Production and Manipulation of Individual Microsclerotia of *Verticillium dahliae* for Use in Studies of Survival

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ABSTRACT

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A method for the laboratory culture of *Verticillium dahliae* and the harvest, isolation, and manipulation of single microsclerotia (MS) is outlined. MS produced in large numbers and stored at several temperatures maintained high, uniform viability for 12 mo or more. Thus, a single lot of MS could be used in long-term experiments on the effect of microbial interaction on MS survival. The MS were harvested from semisolid media by wet sieving, mixed with sand to dry without clumping, and sorted into size classes by dry sieving. After experimentation, single MS were transferred on the tip of a sterile needle into one of 25 squares marked on an agar plate, and germination and colony growth were monitored. The lethal effect of the treatment was reflected by the number of dead MS. Sublethal effects could also be measured as a reduction in colony growth because larger MS (>75 μm) exhibited faster, more synchronous germination and produced larger colonies than smaller MS.

Additional keywords: melanin, ultraviolet irradiation.

During the past few decades, concerns about environmental effects of pesticides have stimulated a greater effort toward the use of biological methods for control of soilborne fungal plant pathogens. Successful implementation of biological control, however, requires a greater understanding of the basic biology of the pathogens, particularly the factors that modulate their survival under field conditions. The survival of sclerotia is of particular interest because for many soilborne plant-pathogenic fungi, such as *Verticillium dahliae* Kleb., these are the structures most responsible for persistence of inoculum in the field (14,19). Reduction of sclerotia populations, therefore, is the primary goal of many diverse disease control strategies.

Although *V. dahliae* is an economically important pathogen worldwide (28), few studies have examined the survival characteristics of *V. dahliae* microsclerotia (MS), most likely because the MS are very small (38–150 μm in diameter) and therefore difficult to manipulate. Hence, there is little published work on the survival of individual MS of *V. dahliae* (4,5,9,19) compared to the work done on larger sclerotia of fungi such as *Sclerotium cepivorum* (8,10,24), *S. rolfsii* (6,17), or *Sclerotinia sclerotiorum* (18,26,27). In previous studies in which survival of *V. dahliae* was investigated, viability tests usually involved spreading a known quantity of inoculum (often soil or plant tissue) on a semiselective medium and counting the number of colonies formed (3,11,15,16,25, 29–31). Since such inoculum could contain an undefined mixture of hyphae, conidia, and MS, it was impossible to know whether the colonies produced originated from single MS or aggregates of all these structures. Even when attempts were made to isolate MS specifically, they frequently clumped together (19,20) or became damaged during processing (1). Use of such preparations generally resulted in unacceptably high within-treatment variability (2), which could potentially mask important differences between treatments. In our experience, variability between experiments was even more problematic because MS produced in the laboratory were often short-lived and their survival differed from preparation to preparation. Since many phenomena of interest in biological control experiments take weeks or months to manifest themselves, this rapid loss of viability made it impossible to monitor long-term survival of MS. Furthermore, the inability of previous methods to detect sublethal effects was a major shortcoming, since these effects are a common and critical feature of biological interactions.

In this study we describe procedures that allow for the production in the laboratory of stable MS populations that can be kept for extended periods without loss of viability. The assay procedure described here can not only quantitatively differentiate the survival of individual MS but can also detect the impact of sublethal effects on MS.

MATERIALS AND METHODS

Strains of *V. dahliae*. The cultures of *V. dahliae* used were isolated from eggplants (*Solanum melongena* L. 'Imperial Black Beauty') grown in field soil collected from the Bean family farm located in Alliston, Ontario. The culture was thus designated V.d.Bean. Cultures were maintained on Czapek-Dox agar (CDX) at 24 C in continuous darkness. The nonmelanized strain was obtained from an albino mutant of *V. dahliae* (Alm-1) provided by M. Wheeler (USDA, College Station, TX).

Media. Initially, CDX agar was used for maintenance of the cultures and for production of MS for experimentation. CDX was prepared by adding 35 g of Difco Bacto CDX broth, 15 g of agar, 10 ml of vitamin stock solution, and 10 ml of mineral stock solution to 1 L of distilled water and then autoclaving the mixture. The vitamin stock solution contained, per liter, 0.02 g each of yeast extract, thiamine HCl, adenosine, and *p*-aminobenzoic acid; 0.01 g of biotin; 0.001 g of folic acid; and 0.005 g each of pyridoxal HCl, nicotinic acid, and riboflavin. The solution was brought to pH 8.5 with NaOH. The mineral stock solution contained 0.308 g of $MnSO_4 \cdot H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.055 g of $CaCl_2$, 0.088 g of $ZnSO_4 \cdot 7H_2O$, 0.004 g of $CuSO_4 \cdot 5H_2O$, 0.0047 g of $CoSO_4 \cdot 7H_2O$, 0.018 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.3 ml of Versenol, and 1 ml of 1 N HCl in 1 L of distilled water. When 500 ml of the vitamin or mineral stock solutions was prepared

and filter-sterilized, the solutions could be stored for several months at 4 C with no contamination. In all the experiments presented below, the protocol for CDX agar was changed to improve MS viability after harvesting. The semisolid modified CDX (MCDX) medium consisted of 35 g of Difco Bacto CDX broth, 0.5 g of dextrose, 2.5 g of pectin, 2.5 g of agar, 10 ml of vitamin stock solution, and 10 ml of mineral stock solution in 1 L of distilled water. Potato-dextrose agar (PDA) was prepared by adding 39 g of Difco Bacto PDA to 1 L of distilled water.

CDX agar plates were cooled and inoculated with a single 0.5-cm plug from an actively growing culture of *V. dahliae* and incubated in the dark at 24 C. MCDX medium was inoculated with several plugs of agar cut from actively growing colonies of *V. dahliae* and shaken well, and 30-ml aliquots were immediately dispensed into petri plates. The plates were stacked inside clear, rigid plastic utility boxes and incubated in the dark at 24 C for 3–6 wk.

The medium used to assay the survival of MS after experimental manipulation was soil-pectate-Tergitol (SPT) agar and was modified from that described by Kantzes (21). It consisted of 1.5 g of KH_2PO_4, 4 g of K_2HPO_4, 2 g of polygalacturonic acid, 15 g of agar, and 25 ml of soil extract. KOH was used to adjust the pH to 7.0, and 1 ml/L of antibiotic solution (50 mg each of chloramphenicol, chlortetracycline, and streptomycin sulfate in 0.5 ml of Tergitol NPX and 1 ml of 95% ethanol) was added after autoclaving. The soil extract was prepared by steaming 1 kg of garden soil in 1 L of tap water for 30 min, then decanting and filtering the suspension through several layers of cheesecloth. Small plastic bottles each containing 25 ml of soil extract were stored at −25 C. To ensure the transparency of the medium and thus optimize the measurement of germination or radial growth of the colonies under the microscope, only 16 ml of SPT agar should be dispensed into each 9-cm-diameter petri plate. In addition, poor quality polygalacturonic acid flocculates and hampers viewing of the colonies.

Production and manipulation of MS. The harvesting procedure for obtaining individual MS was adapted from the homogenizer-screen method of Gordee (13). After growing for several weeks in MCDX medium, the contents of up to five petri dishes were comminuted in a Waring blender with 200 ml of tap water for three or four 10-s pulses, then poured through a nested set of Tyler standard sieves of mesh sizes 150, 200, 270, and 400 (corresponding to openings of 106, 75, 53, and 38 μm, respectively). After the contents of the sieves were rinsed under tap water, the MS were washed back into the blender and comminuted again for three or four 10-s pulses. A sample of MS was viewed on a microscope slide under a stereo dissecting microscope between each rinse to determine if the MS needed more blending. This procedure was repeated until the MS on each sieve were confirmed to be singular and free from mats of hyphal fragments. The final MS preparations were washed off the sieves in a minimal volume of water (2–10 ml) and mixed with approximately 50 g of dry, acid-washed quartz sand to prevent the MS from drying into clumps. The sand was prepared by sieving into particles of 300 μm or larger, soaking overnight in concentrated HCl, and rinsing in tap water until the pH was no longer acidic. The sand/MS mixtures were distributed into 15-cm-diameter watch glasses and air-dried overnight in a laminar flow hood. Difficulties with the MS adhering to the watch glass during drying were alleviated by pretreating the glass with silicon (Sigmacote, Sigma Chemical Co., St. Louis, MO.). When required, the MS could be separated from the sand after drying by placing the sand/MS mixture on a 100-mesh sieve (150-μm openings) and gently rubbing the mixture with a rubber spatula. The sand remained in the sieve, and the MS that fell through the sieve were collected on paper. Once collected, the MS could be further sorted into size fractions by returning the dry MS to the appropriate sieve and gently tapping the sieve or rubbing the MS with an artist's brush to aid the sieving. MS were stored dry, in small plastic tubes with lids (such as Eppendorf microcentrifuge tubes).

To determine how many MS were produced per plate, 12 dishes of MCDX medium were inoculated with *V. dahliae* and incubated at 24 C as previously described. Two dishes were harvested each week for 6 wk after inoculation. The total mass of MS harvested each week was recorded. The MS were then dry-sieved into three size classes—>106, 75–106, and <75 μm—and the mass of MS in each size class was recorded. To determine the approximate number of MS >75 μm per milligram, 1 mg of dry MS was added to 5 ml of sterile distilled water and two drops of Tween 20 and stirred for 15 min on a magnetic stirrer. A 100-μl drop was pipetted onto a microscope slide, and the number of MS was counted under a binocular microscope. The mean of 10 separate counts was calculated and multiplied by 50 to determine the number of MS per milligram.

When dry, MS could be easily manipulated by sprinkling them onto small pieces of paper and moving them around with an artist's brush. The MS were picked up individually on the tip of a sterile hypodermic needle (25G 7/8) under a stereo dissecting microscope according to the method of Ben-Yephet and Pinkas (4). Petri dishes containing SPT agar were placed on a grid of 25 squares, and one MS was placed on the surface of the agar within each square. One or two plates (25 or 50 MS) were used for each treatment, depending upon the experiment. MS could be transferred in the open laboratory because the antibiotics in the SPT agar prevented any contamination by airborne microorganisms. The needle was flamed between treatments to reduce the risk of transferring potential contaminants between treatments. The dishes were incubated at 24 C in the dark. After a minimum of 2 wk of incubation, the number of MS that germinated and formed colonies was counted. Indirect or sublethal treatment effects were assessed by measuring the rate of germination or radial growth of colonies during the first 5 days of incubation.

Rate of germination and colony growth. One hundred MS of each of the three size classes were transferred onto four plates of SPT agar, and 50 MS of each size were plated onto two plates of either CDX agar or PDA. Both CDX agar and PDA received 1 ml/L of the same antibiotic mixture added to SPT agar. The plates were examined daily for a week under a dissecting microscope, and the number of MS that had germinated was recorded. Beginning the second or third day after plating, the diameter of each colony on the plate was measured daily with a micrometer to the nearest 0.1 mm and recorded. The experiment was performed twice.

Storage temperature. In the first of two experiments, MS of the three size classes were put into 1.5-ml capped plastic tubes and placed into sealed coffee cans containing desiccant to control humidity. The containers were stored at 24, 4, −25, and −70 C; a refrigerator freezer was used to provide −25 C. One sample of 25 MS was removed from each tube weekly for 10 wk and transferred onto SPT agar. The number of MS that had germinated to form a colony was counted and recorded after a minimum of 2 wk of incubation. In the second experiment, only MS 75–106 μm in size were used and were stored in a −25 C chest freezer instead of a refrigerator freezer. To avoid repeatedly exposing the MS to rapid temperature changes during sampling, a small subsample of sand/MS mixture was placed into each of 64 plastic tubes and a set of 16 tubes was placed at each temperature in a sealed container with desiccant. At 1, 2, 3, 4, 6, 8, 10, and 13 wk, two tubes were removed from each temperature and 25 MS from each tube were transferred onto SPT agar.

Long-term storage. The survival of MS <53, 53–75, and 75–106 μm in size was monitored over storage periods ranging from 12 to 34 wk. The MS were stored in the dark at 24 C, in small plastic tubes with caps. Every few weeks, two samples of 25 MS each were removed from storage and transferred onto SPT agar. Five groups of MS were monitored, and each group was harvested from a different set of cultures made over a 2-yr period. In two experiments, MS of 75–106 and 53–75 μm were monitored for up to 14 wk. In two other experiments, MS of 75–106, 53–75, and <53 μm were monitored for up to 35 wk. In the fifth group, MS of 75-106 and 53–75 μm were stored at 24 C and their survival was monitored for up to 59 wk.

Irradiation of MS with UV light. The ability to detect the effects of damaging treatments on the survival of MS was tested by

measuring the survival of melanized and nonmelanized MS after UV irradiation. The melanized and albino strains of *V. dahliae* were grown in MCDX medium and the MS was harvested as outlined above. Approximately 25 melanized or albino MS were placed on 1.5-mm squares of plastic, arranged on a tray, and placed in a light-proof box designed for viewing thin layer chromatography plates (Camag UV cabinet 2, Terochem Scientific, Markham, ON). The MS were 15 cm from the UV lamp, which produced UV light of approximately 254 nm. Temperature was controlled by placing the tray of MS on a layer of crushed ice. A control was included by placing melanized or albino MS inside foil-wrapped plastic tubes to prohibit exposure to the UV light but permit exposure to all other experimental conditions. Two squares each of melanized and albino MS were removed after 2, 4, 6, 8, 12, 24, 36, or 48 h of exposure to the UV light. Two of each of the tubes containing control MS were recovered after 6, 12, 24, 36, or 48 h in the box. The MS on each square were transferred to SPT agar as outlined above. After 4 days of growth at 24 C, colony diameters were recorded. The experiment was performed four times.

Burial of MS in soil. The capacity of the assay procedure to measure the survival of MS following burial in soil and to detect the potential effect of microbial interactions with the MS was determined under laboratory conditions. Dry individual MS (harvested by the procedure described above) were placed into small (2 × 3 cm) nylon mesh packets along with some acid-washed quartz sand that prevented the MS from clumping together. The nylon fabric, a material used for silk-screening, had a mesh size of 75 μm, which was small enough to contain the MS after the packets were heat-sealed with a soldering iron or Quik Seal plastic bag sealer (National Instrument Co., Baltimore, MD). Once the experiments were completed, the packets were retrieved and opened and the MS were transferred to SPT agar as described above.

Survival of the MS following burial was evaluated for five different soil types selected for their differing microbial activities and arbitrarily named according to their source: 1) Alliston (sandy loam soil from a field used for potato production), 2) Lundy (sandy loam soil from a field that had been amended yearly with poultry manure), 3) Simcoe (Fox sandy loam soil), 4) sand (coarse silica sand), and 5) greenhouse mix (potting mixture consisting of black muck, peat moss, and sand in a 5:3:2 ratio). Soils 1 and 2 were collected from Alliston in central Ontario and soil 3 was collected from Simcoe in southwestern Ontario. The field soils were stored in plastic bags at 4 C until used.

The survival of MS was monitored in two types of experimental systems. In one system, each soil type was divided among four plastic pots (55 × 80 mm) each containing approximately 300 g of soil. One packet of the sand/MS mixture was buried in each pot at a depth of 25 mm. The pots were placed in a walk-in growth room with 24 C day/20 C night temperatures and 15 h per day of fluorescent lighting. The pots were watered thoroughly every third day with tap water so that the packets were infiltrated with soil water. One packet was removed from each pot after 3 wk of burial. After air-drying for several hours, soil adhering to the surface of the packets was brushed off, the packets were opened, and 25 MS from each packet were transferred onto SPT agar. In some of the soils, the packets were infiltrated by soil particles and organic material. Since such particles made identification of the MS difficult and could carry fungal contaminants, we investigated if rinsing the packets free of such debris altered the outcome of the viability determinations. After 25 MS were transferred from each packet to SPT agar, the remaining sand/MS mixtures from each soil treatment were pooled into one sample and sealed inside new mesh packets. The new packets were placed in a beaker with a cheesecloth cover and rinsed under running tap water for 3 h. The packets were blotted, then air-dried overnight, and 50 MS from each packet were transferred onto SPT agar.

In the second experimental system, each soil type was divided between four clear, rigid plastic boxes (95 × 65 mm, Magenta Corp., Chicago, IL) with lids that permitted air circulation. Each box received 200 g of soil. The water content of each soil was determined by measuring the weight of five 10-g samples of soil before and after oven-drying at 70 C for 12 h. The moisture capacity was measured in the same manner except the soil was saturated with water at the beginning of the procedure. Soil moisture was adjusted to achieve 50% of the soil moisture capacity by adding the appropriate amount of sterile distilled water. One packet of sand/MS mixture was buried in each box at a depth of 5 cm. After 4 wk of incubation at 24 C in the dark, the packets were recovered and 25 MS from each packet were transferred onto SPT agar as previously described. The number of MS that germinated and formed colonies was recorded 3 wk after plating onto SPT agar. Because many of the colonies formed were contaminated with other fungi, each colony was categorized as: 1) *V. dahliae*, 2) *V. dahliae* mixed with another fungus inhibitory to its growth, 3) *V. dahliae* coexisting with another fungus not inhibitory to its growth, or 4) a fungus other than *V. dahliae*. In addition, the number of MS that failed to produce a colony of any kind was recorded.

Statistical analysis. Counts of the number of MS that germinated and formed colonies or those that failed to germinate were analyzed by the chi-square test (χ^2) using the SigmaStat computer package. All experiments were performed at least twice. Data from one representative experiment are presented unless otherwise stated.

RESULTS

Production and manipulation of MS. Over a 6-wk growth period, *V. dahliae* produced an average of 119.7 mg of MS (dry weight, SE = 9.9, $n = 5$) in a single petri dish containing 30 ml of MCDX medium (Table 1). Since the average number of MS per milligram of dry MS was calculated to be 11,865 (SE = 1,171.6, $n = 10$), the total number of MS recovered per plate was approximately 1.4×10^6. Two weeks after inoculation, the smallest MS were most numerous (37%) and there were few MS (6.6%) of the largest size (Table 1). After 2 wk, however, the proportion of larger MS (>75 μm) increased to more than 50% and the proportion of the smallest MS (<53 μm) decreased to approximately 20% (Table 1).

Morphologically, MS were similar in both CDX and MCDX media. Separating MS from solid (1.5%) agar, however, generally required more blending and rinsing than were needed to separate MS from the semisolid MCDX medium, which contained only 0.25% agar. The prolonged blending required to separate MS from solid agar resulted in reduced viability of MS such that percent germination was either low (about 50%) immediately after harvesting or declined rapidly (from 90% to as low as 15%) in the first week after harvesting (*data not shown*). The MS recovered from semisolid MCDX medium exhibited 98% germination even after 46 wk of dry storage at 24 C.

Rate of germination and colony growth. While there was no difference in the final number of MS that germinated, the rate of germination between MS of different size classes differed significantly ($\chi^2 = 146.1$, df = 10, $P < 0.001$) (Fig. 1). The 75–106 μm MS germinated faster than the 53–75 or <53 μm MS, which showed similar germination rates ($\chi^2 = 7.05$, df = 5, $P = 0.217$)

TABLE 1. Total dry weight (milligrams) of microsclerotia of *Verticillium dahliae* recovered per plate of MCDX medium incubated at 24 C

Microsclerotia size (μm)	Time of harvest (wk)[a]				
	2	3	4	5	6
>106	6.3	9.2	53.8	34.4	21.2
75–106	26.5	45.8	39.0	44.6	30.9
53–75	26.9	32.3	26.6	25.5	25.1
<53	35.6	43.1	22.1	30.6	18.9
Total	95.3	130.4	141.5	135.1	96.1

[a]Two plates (60 ml of medium) were harvested each week over a 6-wk period.

(Fig. 1). Virtually all 75–106 μm MS germinated within 24 h after plating on SPT agar, whereas the germination of smaller MS extended over 2–3 days (Fig. 1). On CDX and PDA media, no difference in germination rate was apparent between MS of different size classes because germination of all MS exceeded 90% in the first 24 h. The larger MS (75–106 μm) also produced larger colonies on SPT agar than those formed by the smaller MS (Fig. 2). Analysis of colony diameter distribution (divided into six categories ranging from 1 to 6 mm) revealed that of 4-day-old colonies produced from MS of 75–106 μm size, 70% fell into the 5-mm category (Fig. 3). In contrast, only about 50% of MS 53–75 μm and 25% of MS <53 μm produced colonies that were 5 mm after 4 days of growth (Fig. 3). A greater proportion of smaller colonies was produced by the smaller MS. A significant relationship between MS size and colony diameter after 4 days of growth was confirmed by a chi-square test ($\chi^2 = 39.3$, df = 8, $P < 0.001$). The pattern of colony growth on CDX and PDA media was the same as the pattern on SPT agar (*data not shown*). In a similar experiment done with a different harvest of MS, there was a significant reduction in the rate of both

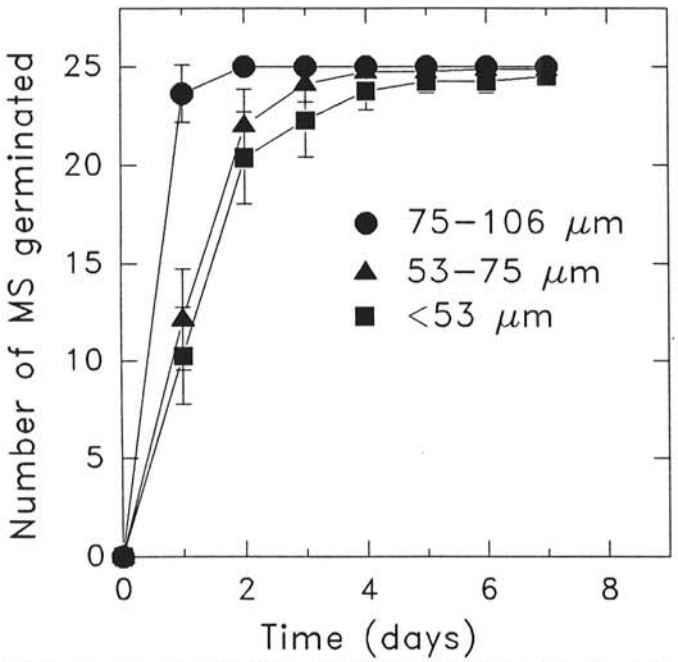

Fig. 1. Mean number of microsclerotia (75–106, 53–75, or <53 μm in diameter) that germinated (±95% CL, $n = 8$) daily on soil-pectate-Tergitol agar.

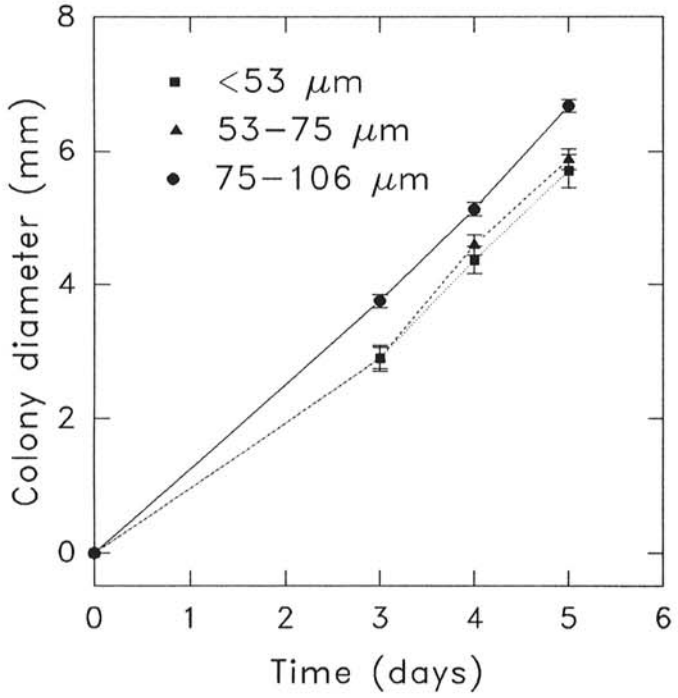

Fig. 2. Mean diameter of colonies (±95% CL, $n = 100$) that were produced by microsclerotia <53, 53–75, or 75–106 μm in diameter.

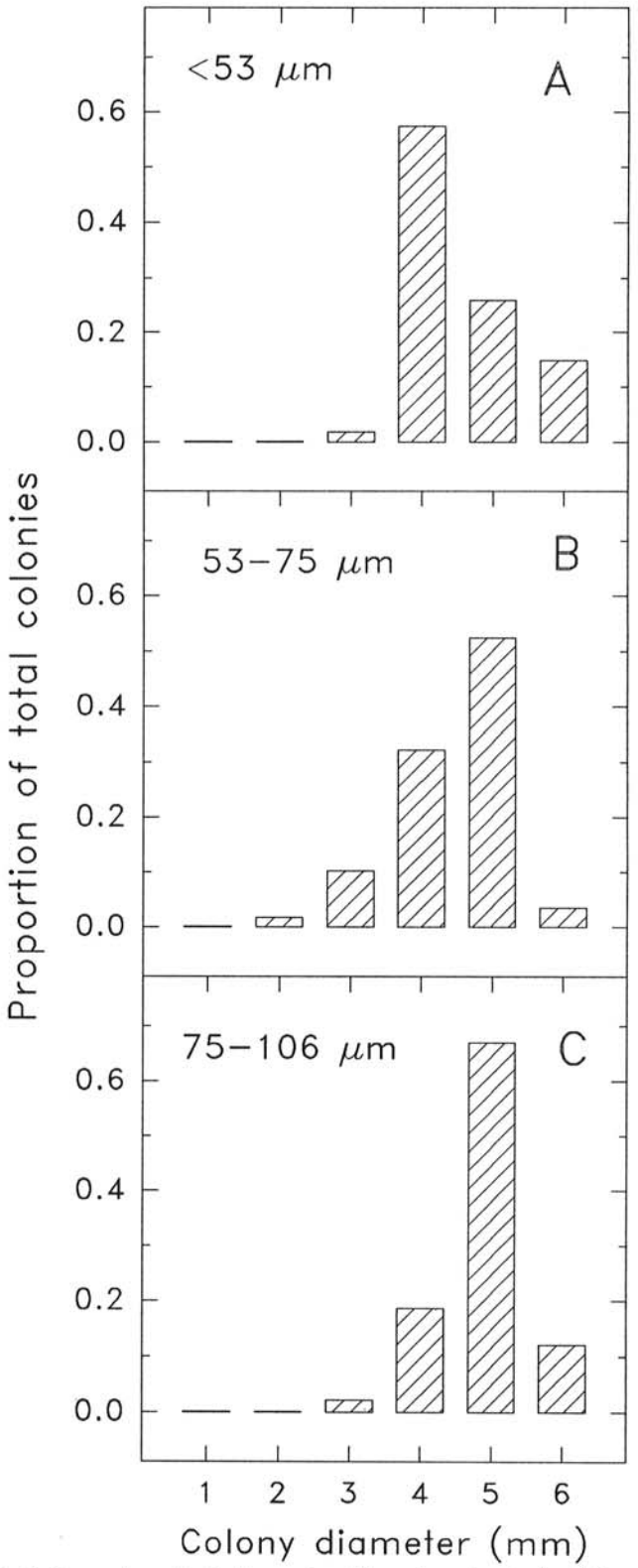

Fig. 3. Proportion of colonies produced by microsclerotia **A,** <53 μm, **B,** 53–75 μm, or **C,** 75–106 μm in diameter after 4 days of growth on soil-pectate-Tergitol agar.

germination and colony growth in MS <53 μm; however, the MS of intermediate size (53–75 μm) performed more like the larger MS (>75 μm) than the smaller MS (*data not shown*).

Storage temperature. The variability in germination of 75–106 μm MS in the first experiment (Fig. 4A) was high compared with that in the second experiment (Fig. 4B), perhaps because the MS were destructively sampled in the second experiment instead of being repeatedly taken out, sampled, and then returned to the storage site, as in the first experiment. Storage of MS in the refrigerator freezer (−25 C) in the first experiment reduced survival in every size class to <20% after 4–5 wk, whereas no reduction in survival occurred when MS were stored in the chest-type freezer at −25 C in the second experiment (Fig. 4B). The pattern of survival of 53–75 μm MS at each temperature (*data not shown*) was similar to that of 75–106 μm MS. Larger MS (>106 μm) survived better than smaller MS over the 10-wk period and exhibited less variability in germination over time (*data not shown*).

Long-term storage. In one experiment, smaller MS (<75 μm) lost viability at a faster rate than MS >75 μm when stored at 24 C for up to 14 wk, yet in another experiment, there was no difference in survival after 12 wk of storage (*data not shown*). In two other experiments, the largest MS (75–106 μm) generally survived better than smaller MS when stored for up to 35 wk at 24 C (Fig. 5). In one case, MS <75 μm began to lose viability after 8–12 wk of storage at 24 C, and by 35 wk, germination of these smaller MS dropped below 40% (Fig. 5A). In another case, MS <75 μm and MS >75 μm showed 96 and 100% germination, respectively, after 35 wk of storage at 24 C (Fig. 5B). Results from a fifth experiment (not shown in Fig. 5) showed that MS of 75–106 and 53–75 μm still exhibited 98 and 90% germination, respectively, after storage at 24 C for 46 wk and 94 and 78% germination after 59 wk.

Irradiation of MS with UV light. Melanin has been suggested to play a critical role in the survival of pigmented fungal structures exposed to UV light. Irradiation with UV light for 6 h or more had a lethal effect on nonmelanized MS (Fig. 6). The germination of nonmelanized MS was reduced by 50% after 2 h of irradiation, whereas the germination of nonirradiated controls remained near 100% throughout the experiment. Irradiation of nonmelanized MS for 2 h or more also caused a marked reduction in the size of 4-day-old colonies compared with nonirradiated MS and melanized MS (Fig. 7). While there was only a slight reduction in the germination of melanized MS after >36 h of exposure (Fig. 6), the colonies produced were less than half the size of those produced by melanized MS not exposed to UV light (Fig. 7).

Burial of MS in soil. When packets of MS were buried in soil and incubated in a growth room for 3 wk, MS germination in each soil type was 98–100%. According to a chi-square test, there was no significant relationship between the soil type and the number of MS that did or did not germinate in either the unwashed packets or the washed packets (*data not shown*). Washing the MS before transferring them to SPT agar did not significantly affect the number that germinated ($\chi^2 = 0.009$, df = 4, $P > 0.999$) but did remove debris from the MS, making them easier to find and reducing surface contamination.

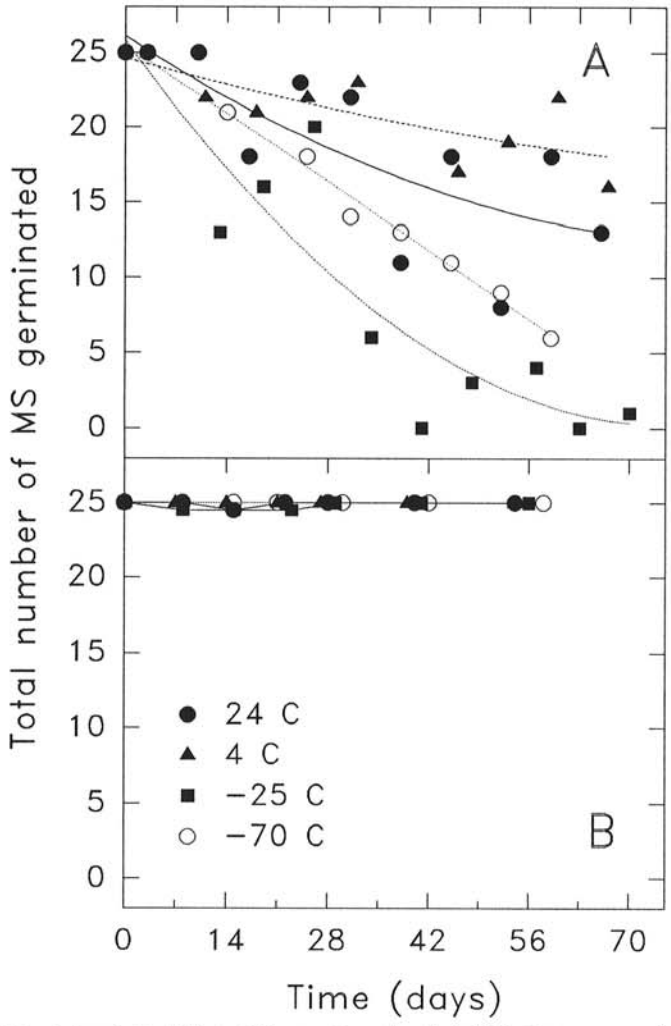

Fig. 4. Survival of 75- to 106-μm microsclerotia (of 25) that were germinated on soil-pectate-Tergitol agar and stored at four different temperatures for up to 70 days: **A,** Microsclerotia that were repeatedly sampled and returned to the storage site (regression lines illustrate trends) and **B,** microsclerotia that were destructively sampled.

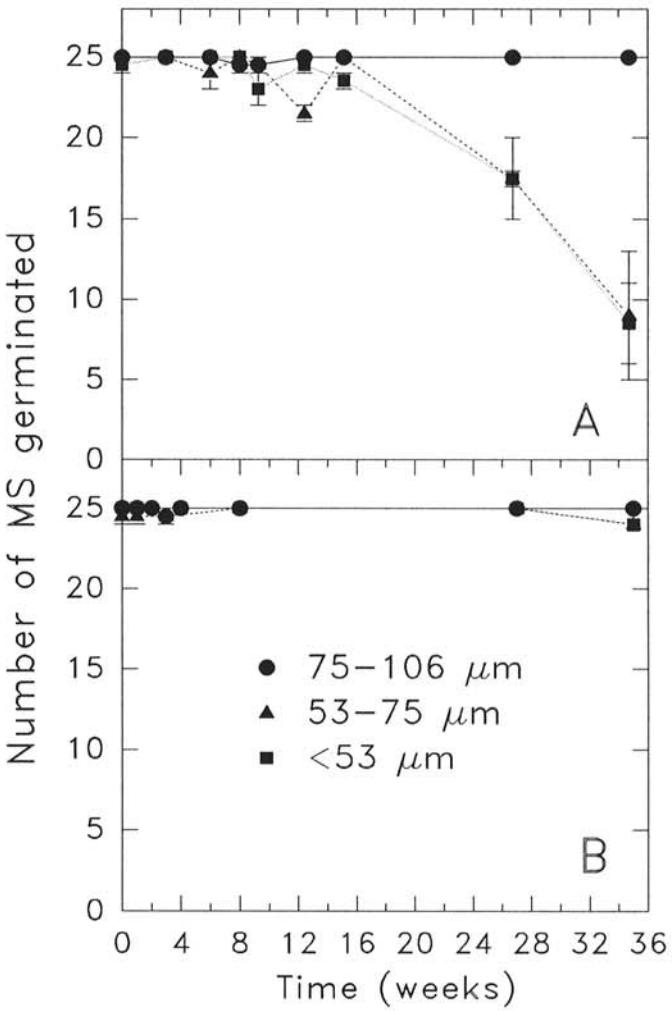

Fig. 5. Survival of three sizes of microsclerotia (of 25) that were germinated on soil-pectate-Tergitol agar and stored at 24 C for 35 wk (mean number ±1 SE, $n = 2$). **A** and **B** represent two separate harvests of microsclerotia.

There was a significant relationship between the soil type and the production of colonies by MS when the MS were buried for 4 wk in Magenta jars ($\chi^2 = 68.96$, df = 4, $P < 0.001$). Burial in sand, greenhouse mix, or Alliston soil had no effect on colony production by MS, whereas the mortality of MS was much higher in Lundy and Simcoe soils than would be expected by chance (Table 2).

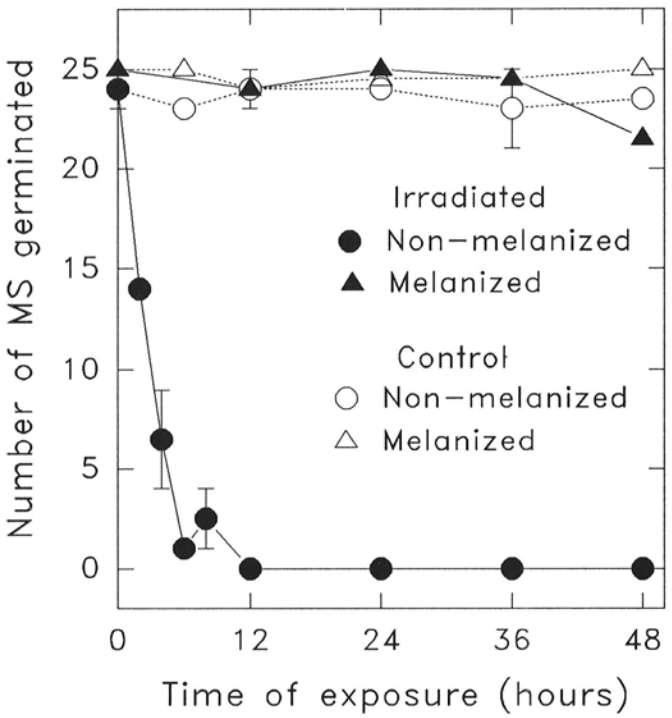

Fig. 6. Mean number of microsclerotia that germinated (±1 SE, $n = 2$) on soil-pectate-Tergitol agar after exposure to short wavelength UV light for up to 48 h.

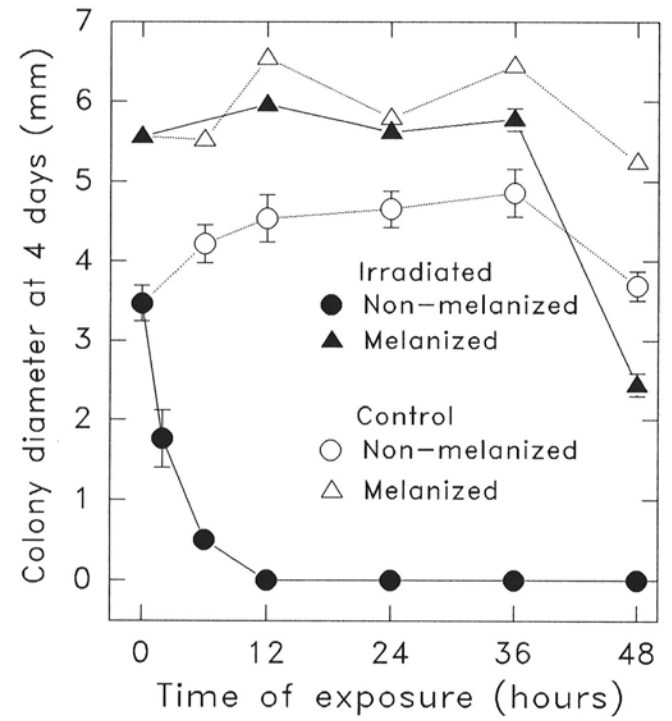

Fig. 7. Mean diameter of colonies after 4 days of growth on soil-pectate-Tergitol agar that were produced by microsclerotia exposed to UV light for up to 48 h (±1 SE, $n = 50$).

After at least 2 wk of growth on plates of SPT agar, the colonies produced by MS were clearly visible and easily distinguishable from other fungi by the presence of melanized MS (Fig. 8). When each colony in Table 2 was categorized as entirely *V. dahliae* or a mixture of *V. dahliae* and another fungus, additional information was revealed (Table 3). The MS recovered from the greenhouse mix (a soil that did not affect colony production, according to results shown in Table 2) produced an unusually low number of colonies composed of *V. dahliae* alone and an unexpectedly high number of colonies of *V. dahliae* associated with another fungus (Table 3). The MS buried in Simcoe soil had very low levels of association with other fungi and had a higher than

TABLE 2. The number of colonies present 5 days after plating arising from microsclerotia of *Verticillium dahliae* buried for 4 wk in soil in Magenta jars

	Colony produced		No colony produced	
Soil type	Observed[a]	Expected[b]	Observed	Expected
Sand	100	93	0	7
Greenhouse mix	100	93	0	7
Alliston	100	93	0	7
Lundy	76	93	24	7
Simcoe	88	93	12	7

[a] One hundred microsclerotia were plated onto SPT agar.
[b] Calculated as (total number of observed)/k, where $k = 5$.

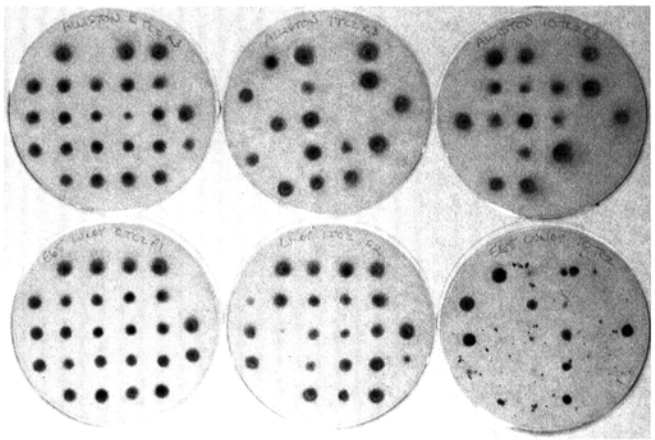

Fig. 8. Plates of soil-pectate-Tergitol agar at least 2 wk after 25 individual microsclerotia were transferred onto each plate: (Left) Typical colonies of *Verticillium dahliae* produced by 96–100% of the microsclerotia, (middle) low rates of germination, and (right) colonies of fungi other than *V. dahliae* produced by many microsclerotia. Other fungi were easily distinguished from *V. dahliae* by pigments or aerial structures.

TABLE 3. The number of colonies of different types produced by microsclerotia of *Verticillium dahliae* buried for 4 wk in soil in Magenta jars

	V. dahliae[a]		Mixed[b]		Coexistent[c]		Other[d]		None[e]	
Soil type	Obs.[f]	Exp.[g]	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Sand	100	82	0	2	0	9	0	6	0	1
Greenhouse mix	56	82	0	2	44	9	0	6	0	1
Alliston	100	82	0	2	0	9	0	6	0	1
Lundy	67	82	9	2	0	9	24	6	0	1
Simcoe	87	82	1	2	0	9	5	6	7	1

[a] *V. dahliae* only.
[b] *V. dahliae* mixed with an inhibitory fungus.
[c] *V. dahliae* coexisting with another fungus.
[d] A fungus other than *V. dahliae*.
[e] No colony formed.
[f] Obs. = observed; 100 microsclerotia were plated onto SPT agar.
[g] Exp. = expected; calculated as (row total)(column total)/grand total.

expected number of MS that did not germinate at all (Table 3). Finally, a significantly higher number of MS buried in Lundy soil gave rise to pure colonies of a fungus other than *V. dahliae* than could be expected by chance. Measurement of colony diameters did not prove useful in this type of experiment because so many of the colonies were not *V. dahliae* and because after only 4 days of growth, it was difficult to tell whether the colony being measured was *V. dahliae* or not.

DISCUSSION

One of the greatest difficulties involved in studying the impact of specific treatments on survival of *V. dahliae* has been the poor reliability of available techniques to isolate individual MS from mixtures of conidia and hyphae. Even when MS were isolated (22,23), it was difficult to be confident that the colonies produced originated from intact, single MS and not fragments or aggregates of MS. If the MS clump together, a colony might still be produced after exposure to specific experimental conditions, even though 90% of the MS are killed. In this situation, attempting to detect interactions of a microbial nature, which would be more subtle than the outright death of the MS, would be futile. Finally, despite subjective observations of differences in viability between larger and smaller MS (19), no consideration has been given to the concept that treatments could impact differently on inoculum composed of MS of various sizes or ages. This could lead to high variability in experimental results, masking the treatment effects.

The procedures outlined here can be used to produce a large number of MS at one time that can be collected, sorted into size classes, and stored easily under several commonly available temperature regimes. The MS produced by this method maintained high, uniform viability for many weeks and in some cases, for over a year, allowing a single batch of MS to be used in many different experiments over a long period of time.

Excellent production of MS was obtained by growing the cultures in the semisolid MCDX medium described. The use of only 0.25% agar allowed blending and wet-sieving of the MS with little damage or reduction in viability. Manipulating the dry, individual MS with a needle was quite simple with the assistance of a good microscope and a steady hand.

Rates of germination and colony growth were affected by MS size class. MS of at least 75 μm in diameter exhibited faster and more synchronous germination than any other size class. They also produced colonies that grew faster than those produced by smaller MS. Huisman and Ashworth (19) similarly found that smaller, immature MS isolated from soil exhibited only 7% germination, while larger, more mature MS had 80% germination. MS may be considered analogous to weed seeds in that seeds of differing size or morphology are produced (sometimes by the same plant) and may exhibit distinct patterns of dormancy, germination, or vigor (7). By using MS of similar size in experiments, then, variability in germination and colony diameter can be better controlled and the within-treatment variance subsequently reduced. Since MS of 75 μm or more are the most abundant and survive storage longer than smaller MS, they are the best candidates for experimental use.

There was little difference between survival of MS of 75–106 μm stored dry at 24, 4, −25, or −70 C. Storage in a refrigerator freezer, however, was detrimental to the MS. It is possible that the frost-free cycle of this refrigerator freezer was responsible for this phenomenon, since MS survived well in the chest-type freezer at the same temperature. Frost-free freezers do not maintain a constant temperature but undergo cyclic temperature fluctuations to prevent frost buildup. Individual MS larger than 75 μm stored dry in a 24 C incubator for more than a year had 94% viability.

A review of the literature on survival of sclerotia revealed that survival data have been analyzed by a wide assortment of statistical methods (9,17,24,26). Most commonly, the number of sclerotia germinated in each treatment was expressed as a percentage, an arcsine transformation was applied, and an analysis of variance was done on the transformed data. Treatments were then compared with a multiple comparison procedure to determine any significant differences. This type of statistical analysis would be particularly useful for more complex experiments of factorial design where more than one factor is being manipulated at the same time. For simpler experiments of completely randomized design where within-treatment variability is low (as it is in the procedure described above, when individual, same-size MS are used), an alternative method of design and analysis can be applied. Because the chi-square test is a nonparametric test and does not require the assumptions implicit in ANOVA, the data need not be transformed. According to Gilligan (12), the potential of the chi-square test has ". . . been little exploited by plant pathologists for the analysis of counts." In the chi-square test, the total numbers of MS that germinated (alive) or remained ungerminated (dead) in each treatment at the end of the experiment were tallied and placed into a contingency table. When the experiment is performed more than once and the results of each trial are calculated to be homogeneous, the data from all the trials can be pooled and reanalyzed together, increasing the sample size (n) and the accuracy of the test (32).

The ability of this method to reflect both lethal and sublethal treatment effects was demonstrated by results obtained following exposure of melanized and nonmelanized MS to UV irradiation. It was also demonstrated that the method was useful for studying the effects of burial of MS in soil under different conditions. A count of the number of MS that were alive or dead at the end of an experiment reflected the lethal effect of a treatment. Reductions in the rate of germination or colony growth, however, reflect sublethal effects of experimental treatments. In essence, a reduction in growth rate compared with untreated MS indicates that the treated MS are responding like smaller structures, which contain fewer cells.

Until now, only methods for indirect study of individual reproductive structures of *V. dahliae* have been available, so it has not been possible to adequately investigate whether such concepts as dormancy apply to MS. The method presented here for the production, isolation, and manipulation of MS can hopefully provide a tool to study these and many other aspects of the ecology of this fungus.

LITERATURE CITED

1. Ashworth, L. J., Jr., Harper, D. M., and Andris, H. L. 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., Waters, J. E., George, A. G., and McCutcheon, O. D. 1972. Assessment of microsclerotia of *Verticillium albo-atrum* in field soils. Phytopathology 62:715-719.
3. Ausher, R., Katan, J., and Ovadia, S. 1975. An improved selective medium for the isolation of *Verticillium dahliae*. Phytoparasitica 3:133-137.
4. Ben-Yephet, Y., and Pinkas, Y. 1977. Germination of individual microsclerotia of *Verticillium dahliae*. Phytoparasitica 5:159-166.
5. Ben-Yephet, Y., Siti, E., and Frank, Z. 1983. Control of *Verticillium dahliae* by metam-sodium in loessial soil and effect on potato tuber yields. Plant Dis. 67:1223-1225.
6. Beute, M. K., and Rodriguez-Kabana, R. 1981. Effects of soil moisture, temperature, and field environment on survival of *Sclerotium rolfsii* in Alabama and North Carolina. Phytopathology 71:1293-1296.
7. Cavers, P. B., and Harper, J. L. 1966. Germination polymorphism in *Rumex crispus* and *Rumex obtusifolius*. J. Ecol. 54:367-382.
8. Coley-Smith, J. R. 1959. Studies of the biology of *Sclerotium cepivorum* Berk. III. Host range; persistence and viability of sclerotia. Ann. Appl. Biol. 47:511-518.
9. Congly, H., and Hall, R. 1976. Effects of osmotic potential on germination of microsclerotia and growth of colonies of *Verticillium dahliae*. Can. J. Bot. 54:1214-1220.
10. Gerbrandy, Sj. J. 1989. The effects of various temperatures during storage in soil on subsequent germination of sclerotia of *Sclerotium cepivorum*. Neth. J. Plant Pathol. 95:319-326.
11. Gilbert, R. G., and Griebel, G. E. 1969. The influence of volatile substances from alfalfa on *Verticillium dahliae* in soil. Phytopathology

59:1400-1403.
12. Gilligan, C. A. 1986. Use and misuse of the analysis of variance in plant pathology. Pages 225-261 in: Advances in Plant Pathology. Vol. 5. D. S. Ingram and P. H. Williams, eds. Academic Press, New York.
13. Gordee, R. S. 1961. Inhibition, physiological, and structural studies on microsclerotia of *Verticillium albo-atrum* R and B. Ph.D. thesis. Purdue University, West Lafayette, IN.
14. Gordee, R. S., and Porter, C. L. 1961. Structure, germination, and physiology of microsclerotia of *Verticillium albo-atrum*. Mycologia 53:171-182.
15. Green, R. J., Jr. 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59:874-876.
16. Green, R. J., Jr., and Papavizas, G. C. 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum* in soil. Phytopathology 58:567-570.
17. Henis, Y., and Papavizas, G. C. 1983. Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. Phytopathology 73:1469-1474.
18. Huang, H. C., and Kokko, E. G. 1989. Effect of temperature on melanization and myceliogenic germination of sclerotia of *Sclerotinia sclerotiorum*. Can. J. Bot. 67:1387-1394.
19. Huisman, O. C., and Ashworth, L. J., Jr. 1974. *Verticillium albo-atrum*: Quantitative isolation of microsclerotia from field soils. Phytopathology 64:1159-1163.
20. Isaac, I., Fletcher, P., and Harrison, J. A. C. 1971. Quantitative isolation of *Verticillium* spp. from soil and moribund potato haulm. Ann. Appl. Biol. 67:177-183.
21. Kantzes, J. E. 1980. A comparative study of selective media for the isolation of *Verticillium dahliae*. M.Sc. thesis. Purdue University, West Lafayette, IN.
22. Lazarovits, G., Hawke, M. A., Olthof, T. H. A., and Coutu-Sundy, J. 1991. Influence of temperature on survival of *Pratylenchus penetrans* and of microsclerotia of *Verticillium dahliae* in soil. Can. J. Plant Pathol. 13:106-111.
23. Lazarovits, G., Hawke, M. A., Tomlin, A. D., Olthof, T. H. A., and Squire, S. 1991. Soil solarization to control *Verticillium dahliae* and *Pratylenchus penetrans* on potatoes in central Ontario. Can. J. Plant Pathol. 13:116-123.
24. Leggett, M. E., and Rahe, J. E. 1985. Factors affecting the survival of sclerotia of *Sclerotium cepivorum* in the Fraser Valley of British Columbia. Ann. Appl. Biol. 106:255-263.
25. Menzies, J. D., and Griebel, G. E. 1967. Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57:703-709.
26. Merriman, P. R. 1976. Survival of sclerotia of *Sclerotinia sclerotiorum* in soil. Soil Biol. Biochem. 8:385-389.
27. Papavizas, G. C. 1977. Survival of sclerotia of *Macrophomina phaseolina* and *Sclerotium cepivorum* after drying and wetting treatments. Soil Biol. Biochem. 9:343-348.
28. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annu. Rev. Phytopathol. 31:111-126.
29. Pullman, G. S., DeVay, J. E., and Garber, R. H. 1981. Soil solarization and thermal death: A logarithmic relationship between time and temperature for four soilborne plant pathogens. Phytopathology 71:959-964.
30. Pullman, G. S., DeVay, J. E., Garber, R. H., and Weinhold, A. R. 1981. Soil solarization: Effects on Verticillium wilt of cotton and soilborne populations of *Verticillium dahliae, Pythium* spp., *Rhizoctonia solani*, and *Thielaviopsis basicola*. Phytopathology 71:954-959.
31. Stapleton, J. J., and DeVay, J. E. 1982. Effect of soil solarization on populations of selected soilborne microorganisms and growth of deciduous fruit tree seedlings. Phytopathology 72:323-326.
32. Zar, J. H. 1976. Biostatistical Analysis. 1974. Prentice-Hall, Inglewood Cliffs, NJ.