A Method of Quantifying Numbers of Microsclerotia of Verticillium albo-atrum in Cotton Plant Tissue and in Pure Culture

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ABSTRACT

Cotton leaves containing microsclerotia of *Verticillium albo-atrum* were blended in water, and the suspension was passed through sieves of different pore sizes. Microsclerotia of different sizes (< 37 μm, 37 to 125 μm, and > 125 μm) were collected and counted microscopically in a Hawksley eel worm counting chamber. Comparisons of different numbers of blending periods (15 seconds) indicated that the maximum number of unbroken microsclerotia (37 to 125 μm fraction) was obtained after two to three 15-second periods (30-45 seconds). The number of microsclerotia in the 37 to 125-μm fraction from a pure culture was determined to be inversely correlated colorimetrically with the percentage of light transmittance at 400 nm. These techniques were useful for quantitative research.

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Additional key words: colorimetry, counting chamber, inoculum.

Microsclerotia of *Verticillium albo-atrum* Reinke and Berth. have been isolated quantitatively from soils by several workers (1, 2, 3, 5, 6). Evans et al. (5) estimated the number of microsclerotia in a plant by photographing stripped pieces of infected tissues from which they estimated that the root and stem of a single cotton plant contained more than 25×10^4 and a leaf 20×10^4 microsclerotia. Gordee and Porter (7) isolated microsclerotia by homogenizing cultures and Isaac et al. (9) by grinding air-dried potato haulms after which the microsclerotia were collected on a 74-μm pore size screen; however, since sizes of microsclerotia range from 11 to 225 μm (2), those less than 74 μm would not be detectable. Hall and Ly (8) measured the quantity of microsclerotia in a pure culture based on the weight of the melanized cell walls after destroying the cells with H_2SO_4. This method would not be satisfactory for measurements of numbers of microsclerotia per gram of tissue due to the nonuniform size of microsclerotia.

We developed a method of quantification of numbers of microsclerotia by blending host tissue and counting (under a dissecting microscope) the microsclerotia collected in several different size fractions on sieves. These data were amenable for the quantitative assessment of the effect of fungicides on the numbers of microsclerotia formed in plant tissues.

MATERIALS AND METHODS.—*Preparation of microsclerotia.*—1) In vitro.—An isolate of the defoliating strain of *Verticillium albo-atrum* (V3H) was grown on sterilized cellophane (Lustro Roll, Zellerbach Paper Co.) which was placed on top of Czapek agar (minus sugar) (1) after the medium had solidified. After 8 weeks of incubation at 25 C in a closed plastic bag, the mycelial mat with microsclerotia was transferred from the agar to a beaker of water. The homogenate was passed through a 125-μm sieve and the filtrate through a 37-μm sieve. The microsclerotia remaining on the 37-μm sieve were used for relating colorimetry to numbers per ml of water determined by use of a Hawksley eel worm counter.

—2) In vivo.—Cotton plants (*Gossypium hirsutum* L. 'SJ1') were grown in 15 × 15 cm plastic pots of sterilized sandy loam soil in a greenhouse for 42 days and inoculated by stem puncture with spores (10^6 per ml) of *V. albo-atrum* (microsclerotial, defoliating isolate V3H) (4). Diseased leaves were collected 8 days later and buried for one month in moist, nonsterilized sandy loam soil. Microsclerotia formed during decomposition in the soil. The leaves were recovered, washed in water, and soaked in 70% ethanol for 24 hours which extracted part of the chlorophyll. Plant tissue was blotted dry on paper towels, and a 1-gram sample was homogenized in 25 ml of distilled water in a Sorvall Omni-mixer (75-ml cup) at full speed for various numbers of 15-second periods of time. Two samples with four observations of each were assayed. One aliquot of the homogenate was passed successively through sieves with 125 μm and 37 μm pore sizes. Three fractions of microsclerotia (> 125 μm, 37 to 125 μm, and < 37 μm) were differentiated and counted microscopically using a Hawksley eel worm counter (Hawksley, England).

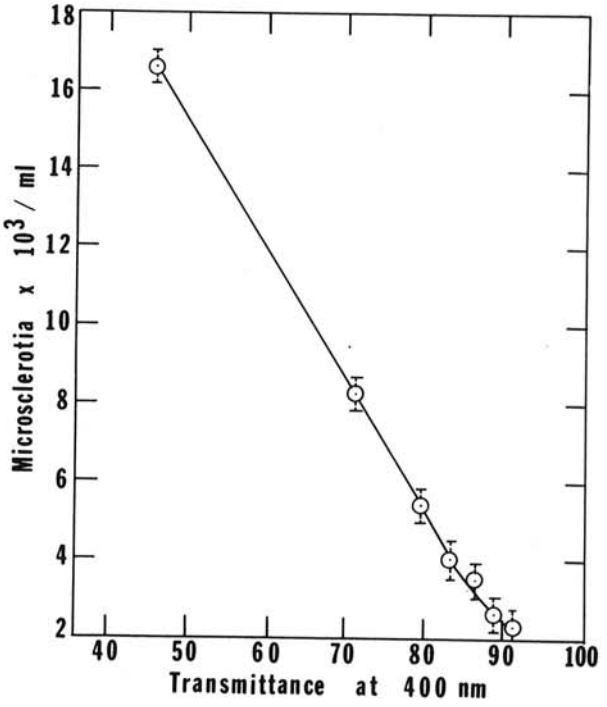

Fig. 1. Inverse correlation of the numbers of microsclerotia (37 to 125-μm fraction) of *Verticillium albo-atrum* in an aqueous suspension with the percentage of light transmittance at 400 nm. Standard deviations among eight observations are indicated by the vertical lines.

Colorimetric measurement.—The microsclerotia in the 37 to 125 -μm fraction obtained from an in vitro culture of *Verticillium* were suspended in sterile distilled water. The number of microsclerotia present in different dilutions was counted microscopically (Wild steroscope) using the Hawksley eel worm counter after recording light transmittance (Beckman Spectronic 20 Spectrophotometer at 400 nm). Samples in a dilution series were replicated twice and four counts of microsclerotia per sample were assessed.

RESULTS AND DISCUSSION.—The density of a microsclerotia suspension (37 to 125 μm) from a pure culture was inversely correlated with the percentage of light transmittance at 400 nm (Fig. 1). The confidence interval obtained from the average of two replicates with four observations of each, indicated little variability.

When the microsclerotia from plant tissue were blended for different periods (15 seconds) of time, the number of microsclerotia in each of three fractions varied with the time of blending (Fig. 2). Blending for one 15-second period resulted in slightly more microsclerotia in the $>$ 125-μm fraction than in the $<$ 37-μm fraction, indicating that one 15-second period was insufficient to release the microsclerotia from the leaf tissue. Microscopically we noted that microsclerotia were still bound in plant tissue. Two 15-second blendings (30 seconds) significantly increased the numbers of microsclerotia in the 37 to 125 μm fraction, but reduced the number of particles in the $>$ 125-μm fraction. After three 15-second periods of blending, the total number of microsclerotia and the number in the 37 to 125-μm and $<$ 37-μm fractions were increased with a corresponding reduction in microsclerotia in the $>$ 125-μm fraction. Four 15-second (60 seconds) periods of blending reduced the number of microsclerotia in the 37 to 125-μm fraction, but increased the number in the $<$ 37-μm fraction although the total number remained the same. Five 15-second (75 seconds) periods of blending reduced the numbers in the 37 to 125 μm fraction as well as the total number of microsclerotia, apparently due to the breakage of microsclerotia into particles too small to be detected.

For convenience, the dominant 37 to 125-μm fraction was considered to be the most important. Blending for three 15-second periods provided data that were appropriately sensitive and accurate for research on the effect of treatments on microsclerotium formation in plant tissue.

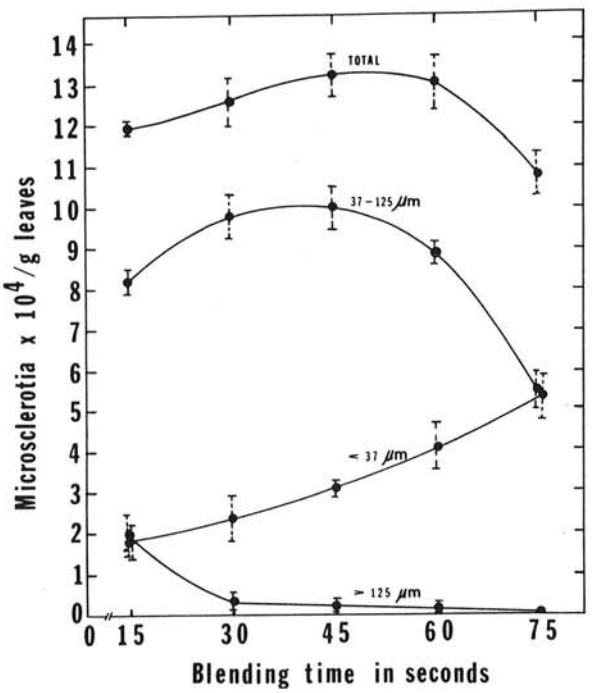

Fig. 2. Effect of blending *Verticillium*-infected cotton leaf tissue for different periods of time (15-second increments) on the numbers of microsclerotia obtained in different sieve size fractions. Standard deviations among 8 observations are indicated by the vertical lines.

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