over a 5 X 5 cm² of washed, sterile cellophane resting on the surface of Czapek's agar, less sugar and amended with 200 $\mu\text{g/ml}$ streptomycin sulfate, in culture plates. The value of this substrate for isolating *V. albo-atrum* was discovered while assaying soils for *Stachybotrys atra*, a root pathogen of cotton (1) which is highly cellulolytic (5). First, the typical conidiophores of *V. albo-atrum* were observed (Fig. 1-B). Later, groups of microsclerotia (Fig. 1-C, D) were evident, at a magnification of 25 times, on plates from which soil particles were washed after they were incubated 6 days at 26 C. The propagules that gave rise to the discrete groups of microsclerotia on cellophane film (Fig. 1-C, D) were presumed to be microsclerotia in soil particles. It is not likely that conidia were involved since the soils were air-dried 2 to 3 weeks before the assays were made (11).

In all, soils of 32 fields were assayed for viable propagules of the fungus. Viable propagules were detected in samples from 16 of 32 fields. While the groups of microsclerotia that developed on cellophane were readily observed, they were few in number. The greatest count was 14.3 viable propagules/g soil; one count was 5.0/g, and the other 14 samples ranged from 0.2 to 2.0 propagules/g. An increase in the number of 0.5-g samples from three to ten for each replicate increased the number of positive samples from 16 of 32 to 19 of 32, but did not result in higher sclerotium counts within samples. In addition, in some fields that were negative for *V. albo-atrum* by this assay, plants had symptoms of Verticillium wilt. Likewise, the standard errors of the means of these assays were about equal to their arithmetic means, indicating that the assays had only qualitative value, due principally to the inadequate size of soil samples.

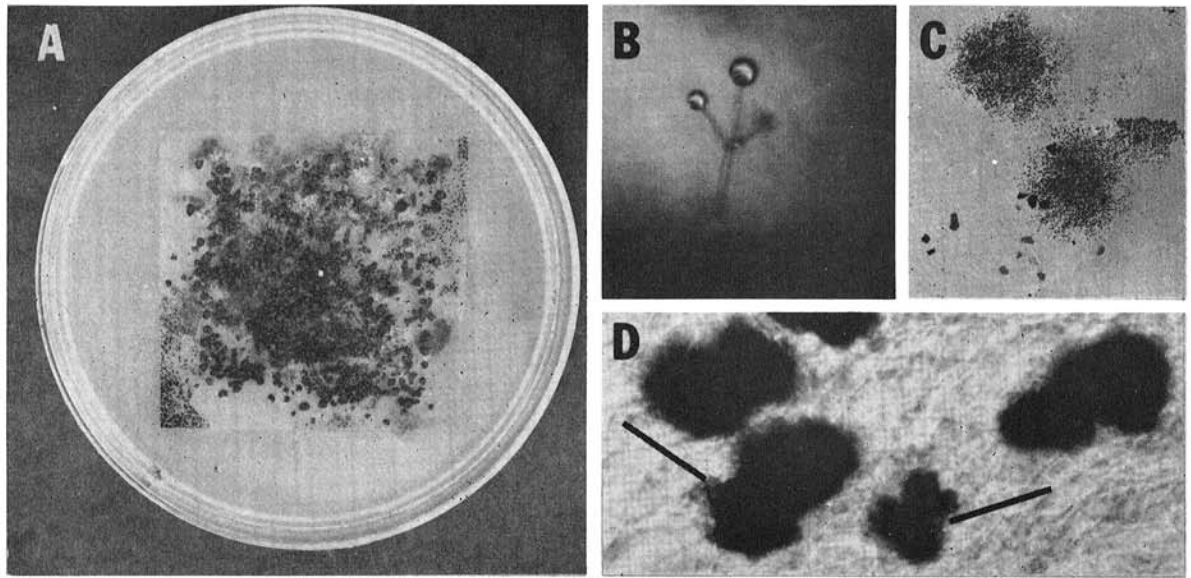


Fig. 1. A) Cellophane agar plates coated with soil particles. B) Conidiophores of *Verticillium albo-atrum* commonly observed on cellophane agar plates. C) Microsclerotia in groups on the surface of a cellophane agar plate. D) A photomicrograph showing light-colored cells of microsclerotia.

In connection with the tests just described, groups of microsclerotia were dissected from cellophane film, air-dried, disinfested 30 sec in a 1:100 aqueous dilution of commercial sodium hypochlorite bleach solution, and transferred to potato-dextrose agar slants. Ten isolates of *V. albo-atrum* obtained in this manner were tested for pathogenicity to young cotton plants (*Gossypium hirsutum* L. 'Acala S J-1'). A droplet of a dense suspension of conidia was injected into each of 24 plants for each of the 10 isolates of the fungus, all of which were typical mycelium-sclerotium (MS) types (11). A droplet of sterile water was injected into the stem of each of 55 control plants. Control plants were free from symptoms of infection after 2 weeks. Seven of the isolates were highly virulent, inducing systemic wilting in 19-24 of 24 inoculated plants; two isolates induced wilting in 5 and 13 of 24 plants. One isolate apparently was avirulent because it neither caused symptoms of infection nor was isolated from inoculated plants.

Origin and nature of propagules in field soils.—Triplicate 2-g samples of nine field soils, air-dried for 2 to 3 weeks, were washed over a series of tandem sieves of 2-mm, 1-mm, 495-, 250-, 125-, 74-, 53-, 37-, and 18- to 22- μ sizes. The filtrate was collected and filtered through a 0.45 μ Millipore filter. All residues except those of the 2-mm and 1-mm sieves were washed into separate vials, and these suspensions were spread in 1-ml portions on the cellophane-agar plates described above. Additional water was added to vials, and transfers were continued until all obvious residues of samples were transferred to plates. Three to five plates were

required for each residue. The following day, plates were examined directly. Clusters of typical microsclerotia were observed in bits of organic debris in plates having soil residues arrested by the 495-, 250-, and 125- μ sieves. Occasional microsclerotia also were observed in the residues of the other sieves; in each case, they were free from plant debris and difficult to distinguish among the dark-colored amorphous residue particles of similar size. No microsclerotia were observed in the plates bearing residues from the Millipore filter pads. After 6 days, the residues were washed from the cellophane-agar plates, and the numbers of groups of microsclerotia were determined as before.

Viable propagules were detected in all nine soil samples, three of which were negative in qualitative tests. The numbers of viable propagules, a summation of those arrested on all sieves, ranged from 4 to 45/g soil. This procedure obviously was more effective than the soil particle method, since three previously negative samples were positive in this test and the average increase in the amount of viable propagules was about 10-fold.

No groups of microsclerotia were present in plates from the residues that passed the 18- to 22- μ sieve, indicating that viable conidia and microsclerotia smaller than 18 to 22 μ were absent from the soils. Few viable propagules were arrested by the 495-, 250-, and 125- μ sieves (ca. 2%, 2%, and 4% of the total propagules, respectively). The groups of microsclerotia that developed in cellophane-agar plates seeded with residues from these sieves originated largely from clusters of microsclerotia imbedded in bits of infested plant debris, and which

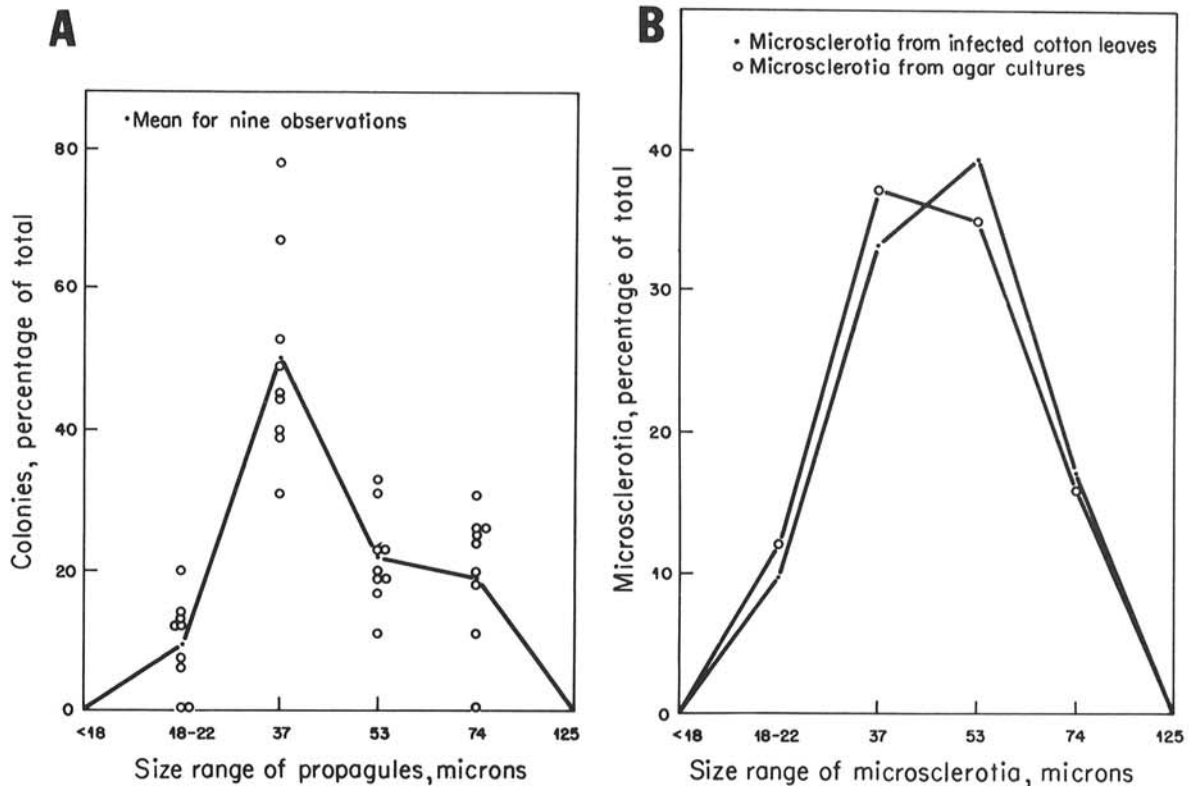


Fig. 2. A) The percentage of the total viable propagules of *Verticillium albo-atrum* from nine field soils arrested by sieves of various sizes. B) The percentage of total microsclerotia of *V. albo-atrum* from cotton leaves and from agar cultures arrested by sieves of various sizes.

were readily detected in residues examined directly.

About 90% of all viable propagules in the nine soil samples were arrested by the smallest (74-, 53-, 37-, and 18- to 22- μ) sieves. The data are summarized in Fig. 2-A. The 37- μ sieve arrested an average of ca. 50%, with a range of 30 to 80%, of the viable propagules free of plant debris that developed on the plates. The fewest viable propagules free from plant debris were arrested by the 18- to 22- μ sieve; thus, about 90% of the viable propagules free from plant debris was arrested by the 37-, 53-, and 74- μ sieves. Results of these tests suggested that the propagules retained by the small sieves, while varying with regard to prevalence from sample to sample, were probably microsclerotia, since the size range agrees with reported sizes of microsclerotia (4). These results were compared with the sieving characteristics of bonafide microsclerotia in another test.

Infected cotton leaves were collected from plants in the field and incubated 7 days at 26 C when mature microsclerotia in the leaves became evident. The leaves were air-dried and ground to pass a 20-mesh screen with a Wiley mill. The material resting on a 1-mm sieve was discarded, and the microsclerotia were separated from the smaller debris by the flotation procedure described by Evans et al. (2). The surface mats of four isolates of *V. albo-atrum* were cut from slant cultures and blended with water for 5

min in a Waring Blendor. Microsclerotia from both sources then were sieved through the series of sieves, and the residues passing the 18- to 22- μ sieve were arrested on coarse filter paper. The residues were transferred to vials. Those passing the 18- to 22- μ sieve and arrested on filter paper were suspended in 1 ml of water, and others from cotton leaves and cultures were suspended, respectively, in 5 and 10 ml of water.

The suspended residues of particles arrested on filter paper were examined at a magnification of 190 times; they were free from typical microsclerotia, although clusters of 3 to 6 dark-colored cells were observed in the residues from agar-grown cultures. A hemacytometer was used to quantize the numbers of microsclerotia in the other suspended residues, with 10 samples of each residue being counted. Then the mean percentage of the total microsclerotia represented by each size group was calculated (Fig. 2-B). No microsclerotia from either cultures or cotton leaves were arrested on the 495- and 250- μ sieves. The 125- μ sieve bore aggregates of microsclerotia, in the case of material from culture, and of sclerotia in bits of leaf tissue in the case of material from cotton leaves. The sieving characteristics of microsclerotia in this test (Fig. 2-B) agree with the data on viable propagules from naturally infested field soils (Fig. 2-A), indicating that the viable propagules in soil

TABLE 1. The influences of differences in soil preparation and sieving procedures upon recovery of microsclerotia of *Verticillium albo-atrum* on cellophane-agar plates

Sieve size	Microsclerotia in soil			
	Dry-sieved soil		Wet-sieved soil	
	Pulverized ^a	1-mm particles ^b	Pulverized	1-mm particles
μ	No./g	No./g	No./g	No./g
74	11.0	9.1	9.7	4.6
53	11.5	6.6	7.3	5.4
37	30.4	20.0	17.7	10.4
LSD = .05	21.2			

^a Soil pulverized with a mortar and pestle.

^b Soil particles passing a 2-mm sieve and resting on a 1-mm sieve.

were microsclerotia free in the soil.

Quantitative procedure for determining the number of microsclerotia free in soil.—Results of a test described above suggested that about 10 times more microsclerotia were recovered from residues obtained by the washing of soil through a series of sieves than from soil particles arrested on a 1-mm sieve. Further experiments were made to determine an efficient sieving procedure for soil. We determined the number of microsclerotia recovered on 74-, 53-, and 37- μ sieves by wet- and dry-sieving procedures. In addition, the amount of microsclerotia recovered from soil pulverized with a mortar and pestle versus particles of soil arrested on a 1-mm sieve was determined. In this test, five 2-g replicates for each treatment were made from a single soil sample. Twenty plates, each with 0.5 g of 1-mm soil particles, were used as a control for the experiment. The control series showed the microsclerotium content of the soil to be 6.0/g, but greater numbers of microsclerotia were recovered in all other treatments (Table 1). In other comparisons, more microsclerotia were recovered from pulverized samples than from samples made up only of the 1-mm fraction of soil, whether the samples were wet- or dry-sieved. Likewise, more microsclerotia were recovered from dry-sieving treatments than from wet-sieving treatments. But while these differences were indicative of relative efficiency of methods, a difference at the 0.05 level of confidence occurred only between dry-sieved pulverized samples and wet-sieved samples of 1-mm soil particles (Table 1).

The quantitative assay for viable microsclerotia of *V. albo-atrum* in field soils, now used routinely in our laboratory, is given below. This assay, based upon the experiments described above, utilizes the wet-sieving procedure because of convenience, with the realization that it might be somewhat less effective than the dry-sieving procedure. Triplicate 15-g samples of air-dried soils were sieved through 125-, 74-, 53-, and 37- μ sieves, and the residues arrested by the last three sieves were bulked. The composite residues were suspended in about 10 ml of water in

test tubes, and 1-ml portions were transferred to plates whose entire agar surfaces (about 180 mm²) were covered with cellophane. Additional water was added as before to remove all obvious residue from tubes. Each 15-g replicate required 15 to 16 plates, and thus each sample required a total of 45 to 48 plates.

Soil samples were collected from 32 cotton fields during June to September 1971. They were air-dried, as before, then assayed for microsclerotia as described above. No microsclerotia were detected in soils of two fields. Soils of five fields contained from 0.03-0.08 microsclerotia/g. In these cases, the standard errors of means were ± 0.02 to 0.07, indicating that results were not reliably quantitative. However, results of soil assays of the remaining 25 fields were highly quantitative, ranging from 0.13 ± 0.02 to 46.97 ± 2.11 . The mean coefficient of variation for all samples was 15.4%.

In addition to this procedure's being quantitative, the use of the substrate allowed microsclerotia of *V. albo-atrum* to be counted without conflict due to overgrowth by other microorganisms. This is the principal advantage of our substrate over others (2, 9, 10) used for this purpose. While *V. albo-atrum* is not a highly cellulolytic fungus (14), it competed very well on this substrate in which cellophane was the principal carbon source. The procedure was unaffected by soil types which ranged in texture from sandy loams to clay loams taken from 32 cotton fields in central California. Our principal difficulty was in assaying soils having 20 or more microsclerotia/g. In such cases, greater precision would be possible with an increase of the number of plates on which the residues are spread, thus increasing objectivity in determining the centers of individual groups of microsclerotia on plates (Fig. 1-C). A second difficulty was caused by the cellulolytic activity of microorganisms in the soil samples which, in time, disintegrated the cellulose film. This problem was overcome by washing residues from plates after 6 days and by storage of washed plates at 2 to 5 C until counts were made.

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