Reassessment of Soil Assays for Verticillium dahliae

E. J. Butterfield and J. E. DeVay

Department of Plant Pathology, University of California, Davis, CA 95616.
Present address of senior author: Boyce Thompson Institute, 1086 North Broadway, Yonkers, NY 10701.
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ABSTRACT

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Modifications in the Anderson Sampler technique of soil plating increased the accuracy and sensitivity of soil assays for microsclerotia of *Verticillium dahliae*. This modified technique was compared with a wet-sieving technique for eight different soils and it yielded approximately 2.8 times more propagules per gram of soil. Possible reasons for the difference in efficiency between the two techniques are discussed.

An accurate quantitative assay for natural soilborne populations is necessary in studies on the epidemiology of wilt diseases caused by *Verticillium dahliae* Kleb. The slow growth characteristics and relatively small number of *V. dahliae* propagules in soil limits the usefulness of traditional soil-dilution techniques in the absence of selective isolation media. The ethanol-streptomycin medium of Nadakavukaren (13) was useful for dilution-counting of propagules in artificially infested soils but it has been of limited use for counts in naturally-infested soils. Green (9) and later Jordan (12) reported the successful use of soil-dilution techniques with naturally-infested, fresh soils but these techniques have proven to be inadequate for our work which was focused on the persistent propagules remaining in air-dry soil.

Two techniques have been described which are useful for analyzing the number of propagules of *V. dahliae* that persist in air-dry soil. Ashworth et al. (4) and Huisman and Ashworth (11) described wet-sieving techniques and a sodium pectate substrate which enabled them to make quantitative assessments of *Verticillium albo-atrum*, MS form, (= *V. dahliae*) in field soils. Harrison and Livingston (10) and DeVay et al. (8) have described the use of an Anderson air sampler for distributing soil on suitable agar substrates for assays of *Verticillium*. The wet-sieving techniques appear to offer sensitivity and low variation in estimates of propagule numbers due to the large sample size that can be used; however, there is difficulty in assaying soils with high propagule counts. The Anderson Sampler technique was more effective than other techniques for assaying soils with high numbers of propagules, although the small sample size (10 mg) limited the sensitivity when the populations of *V. dahliae* were low (< 30 propagules/g).

The purpose of this study was to reassess the differences between the wet-sieving and Anderson Sampler techniques and to analyze the effect of procedures used in these assays on the number of propagules of *V. dahliae* found. A preliminary report of some of this work has been made (6).

MATERIALS AND METHODS

Soil collection and preparation.—Soil samples used in this study were taken in the Sacramento and San Joaquin valleys of California from cotton fields with a history of Verticillium wilt. The soils were Panoche clay loam from the Westside Field Station at Five Points; Hanford sandy loam from Lemoore; and Yolo loam from Davis. When making a collection, the surface 5-8 cm was removed and a 2.5-cm-diameter soil tube was used to remove a 25-cm soil core. Cores from each sampling area were bulked and hand-mixed. Unless indicated otherwise, the samples were air-dried in paper bags for 4-6 wk at 30-50% relative humidity and 20-24 C to eliminate propagules sensitive to desiccation. In most experiments, the dried soil was homogenized in a revolving jar mill for 20 min with the largest available stones (3.0 cm × 3.0 cm). Samples not analyzed immediately were stored at 4 C until analyzed.

Soil analyses for Verticillium dahliae.—We used the wet-sieving technique of Huisman and Ashworth (11). The soil residue remaining on the 37-μm (400-mesh) sieve (37-μm pore size) was suspended twice for 5 sec each in 0.5% NaOCl, rinsed with tap water, transferred to large test tubes (2.5 × 20 cm), and allowed to settle for 20 min. The excess water was removed by aspiration and the wet residue was dispersed on the surface of sodium pectate agar. In some tests the treated residue was washed into sterile glass petri plates and allowed to settle 1 hr. The water was removed by aspiration and the residue was air-dried overnight. The dry residue then was dispersed onto the sodium polypectate substrate.

Our Anderson Sampler technique was that of Butterfield and DeVay (6), except that a sampling tower was added and a circular deflector (1 cm in diameter) was placed above the first sieve plate (Fig. 1). The cellophane film-soil extract agar used by DeVay et al. (8) was replaced by a modification of the sodium pectate substrate of Huisman and Ashworth (11). It was prepared

as follows: 10 ml of 1.0 M guanidine·HCl, 3.5 ml of 2.0 M KCl, and 7.0 ml of 1.0 M KH_2PO_4 were added to 500 ml of distilled water in a blender jar. While the solution was blending slowly, 0.5 ml of Tergitol-NPX, 5 g of sodium polygalacturonate, and 50 mg each of streptomycin sulphate, chloramphenicol, and chlorotetracycline·HCl

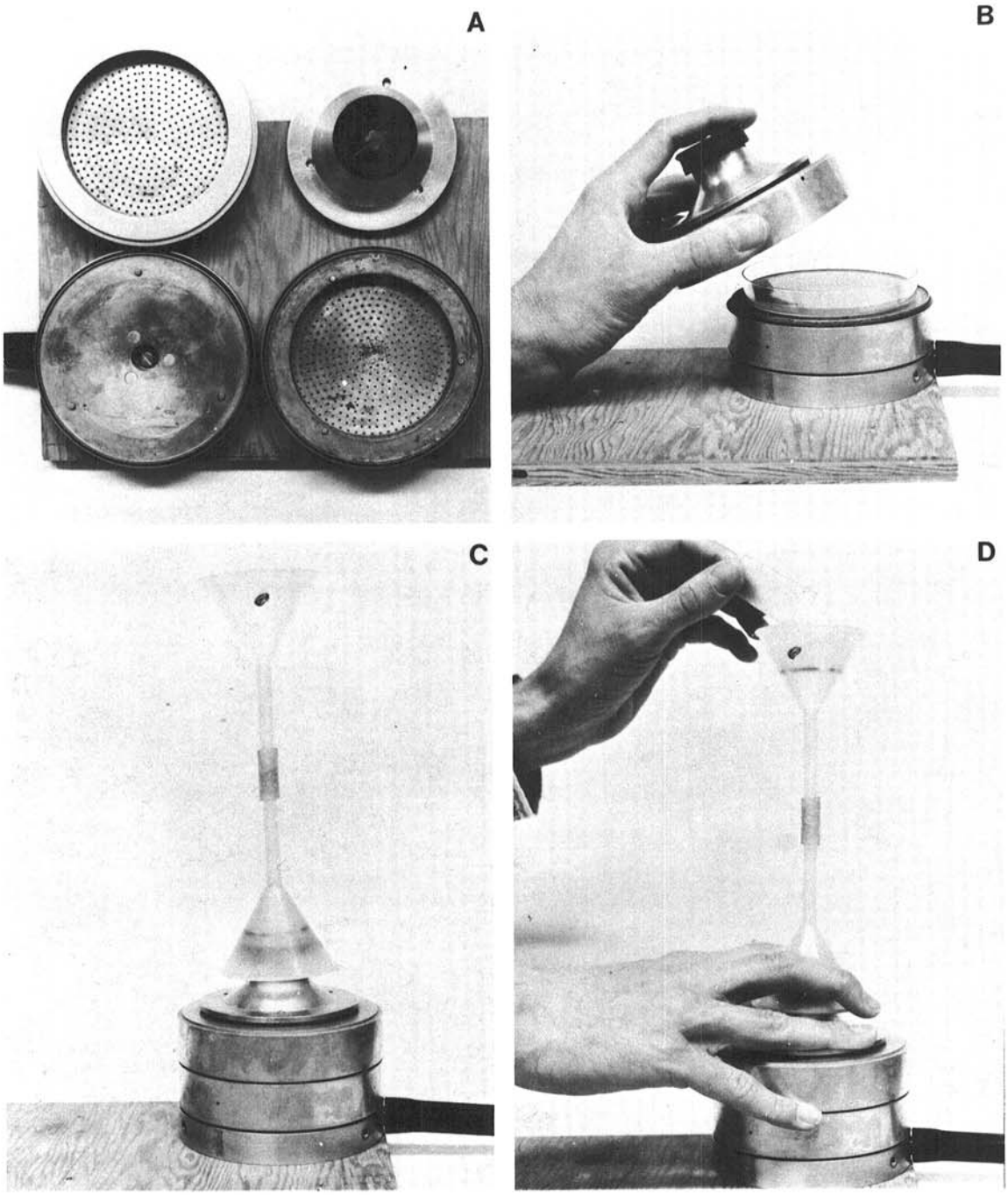

Fig. 1-(A to D). The modified Anderson Sampler used in analyses of soils for propagules of *Verticillium dahliae*. **A)** The components of the sampler; the sieve plates have pore sizes of 1.18 and 0.81 mm. **B)** The partially assembled sampler; petri plates are placed beneath each sieve plate. **C)** The assembled sampler; air is drawn through the tower, over the petri plates, and out through the suction tube at the lower right at 28 liters/min. **D)** Soil samples are dumped from glassine weighing paper into sampling tower while air is being drawn through the sampler.

were added. Fifteen g of agar (Difco Agar Flake) were added to 500 ml of distilled water in a separate flask and the two suspensions were heated for 3 min at 121 C. The resulting solutions were then mixed and dispensed, 20 ml per disposable petri plate (100 × 15 mm).

The modified Anderson Sampler (Fig. 1) was used to distribute the soil on the surface of the agar substrate. In a typical analysis, five 100-mg subsamples were distributed onto five plates in the top position. A sixth plate usually was placed in the second position in the sampler. The top plate was changed for every 100-mg subsample and the plate in the second position was removed after the five subsamples had been distributed. More than 95% of the propagules were retained on the upper plate and the remaining propagules trapped on the second plate. With both techniques, microsclerotial colonies of *V. dahliae* were counted with the aid of a stereo-dissecting microscope after 14 days of incubation at 24 C. The soil on the agar substrate was removed by washing immediately prior to counting.

RESULTS

The influence of substrate and sample size.—Water agar plus streptomycin sulphate, chloramphenicol, and chlorotetracycline·HCl (14); soil extract agar overlain with a cellophane film (8); and sodium pectate agar (11) were all tested with the Anderson Sampler for effectiveness in enumerating propagules of *V. dahliae* from soil. As previously described (8), the efficiency of recovery decreased rapidly with increasing sample size when the cellophane-soil extract substrate was used (Fig. 2). The same effect also was observed when the water-agar antibiotic substrate was used. In contrast, the sodium-pectate-agar allowed efficient recovery of propagules with sample sizes up to 100 mg for the Yolo loam soil. However, for other soils, the upper limit of the sample size per plate that permitted maximal recovery of propagules varied from 50 to 250 mg depending on the soil type and the time of collection.

The influence of milling.—Ashworth et al. (1), demonstrated that milling air-dry soil significantly altered the apparent number of propagules of *V. dahliae*; a high-speed micromill and a mortar and pestle, as used in previous studies (10, 14), both caused fracturing of microsclerotia. However, they did not examine the effect of a revolving jar mill used by DeVay et al. (8). The jar mill combines the processes of reducing soil particle size and thorough mixing which makes it a valuable tool in preparing soil for analysis with the Anderson Sampler.

The effect of the jar mill on number of propagules detected in soil samples was examined in the present study. Batches of soil were air-dried 24 hr, hand-mixed, passed dry through a 0.71-mm (25-mesh) sieve, and then further air-dried for 6 wk. These soils were then mixed without stones in the jar mill for 20 min. A zero-time sample was withdrawn, the stones added and the samples drawn after 1, 5, 10, 20, 40, and 60 min of milling. The samples were plated with the Anderson Sampler which captures all propagules independent of size; thus any fracturing of microsclerotia should be evident by increases in numbers of propagules. There was a small but significant increase in the number of propagules in the 5- and 10-min milling period, but not for longer milling periods (Fig. 3).

The influence of drying time.—Our soils were routinely dried to eliminate short-lived propagules (free conidia and hyphal cells), but at least one report (2) indicated that microsclerotia also may be susceptible to desiccation. To determine the effect of length of drying, three different soils were assayed for numbers of propagules with the Anderson Sampler technique and water contents were determined during a 7-wk drying period. The propagule counts initially were low then fluctuated until they stabilized after 5 wk of drying (Fig. 4). This fluctuation in propagule numbers was not observed in samples assayed with the wet-sieving technique.

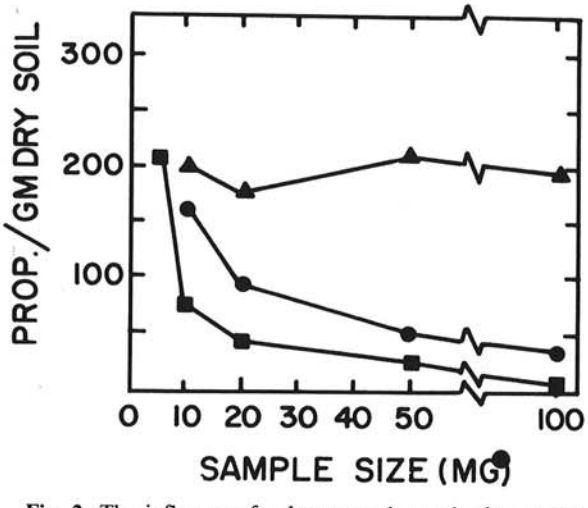

Fig. 2. The influence of substrate and sample size on the recovery of *Verticillium dahliae* propagules from Yolo loam soils. The substrates were soil extract agar plus cellophane (■ - ■), water agar plus three antibiotics (● - ●), and pectate agar (▲ - ▲).

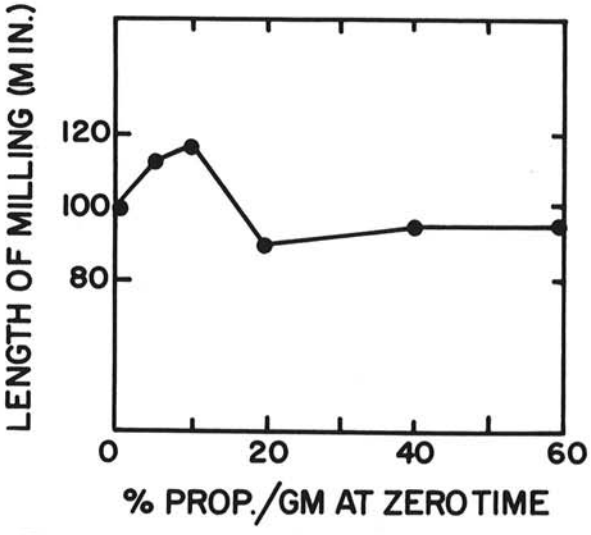

Fig. 3. The influence of milling time on recovery of propagules of *Verticillium dahliae* from field soils (average of three tests).

The influence of the Anderson Sampler.—Earlier reports indicated that a substantial portion of the soil introduced into the sampler was not impacted on the agar substrate. With 100-mg samples only 63.6% of the soil was trapped on the petri plate; the rest of the soil remained on the walls of the sampler and the filter at the vacuum source (8). This latter soil was extremely fine and only occasional propagules could be recovered from it. This indicated that the sampler, as modified for soil analysis, efficiently impacts propagules of *V. dahliae* onto the agar substrate.

It is also possible that the microsclerotia could be fractured during passage through the Anderson Sampler. However, reanalysis with the Anderson Sampler of soil previously passed through the Sampler indicated no increase in the number of propagules.

Comparison of wet-sieving with the Anderson Sampler technique.—The efficiency of the wet-sieving and the Anderson Sampler techniques in recovering propagules of *V. dahliae*, was compared directly by analyzing comparable samples from eight soils of different numbers of propagules (Table 1). In every case, the Anderson Sampler technique gave higher estimates of propagule numbers which averaged 2.8 times the number of propagules found with the wet-sieving technique. The consistency of the higher numbers detected with the Anderson Sampler technique indicated that it is more efficient and that the higher numbers were not the result of variation due to the small sample as previously suggested (1). Since examination of the procedures used in the Anderson Sampler technique did not reveal any significant increase in propagule numbers, an examination of the procedures used in wet-sieving could reveal a loss of propagules.

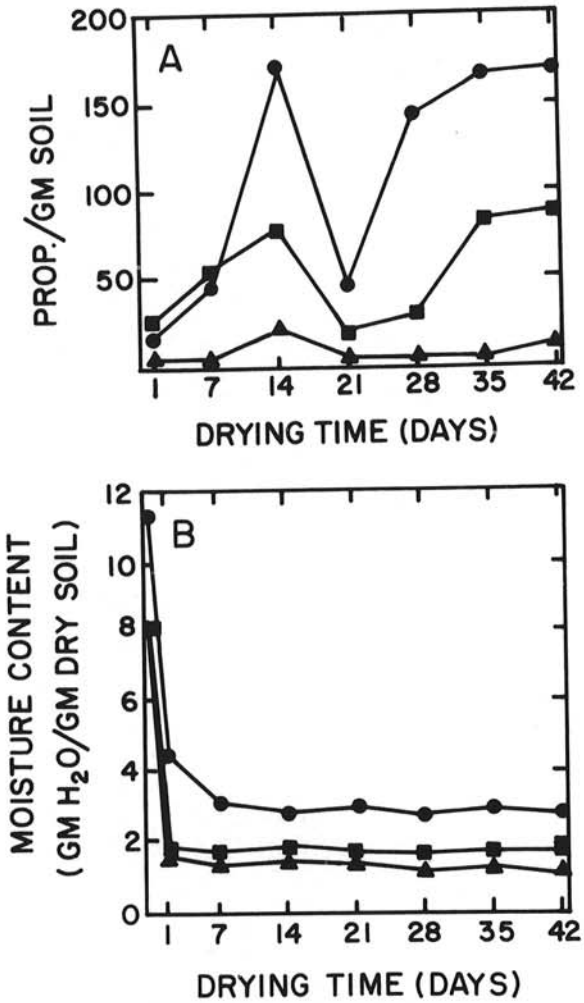

Fig. 4-(A, B). The influence of drying time on recovery of propagules of *Verticillium dahliae* with the Anderson Sampler technique. **A)** The influence of drying on the number of propagules recovered from Yolo loam soil (● - ●), Panoche clay loam (■ - ■), and Hanford sandy loam (▲ - ▲). **B)** The moisture content of the same three soils during air drying.

Influence of sieving on numbers of propagules detected.—Ashworth et al. (4) indicated for the wet-sieving technique approximately 10% of the propagules passed the 37-μm sieve. We assayed the fraction that passed the 37-μm sieve by collecting the first 2 liters of water washed through the sieve and concentrating the residue by gravity sedimentation. When this residue was plated wet, approximately 10% of the propagules had passed the sieve. If the residue was dried overnight, then plated, the apparent number of propagules was increased. The increase indicated that 30-50%, not 10%, of the total number of propagules in soil may pass the 37-μm sieve. This percentage of propagules accounted for a significant protion of the difference in propagule counts between the wet-sieving and Anderson Sampler techniques.

Nature of the propagules.—Direct microscopic examination of assay plates immediately after plating with the Anderson Sampler, and after 10 days of incubation, revealed that most colonies of *V. dahliae* arose from identifiable microsclerotia. The microscelerotia resembled, in size and appearance, those described by other workers (4, 8, 9, 10, 11, 12, 14). The microsclerotia from soil, as observed on the agar medium, were dark in color. The pigmentation of the individual cells was variable, even within a single microsclerotium. Due to the presence of soil particles and the pigmentation

TABLE 1. Comparison of the Anderson Sampler and wet-sieving techniques for estimation of propagules of *Verticillium dahliae* in field soils

Sample no.	Anderson Sampler no.	Wet-sieving no.	Ratio
1	40[a]	21.4[a]	1.9[b]
2	68	29.3	2.3
3	122	47.2	2.6
4	144	53.0	2.7
5	116	30.6	3.8
6	160	48.4	3.3
7	178	53.5	3.3
8	224	83.4	2.7
	...	...	...
Avg.	131.5	45.8	2.8

[a]Propagules/g air-dry soil.

[b]Number of propagules estimated by the Anderson Sampler technique divided by number of propagules estimated by the wet-sieving technique.

of the massed cells, it was difficult to observe which cells within the microsclerotium gave rise to the multiple germ hyphae. A variable but large number of propagules were either free or were contained in small pieces of decomposed plant debris. The remaining portion, varying from 0 to 10%, were in large (> 500 μm) pieces of plant debris.

DISCUSSION

The number of articles concerned with assaying populations of *V. dahliae* in soil emphasizes both the importance of this widespread plant pathogen and the necessity for quantitative techniques for estimating the abundance of its propagules. Both techniques studied here can satisfy this need, but the effects of the procedures involved should be understood when interpreting data.

Milling of air-dried soil may increase the apparent number of propagules (1) but the use of the revolving jar mill minimized this problem. We did observe a significant increase in propagule numbers during the first 5-10 min of jar milling. It is not known whether this increase was due to release of microsclerotia from small pieces of plant debris or from fracturing of individual microsclerotia. However, when longer jar milling times were used (20-60 min), the initial increase in numbers of propagules decreased to counts near the original concentration of propagules.

The Anderson Sampler uniformly distributes soil particles over the agar plates; it gives maximum separation of the propagules of *V. dahliae* from each other and from propagules of other microorganisms. It is essentially similar to Warcup's soil-plating technique (15) and increases the probability of recovering the propagules of slow-growing organisms present in low numbers. Buxton and Kendrick (7) also observed significant increases in propagule numbers of *Pythium* spp. and *Fusarium oxysporum* when they compared the Anderson Sampler to a soil dilution method to assay soil samples. Since the propagules of *Pythium* and *F. oxysporum* are assumed to be single-cell units, an increase in their recovery could not be attributed to fractured propagules. Buxton and Kendrick (7) indicated that the apparent increase in propagule counts resulted from separation of the propagules from antagonists.

The experiments in this study indicated the main sources of variation in propagule counts observed between the two techniques and the reasons why the Anderson Sampler technique gave consistently higher (2.8-fold) propagule counts; it appears that both techniques count essentially the same type propagules, but in different proportions.

The wet-sieving technique is especially useful for assaying soils with very low inoculum density because it gains sensitivity from the large sample size used. It requires a large amount of agar substrate and 45 petri plates per sample. The Anderson Sampler technique is more useful for soils with high inoculum density where overlapping of colonies might be a problem with the wet-sieving technique. At low propagule densities (0-10 propagules/g) the variability of counts with the Anderson Sampler is high. At higher propagule densities the variability decreases rapidly so that above 20 propagules/g the standard deviation is usually less than 10% of the mean propagule number. The Anderson Sampler technique requires less substrate and only six petri plates per sample.

Ashworth et al. (3) have reported an apparent copper-induced fungistasis that affects microsclerotia of *V. albo-atrum*. They indicated that this fungistasis appeared suddenly in November 1972. We also sampled a number of fields in the San Joaquin Valley during this period and assayed the soils by the Anderson Sampler technique, but did not observe this phenomenon. Propagule counts in soil samples taken in October 1972 and April 1973 from fallowed soils or soils which had been cropped to non-host plants were virtually identical. Propagule counts for soils which had been cropped to cotton increased substantially between the two sampling dates.

We have observed apparent fungistatic effects under different circumstances than those reported by Ashworth et al. (3). As demonstrated in Fig. 4, many propagules failed to initiate colony formation until the soil had been air-dried for 5 wk. This is considerably longer than the drying period used by Ashworth et al. (4). We have further observed that air-drying of soil partially removes the requirements for NaOCl treatment when soils are analyzed by wet-sieving. Air-drying of soil causes significant microfloral changes which are readily apparent by examining assay plates inoculated with soil dried for various periods of time.

We also observed the apparent reduction in numbers of propagules when larger amounts of soil are applied to assay plates. This reduction is almost certainly due to microbiological effects since it is reversible by the use of a more selective substrate (Fig. 2). The presence of antibiotics and select nutrients in the substrate must restrict the growth of many potential competitors. We have made a preliminary report of the apparent involvement of a bacterium in the inhibition of germination of *V. dahliae* microsclerotia (5). This bacterium can reproduce at least some of the properties of fungistasis and is sensitive to streptomycin sulphate and NaOCl.

Finally, we have observed reductions of more than 75% in propagule counts when sodium polygalacturonate from a different source was used. The inhibitory effect could not be removed by washing the polypectate with 95% ethanol.

The usual source of sodium polygalacturonate was Sigma Chemical (Grade II) but material supplied by Pfaltz & Bauer, Inc. 375 Fairfield Ave., Stamford, CT 06902 was equally effective.

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