Verticillium albo-atrum: Quantitative Isolation of Microsclerotia From Field Soils

O. C. Huisman and L. J. Ashworth, Jr.

Assistant Professor and Plant Pathologist, Department of Plant Pathology, University of California, Berkeley 94720.

Accepted for publication 26 March 1974.

ABSTRACT

Microsclerotia of *Verticillium albo-atrum* were quantitatively isolated from field soils by a density flotation procedure. Organic residues were obtained by wet-sieving soil samples and subjecting the 37 to 125 μm soil fraction to density flotation in 65% sucrose (w/w). The sucrose flotation was less effective (70%) and gave more variable results in determining populations of *Verticillium* in soils than the direct plating of wet sieved residues, but the flotation method allowed direct isolation of microsclerotia. With the aid of a dissecting microscope, microsclerotia-like structures were individually removed from the sucrose flotation residue for separate plating. Nearly 100% of the *Verticillium* detected by our assay was present as microsclerotia. All the microsclerotia had small bits of plant organic material associated with them. A significant number of the microsclerotia were also present in small clusters of two or more. Assuming that all microsclerotia-like structures were *Verticillium*, minimum viability values ranged from 5 to 50%.

Phytopathology 64:1159-1163

Additional key words: Gossypium hirsutum.

Dark-colored microsclerotia of *Verticillium albo-atrum* R. & B. (*V. dahliae* Kleb.) generally are considered to account for the persistence of the fungus in field soil (3, 11, 12), but Schnathorst (13) and DeVay et al. (4) report that hyaline structures, atypical of mature microsclerotia, are important survival structures of the fungus. Evans et al. (5) have isolated small numbers of microsclerotia directly from field soils. Further evidence is reported here that microsclerotia are the persistent propagule of *V. albo-atrum* in soil. Development of a method for direct isolation of large numbers of microsclerotia from field soils was mainly responsible for this conclusion. Portions of the work have been reported previously (6, 7).

MATERIALS AND METHODS.–Soil samples were collected from a number of cotton fields in the lower San Joaquin Valley in California during 1972. Samples were taken at 0- to 10-cm depth from 20-30 locations in each field. The subsamples were bulked and air-dried for 2 days at 20-27 C. The dry soil was pulverized in a soil mill (1) and mixed in a V-shaped mixer (Patterson-Kelley Co., East Stroudsburg, Pa.). Soils were stored, following mixing, at 4 C until used. Assays for *V. albo-atrum*, unless otherwise stated, were performed on three replicate 15-g soil samples.

Two assay procedures were used. The direct assay procedure in which soil particles 37-125 μm in size were isolated by wet sieving and cultured on pectate media (8), and a sucrose flotation procedure. The initial steps of the sucrose flotation procedure were the same as for the direct assay procedure: the soil residue retained by the 37 μm sieve was collected in 30-ml centrifuge tubes, centrifuged for 5 min at 1,600 *g*, and following centrifugation, all except 1-2 ml of the covering water was removed by aspiration. The residue was thoroughly suspended in approximately 20 ml of 65% sucrose (w/w) and centrifuged for 20 min at 1,600 *g*. The supernatant solution was decanted into a beaker and saved along with water used to rinse the walls of the inverted centrifuge tube. The precipitate was resuspended in 60% sucrose solution, centrifuged as before, and this supernatant was bulked with the first. The pooled supernatant solutions were mixed with approximately 300 ml of sterile water and passed through a Duralon Millipore filter (14-μm pore size) under suction. The final residue collected on the filter pad was either suspended in sterile water and cultured by spreading over 10 plates of pectate media, or microsclerotia were isolated individually from the filter pad as described below.

Individual microsclerotia were isolated directly from the residue retained on the Millipore filter, using a dissecting microscope (× 80) and fine tweezers used in electron microscopy. The microsclerotia were cultured separately on pectate media. Filter pads imprinted with a grid simplified systematic removal of microsclerotia.

Cultures from 20 different colonies of *Verticillium* appearing on sucrose flotation assay plates were tested for pathogenicity to cotton, cultivar Acala SJ-1. Ten plants for each isolate were inoculated by injection of 5-10 μl of a suspension of conidia in water.

Microsclerotia used in germination tests were isolated from residues of naturally infected cotton plants. The residues were collected during November, 1972, from a cotton field in which essentially all plants were infected by the fungus. The residues were stem segments that had been incorporated into the soil with a disk harrow 2 wk prior to collection. The residues were air-dried, then microsclerotia were freed from tissues by pulverizing the residue in a Wiley mill fitted with a 240-μm (60-mesh) screen.

RESULTS.–*A comparison of direct and sucrose assay procedures.*–Experiments with microsclerotia obtained from *Verticillium* grown on potato-dextrose agar showed that microsclerotia were buoyant in sucrose solutions with minimum density of 1.23 (50% sucrose w/w). A 65% sucrose solution (w/w) was selected for routine flotation work as this provided a

PHYTOPATHOLOGY for August (64:1049-1158) was issued 19 August 1974.

solution dense enough to insure flotation of microsclerotia without presenting excessive viscosity problems.

The flotation method allowed a significant reduction in sample size. From the original 15 g of dry soil a final yield of 3-10 mg (dry weight) residue was obtained. This represented a 1,500- to 5,000-fold concn of microsclerotia compared to a 3- to 8-fold concn of microsclerotia for the direct assay procedure. The final residue obtained from soil samples with the sucrose flotation procedure consisted of small fragments of organic debris and an assortment of microbial propagules. Comparisons of relative efficiency of the two assays were made with a variety of field soils. Overall, the sucrose flotation assay was about 70% as efficient as the direct assay in measuring populations of *Verticillium* in field soils (Table 1). The sucrose flotation assay gave reproducible quantitative measurements but the variability was larger than that for the standard assay. Between replications of a given assay, the mean variability was 11%. However, the mean variability increased to 41% between assays of the same stored soil lot performed at different times.

Isolation and germination of microsclerotia from soil and plant tissues.—To determine whether microsclerotia could account for all the *Verticillium* detected by our assay technique, residues obtained from the sucrose flotation procedure were systematically searched for microsclerotia. The small size of the final sample made this feasible. All microsclerotia-like structures were removed and plated separately (Fig. 6), and the residue remaining after the removal of the microsclerotia was then cultured to determine propagules of *Verticillium* missed during the isolation step. In the initial experiments, 80 to 95% of the *Verticillium* present was recovered as microsclerotia. In later experiments, as the proficiency in the technique improved, nearly all of the *Verticillium* could be recovered as microsclerotia (Table 2). Often other fungi developed from isolated microsclerotia.

The technique described permitted estimation of the minimum viability of the microsclerotia present in soil. Values ranging from 5 to 50% viability were thus obtained (Table 2).

For comparative purposes, an experiment on germinability was performed with microsclerotia obtained from *Verticillium*-infested cotton residues collected from the field. The residue was first pulverized, wet-sieved, and processed by the sucrose flotation procedure. The final residue was systematically searched for microsclerotia, which

TABLE 1. Recovery of *Verticillium* from soil by direct plating and sucrose flotation procedures

Soil	Colonies detected (no./g dry soil) Direct method	Sucrose method	$\frac{\text{Sucrose}}{\text{Direct}} \times 100$
95A	40.6	25.8	63
95B	21.6	19.6	91
42B	20.8	17.5	84
WS-3B	69.8	68.7	98
86	8.2	1.7	21
87	18.0	9.0	50
88	2.0	1.3	65

TABLE 2. Recovery of *Verticillium* microsclerotia from field soils and infested cotton residues. The values given are those obtained from single 15-g soil samples. The soils used represented a wide variety of soil types from the lower San Joaquin Valley, California, and also represented a range of *Verticillium* population levels.

Source	Total number of: Isolations	Viable *Verticillium*	Viable misc. fungi	Percent of: *Verticillium* recovered[a]	Minimum viability[b]
Soil					
WS-3B	1920	1024	71	99	53
95	760	381	58	99	50
89	386	202	24	95	52
86	276	106	28	99	38
42A	230	54	50	95	23
SH	450	27	50	100	6
88	202	11	35	100	5
83	20	1	5	100	5
Cotton residue					
Systematic search	397	179	30	99	45
Selected mature ms[c]	104	82	2	—	79
Selected immature ms[c]	53	4	0	—	7

[a]Percent of *Verticillium* isolated relative to the total *Verticillium* detected.

[b]Percent of *Verticillium* isolations relative to the total isolations made.

[c]ms = microsclerotia; "Mature" microsclerotia were well developed and pigmented, while "immature" microsclerotia were small and poorly pigmented.

were removed and cultured separately. A viability of 45% was calculated for these microsclerotia (Table 2). When microsclerotia were removed more selectively (Table 2), the mature (well-developed and pigmented) microsclerotia exhibited a far greater viability (80%) than the immature (small and poorly pigmented) microsclerotia (7%). These results indicated that the viabilities of microsclerotia isolated from soils do not differ sharply from those still embedded in intact cotton residues.

Morphology of microsclerotia isolated from soil.—Microsclerotia isolated by the procedure described above were mounted on slides and examined with a compound microscope. These microsclerotia from soil (Fig. 1-5) appeared identical in size and morphology to those obtained in culture (9, 14) and from infected cotton debris (5). The microsclerotia were variable in size with the distribution range (37-100 μm) similar to that reported by Ashworth et al. (3). The majority of the structures were mature and well pigmented (Fig. 1-5) but small, lightly pigmented structures were also observed (Fig. 2). All the microsclerotia were found to be embedded in small pieces of organic matter (Fig. 1-5). Occasionally microsclerotia were apparently free of associated organic debris; however, with dark-field illumination small remnants of adhering plant tissue could be detected. Due to the soft compressible nature of this associated debris, it is unlikely that the sieving characteristics of microsclerotia would be significantly affected. Presumably this material represented the final remnants of the cotton residue in which the microsclerotia were originally formed. Many of the microsclerotia also occurred in small clusters (Fig. 1, 3 and 5), and in most soils such clusters constituted up to half of the isolated units. Such clusters either were closely associated microsclerotia (Fig. 3 and 5) or were held together by the surrounding organic matrix (Fig. 1 and 3).

The pathogenicity of the microsclerotia of *Verticillium* detected by our assay in soils was tested by inoculation of cotton plants. Twenty random isolates were obtained from colonies of *Verticillium* appearing on the sucrose flotation assay plates from a number of different soils. All isolates proved to be pathogenic, and induced defoliating wilt symptoms in the test plants.

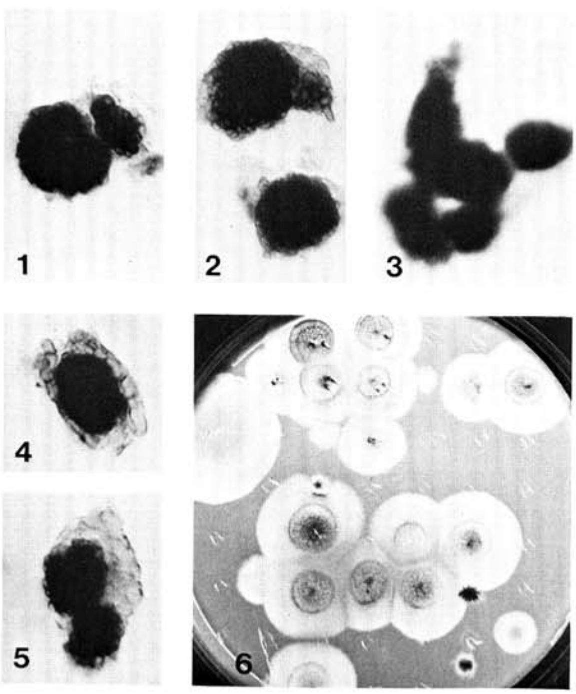

Fig. 1-6. 1-5) Representative microsclerotia and microsclerotial clusters of *Verticillium albo-atrum* isolated from naturally infested field soils by the method described in the text. The organic debris associated with the microsclerotia is clearly visible in Figs. 4 and 5. [Fig. 1, 2, 4, 5 (× 280), and Fig. 3 (× 200)]. **6)** Representative 14-day-old agar plate onto which had been plated microsclerotial (*Verticillium*) structures as detailed in text. All aerial mycelium and spores have been removed by gentle rubbing of the agar surface under running water. The *Verticillium albo-atrum* colonies can be identified by the peppery appearance of microsclerotia at the center of the colony. Several miscellaneous fungi are visible. Locations where nonviable propagules were deposited are identified by the puncture marks left in the agar by the tweezers.

DISCUSSION.—The sucrose flotation procedure is based on the large difference in density between the inorganic fraction of the soil and the particulate organic fraction which includes the microsclerotia of *Verticillium*. Although we have not worked with other organisms, the procedure described for *Verticillium* may be useful for recovering similarly sized propagules of other fungi on a quantitative basis from soils. Lingappa and Lockwood (10) have reported successful recovery of *Glomerella cingulata* conidia from inoculated soils by a density flotation method utilizing glycerol.

The sucrose flotation was less effective and gave more variable results in determining populations in soils than the direct plating method; but the flotation method allowed direct isolation of microsclerotia. Thus the direct plating method is preferrable for routine assays for *V. albo-atrum* in soils. However, with most soils the flotation method gave sufficient recovery (50-70% relative to the direct plating assay) to warrant the view that the *Verticillium* structures thus obtained were representative of the populations of *Verticillium* detected by our direct plating assay.

Microsclerotia have been reported as the main survival structures of *Verticillium* in soil by other workers (11, 12). Evans et al. (5) reported the direct isolation of a small number of microsclerotia from field soil. These workers reported single microsclerotia to be predominant, but noted the presence of microsclerotia embedded in microscopic bits of plant debris. In earlier work Ashworth et al. (3) reported that individual microsclerotia represented the viable propagules of *V. albo-atrum* in field soils. This conclusion was based on the identical sieving characteristics of microsclerotia from culture and the viable propagules of *Verticillium* present in field soils. Although not all microsclerotia were present as individual units, the work here substantiates the earlier conclusion. By individually removing all microslcerotia-like structures from the organic residues from a number of different soils, nearly 100% of the *Verticillium* detected by our assay could be removed from the residues (Table 2). From this we concluded that all of the persistent propagules of *Verticillium* in field soils detected by our assay were present in the form of microsclerotia.

The viability values determined for microsclerotia isolated from soil should be viewed as approximations as there are at least two possible sources of error. If the possibility is taken into account that not all structures isolated were necessarily microsclerotia of *Verticillium*, the calculated viability values (5 to 50%) would represent minimum values. Most of the miscellaneous fungi observed on the plates (Table 2, Fig. 6) probably arose from spores adhering to the microsclerotia since both *Verticillium* and other fungi were occasionally observed arising from the same propagule. Some of these fungi could also have originated from propagules resembling microsclerotia of *Verticillium* which were mistakenly selected. The small aggregates of microsclerotia, on the other hand, may have inflated the viability figures somewhat. Only one viable microsclerotium need be present in each such aggregate to give a positive viability count for that unit.

The higher viability values were found in soils with relatively high populations of *Verticillium*. These values compared favorably with those observed for microsclerotia obtained from field collected infected cotton residues (Table 2), and are in agreement with the 60 to 70% figures reported by Evans et al. (5) for the microsclerotia they isolated from cotton residues and from field soil. The low viability values calculated for the soils with low levels of *Verticillium* (Table 2) may reflect a greater tendency to retrieve non-Verticillium propagules from the residue. The ability to discriminate between *Verticillium* and miscellaneous propagules should improve with practice. It is also possible that viability of microsclerotia in these soils is indeed significantly lower.

The microsclerotia isolated from soil differed from those obtained in culture in that most were still embedded in small bits of plant debris (Fig. 4 and 5). This finding is in agreement with the suggestion by Evans et al. (5) that microsclerotia originate in host tissue. Whether this halo of plant tissue can serve as an external source of potential nutrition for the microsclerotia is not known. It is possible that this material represents the final remnant of plant tissue from which all readily available nutrients have been exhausted during decomposition.

A large fraction of the *Verticillium* in the soil exists as small aggregates of microsclerotia. This observation has relevance to the concept of free and bound inoculum. Menzies (11) distinguished between effective and potential inoculum. The clusters of microsclerotia we observed probably behave as single units of inoculum in the soil (i.e., units of effective inoculum), as suggested by Menzies (11). Each cluster also represents a significantly higher potential inoculum in that further dispersion of the microsclerotia in these aggregates through tillage or any other means would result in an increase in effective units.

Any propagules of *Verticillium* lost during the wet-sieving step could represent a population of *Verticillium* not accounted for in our work. For most of the soils tested, however, from 10 to 20% of the detectable *Verticillium* was not retained by the 37-μm sieve. Such figures agree well with the sieving properties reported for microsclerotia of *Verticillium* (3). We did not detect any of the hyaline structures reported by DeVay et al. (4) and by Schnathorst (13). But other data (1) indicated that their soil handling and assay procedures resulted in breakage of microsclerotia into small, but germinable, units. The good correlation between population levels of *Verticillium* detected by our assay and disease incidence (2) discounts the importance of other propagules in the epidemiology of Verticillium wilt.

LITERATURE CITED

1. ASHWORTH, L. J., JR., D. M. HARPER, and H. L. ANDRIES. 1974. The influence of milling of air dry soil upon apparent inoculum density and propagule size of Verticillium albo-atrum. Phytopathology 64:629-632.
2. ASHWORTH, L. J., JR., O. D. MC CUTCHEON, and A. G. GEORGE. 1972. Verticillium albo-atrum: the quantitative relationship between inoculum density and infection of cotton. Phytopathology 62:901-903.
3. ASHWORTH, L. J., JR., J. E. WATERS, A. G. GEORGE, and O. D. MC CUTCHEON. 1972. Assessment of microsclerotia of Verticillium albo-atrum in field soils. Phytopathology 62:715-719.
4. DE VAY, J. E., L. L. FORRESTER, R. H. GARBER, and E. J. BUTTERFIELD. 1974. Characteristics and concentration of propagules of Verticillium dahliae in air-dried field soils in relation to the prevalence of Verticillium wilt in cotton. Phytopathology 64:22-29.
5. EVANS, G., W. C. SNYDER, and S. WILHELM. 1966. Inoculum increase of the Verticillium wilt fungus in cotton. Phytopathology 56:590-594.
6. HUISMAN, O. C., and L. J. ASHWORTH, JR. 1972. Separation of microsclerotia of Verticillium albo-atrum from soil residues by density flotation. Phytopathology 62:766-767 (Abstr.).
7. HUISMAN, O. C., and L. J. ASHWORTH, JR. 1973. Quantitative recovery of Verticillium albo-atrum. Page 23 *in* Proc. Beltwide Cotton Res. Conf. Phoenix, Arizona.
8. HUISMAN, O. C., and L. J. ASHWORTH, JR. 1974. Quantitative assessment of Verticillium albo-atrum in field soils: procedural and substrate improvements. Phytopathology 64:1043-1044.
9. ISAAC, I. 1949. A comparative study of pathogenic isolates of Verticillium. Trans. Br. Mycol. Soc. 32:137-157.
10. LINGAPPA, B. T., and L. LOCKWOOD. 1961. The nature of the widespread soil fungistasis. J. Gen. Microbiol. 26:473-485.
11. MENZIES, J. D. 1970. Factors affecting plant pathogen population in soil. Pages 16-21 *in* T. A. Toussoun, R.

V. Bega, and P. E. Nelson, eds. Root diseases and soil-borne pathogens. Univ. Calif. Press, Berkeley and Los Angeles.

12. POWELSON, R. L. 1970. Significance of population level of Verticillium in soil. Pages 31-33 *in* T. A. Toussoun, R. V. Bega, and P. E. Nelson, eds., Root diseases and soil-borne pathogens. Univ. Calif. Press, Berkeley and Los Angeles.

13. SCHNATHORST, W. C. 1965. Origin of new growth in dormant microsclerotial masses of Verticillium albo-atrum. Mycologia 57:343-351.

14. WILHELM, S. 1951. Is Verticillium albo-atrum a soil invader or a soil inhabitant? Phytopathology 41:944-945 (Abstr.).