The Influence of Milling of Air-Dry Soil Upon Apparent Inoculum Density and Propagule Size of Verticillium albo-atrum

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This research was supported in part by Cotton, Incorporated and California Planting Cotton Seed Distributors.
Accepted for publication 5 November 1973.

ABSTRACT

Microsclerotia of *Verticillium albo-atrum* in air-dry field soil equilibrated in 1 week at 80% relative humidity (RH), and in soil air-dried for 48 h at 22-25 C and 40% RH, were unaffected by various soil milling procedures. But microsclerotia in soil stored at 40% RH (room conditions) for 4.5 mo before milling, were broken into smaller units regardless of milling procedure. Milling procedures in decreasing order of microsclerotium breakage were mortar and pestle, high-speed micromilling (20,000 rpm), and a 2-mm sieving. The data indicate that microsclerotium breakage, depending upon soil storage conditions and milling procedures, can result in apparent inoculum densities greater than the actual inoculum densities of field soils.
Phytopathology 64:629-632.

Additional key words: soil-borne pathogens.

Views among investigators vary on the numbers of microsclerotia (ms) of *Verticillium albo-atrum* Reinke and Berthe. (*V. dahliae* Kleb.) in naturally infested soils. But most reports indicate that they are relatively scarce, ranging from less than 1 ms/g soil to 250 ms/g soil (1, 4, 5, 6, 8, 10, 11). The data of these reports are qualitatively similar but differ quantitatively, particularly at the threshold detection level. Ashworth et al. (1) reported a quantitatively detectable threshold of about 0.15 ms/g air dry soil; other workers reported minimal inoculum densities of 2 to 25 ms/g soil (4, 5, 8, 10, 11). Differences in soil sample size may account for these discrepancies. Sample size in most instances has ranged from about 10 mg (3, 8, 10, 12) to 50 mg (5) or 100 mg (6) and the fungus was not always detected in situations where disease symptoms were obvious (3) (M. D. Harrison, *personal communication*). This indicates that chance influences detection of the fungus in small samples of soils having low inoculum densities. It follows that mean values influenced by chance may become greater than actual when they are multiplied by dilution factors of 10 to 100 in order to convert data to numbers of ms/g soil. On the other hand, our sample size (15 g) ranged from 150 to 1,500 times larger than those of other workers and the fungus was quantitatively detected in all soils where diseased plants were observed (1, 2), indicating that chance was not an important factor in detection of the fungus. In addition, a log-log relationship between numbers of ms and infected plants was observed (1).

Two recent reports (3, 12) are at odds with earlier work. These workers reported up to 3,000 multicellular propagules (atypical of microsclerotia)/g naturally infested soil. It seems unlikely that sample size accounts for this difference since other workers used 10-mg soil samples (8, 10) like De Vay and Forrester (3) and Schnathorst and Fogle (12). We thus investigated and report here the roles of soil conditioning and of milling procedures on apparent inoculum densities.

MATERIALS AND METHODS.—Soils stored for 4.5 mo at 22-27 C and 40% RH were used as controls in all experiments. These conditions approximate the storage at room temp employed by other workers (J. E. De Vay, G. Evans, M. D. Harrison, and W. C. Schnathorst, *personal communications*). We compared this soil storage practice with those used in our laboratory. We routinely air-dry soil in 48 h at 22-25 C and 40% RH following collection. Soils then are assayed for viable ms immediately or, on occasion, they are stored at 4 C and 80% RH for up to 1 wk before being assayed. Soil stored under room temp conditions, as described above, was first passed through a 6.4-mm (0.25-in) screen. Then for comparative purposes, a batch of this soil was wetted to field capacity using a twin-shell blender to obtain uniform distribution of moisture. The soil was then air-dried 48 h at 22-25 C and 40% RH. Portions of the soil were assayed immediately and other portions were stored at 4 C and 80% RH for 1 wk before being assayed.

The influence of three milling procedures upon apparent inoculum densities of soils stored as described above was determined. The mills were a low-speed (1,725 rpm) flail-type mill that reduces soil aggregates to particles 2 mm or less in size; (this mill was custom-built for us by Huron Welding and Machine Works, Huron, California 93234), a high-speed (20,000 rpm) micromill as used by Harrison and Livingston (8): and a mortar and pestle as used by Schnathorst and Fogel (12). Soil sieved through a 6.4-mm screen was used as controls for all tests.

Approximate propagule sizes were determined in one experiment. Soil stored 1 wk at 4 C and 80% RH and passed through a 6.4-mm screen, was milled with the flail-type mill, or by mortar and pestle. Propagules were separated from sieved soil residues by density flotation, using 65% sucrose solution (9), then segregated into groups of various sizes as follows: those $< 125\ \mu > 37\ \mu$, the size range we routinely culture (1, 2); those $< 37\ \mu > 18\ \mu$; and those $< 18\ \mu > 14\ \mu$. Sieves were used to separate segregates 18 μ or more in size, and a 14-μ Millipore filter was used to separate the smaller segregates.

RESULTS AND DISCUSSION.—In an initial experiment, all milling procedures resulted in decreases in the apparent inoculum densities of soils equilibrated with 40% RH for 4.5 mo. But only extended milling periods

TABLE 1. Influence of soil drying, sieving, and milling procedures on apparent *Verticillium albo-atrum* microsclerotium content of naturally infested clay loam soil

Soil milling procedures	Apparent numbers of microsclerotia (No./g soil)[a]		
		Soil moistened, stored 48 h, 4 C and dried 48 h, 22-25 C[b], 40% RH[c]	
	Assayed immediately	Assayed immediately	Assayed after storage 7 days, 4 C, 80% RH
6.4 mm sieve	23.3	19.3	23.1
Sieved, <2 mm, >1 mm	7.1	19.0	27.2
U.C. flail-type mill	11.5	24.6	23.4
Micromill, 2 s	5.2	21.9	21.2
Micromill, 5 s	3.8	15.0	17.3
Micromill, 15 s	4.0	9.1	5.9
Mortar and pestle	4.8	20.2	16.1

[a] Bulk soil equilibrated at 40% relative humidity, 22-27 C for 4.5 mo.
[b] 6-7% moisture, wet weight basis.
[c] Relative humidity.

TABLE 2. Influence of milling procedures on apparent *Verticillium albo-atrum* microsclerotium content of soils

Soil milling procedures[a]	Microsclerotia in air dry soil (No./g)	
	Clay loam soil	Sandy loam soil
6.4-mm sieve	10.0	4.8
U.C. flail-type mill	4.1	2.1
Micromill, 5 s	2.4	--
Micromill, 15 s	0.4	--
Micromill, 30 s	0.5	--
Mortar and pestle	0.5	1.1

[a]Soils equilibrated at 30-40% relative humidity, 22-27 C.

with the micromill caused apparent decreases of inoculum densities in soil stored under conditions we employ, Table 1. Microsclerotia of soil stored under humid conditions appeared to have an elasticity which permitted milling without fragmentation. The influence of milling procedures upon apparent inoculum densities of a second clay loam soil, and a sandy loam soil equilibrated at 40% RH was determined in another experiment. While all milling procedures resulted in decreases in apparent inoculum densities of the soils, the effects of the micromill at 15 s and 30 s and the mortar and pestle were more severe than the other treatments, Table 2. Damage to ms from milling was greater with the clay loam soil than with the sandy loam soil of this experiment, and with another clay loam soil (see Table 1). These observations indicate that damage to ms cannot be expected to be constant between soil types.

Approximate propagule sizes and their relative contribution to inoculum density were determined in an experiment in which soils were milled with the flail-type mill and by mortar and pestle. The data show that the mortar and pestle, when all segregates are cultured, resulted in an increase in apparent inoculum density, in this case from 2.2 to 12.3 propagules/g soil. The apparent increase appears to result from breakage of ms into smaller but still viable units, because about 40% of the viable propagules of the soil milled by mortar and pestle were smaller than 18 μ in size, Fig. 1. Results of this test show that breakage of microsclerotia by milling procedures is more pronounced than indicated by tests in which only propagules larger than 37 μ in size were cultured, Tables 1, 2. In this and 10 other tests (L. J. Ashworth, et al; *unpublished*), however, no propagules smaller than 18 μ were detected in soils milled with the flail-type mill.

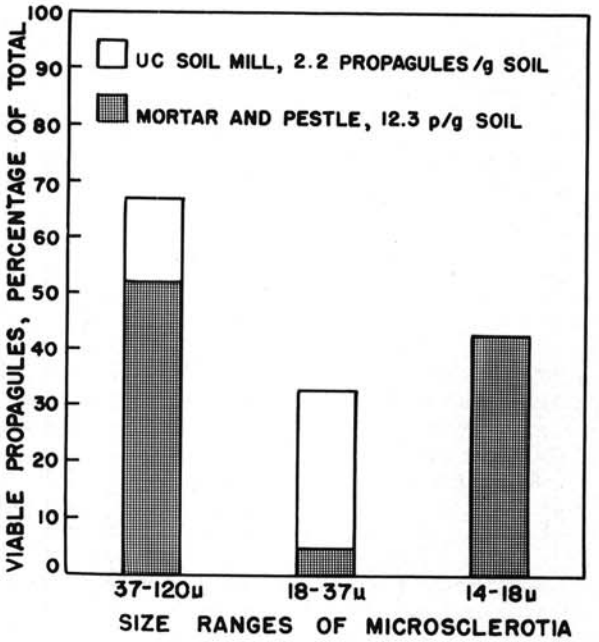

Fig. 1. The influence of soil milling procedures upon size of viable propagules of *Verticillium albo-atrum*.

Ultrasonic oscillation of ms is known to reduce microsclerotia to smaller viable units (7, 13). The small propagules detected in tests reported here may account for atypical propagules of the fungus described recently by De Vay and Forrester (3) and by Schnathorst and Fogel (12), and by Gordee and Porter (7) who reported similar structures derived from ultrasonically oscillated ms suspensions. More importantly, however, the data help us to understand the divergent opinions about numbers of propagules in soil. Harsh milling procedures of air dry soils stored under room conditions enhance the odds of detecting the fungus in small (10-100 mg) soil samples. Both factors can result in distorted views of actual numbers of ms in soil. This is very important, as illustrated by analysis of data of Evans et al., who used 50 mg soil samples (5). Their data are highly significant, but the LSD ($P = 0.05$) = 70 ms/g soil (our calculation from their data), indicating presence of a large experimental error. On the other hand, the quantitative threshold detection level with 15-g soil samples was about 0.15 ms/g soil with a coefficient of variation of about 20% (2).

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