Reduced Number of Microsclerotia Formed by Verticillium dahliae in Cotton Tissue Exposed to Systemic Benzimidazole Fungicides and Desiccation

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ABSTRACT

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Application of benomyl, carbendazole, thiophanate M, or folcidin to cotton plant tissue, infected with *Verticillium dahliae,* suppressed microsclerotia production markedly in leaf tissue and to a lesser extent in petiole or stem tissue. Addition of a paraffinic oil increased the effectiveness of the fungicides in some experiments. Drying infected leaves of cotton reduced the number of propagules of *V. dahliae* from 6.81×10^4/g in nondried (81.8% moisture) plant tissue to 1.66×10^4/g in dried tissue (9.5% moisture). Application of 2,500 μg/ml benomyl or folcidin to infected leaves, stems, and petioles for different periods of drying prior to incubation in moist soil, reduced the number of microsclerotia markedly in leaves treated for 0.01 and 1 hr. Microsclerotia were suppressed in petioles and stems only after treatment for 3 to 5 hr. The degree of reduction of microsclerotia in the plant tissue was greatest when benomyl was applied to infected leaves, petioles, and stems followed by different periods of drying before incubation in moist soil. Application of benomyl and methyl 2-benzimidazolecarbamate·HCl with oil on infected plants in the field suppressed 70% of the microsclerotia production in leaves.

Additional key words: inoculum density, adjuvant.

Verticillium dahliae Kleb. has little or no competitive saprophytic capability in colonizing noninfected tissue (28). However, microsclerotia are produced saprophytically in infected cotton leaves (4) and in the xylem and pith of the lower stem and roots of cotton plants *Gossypium hirsutum* L., but only after death of plant tissues (15). Evans et al (15) suggested that removal or destruction of stalks and roots prior to decomposition in soil might result in the reduction of soil-borne inoculum. Schnathorst (22, 23) considered that hyaline, torulose cells of *V. dahliae* produced in plant tissue were important sources of primary inoculum and suggested that fragmentation of plant material before incorporation into soil might be an effective control measure.

Ashworth et al (2, 3) reported a direct correlation between numbers of microsclerotia in the soil (wet-sieve method of assay) and incidence of Verticillium wilt of cotton (based on vascular discoloration). In one report the numbers of microsclerotia in soil (air dried for 6 wk) from 26 cotton fields estimated by the Anderson air sampler method (18), were not correlated with incidence of foliage symptoms of Verticillium wilt (11). Subsequent research (7, 8, 9) in the same laboratory showed that microsclerotia density in soil from areas within a single field was related to disease incidence and should be considered important in long range control.

The use of chemicals to suppress the saprophytic phase of other pathogens has been reported. Perithecial formation by *Venturia inaequalis* (Cke.) Wint. was prevented by spraying infected apple leaves just before leaf fall with benomyl (10, 21). Benomyl suppressed ascocarp formation in *Gloeotinia temulenta* (Prill and Del.) Wilson, Noble, and Gray and in *Claviceps purpurea* (Fr.) Tul. on grasses at the soil surface (16, 17).

If microsclerotia from previously infected tissue are the primary inocula for Verticillium wilt, the suppression of microsclerotia in infected plant tissue should reduce the inoculum density of *V. dahliae* in soil. The Verticillium-wilt disease is a single cycle or "simple interest" disease (27) in which disease incidence in one year depends on the amount of viable inoculum from the previous season or seasons. Van der Plank (27) theoretically showed that reduction of inoculum of a single cycle disease was more efficacious for control than with a multicycle disease such as late blight of potato caused by *Phytophthora infestans* (Mont.) d By.

Evans (1973 *unpublished,* cited in 12) reported that benomyl-treatment of plant tissue infected with *V. dahliae* prior to incubation in soil suppressed the formation of microsclerotia in plant tissue. We report here that application of some benzimidazole fungicides to infected cotton plant tissue as well as desiccation of plant tissue reduced the number of microsclerotia produced by *V. dahliae*. Certain aspects of this study were briefly discussed in relation to the control of vascular wilt pathogens (12) and in an abstract (14).

MATERIALS AND METHODS

The field in Tulare County, California, in which naturally-infected cotton plant (cultivar SJ-2) material

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was collected was heavily infested with *V. dahliae*. The incidence of Verticillium wilt had been almost 100% every year since 1966. The predominant pathotype from infected plants caused defoliation of cotton. In another experiment, plant material was collected from an experimental plot at the U.S. Department of Agriculture Cotton Research Station, Shafter, California in which the incidence of Verticillium wilt was about 50%. Stem, petiole, and leaf tissues were harvested in some experiments from 2-mo-old plants that were stem-inoculated in the greenhouse with the defoliating, microsclerotia producing isolate V3H.

Fungicides tested included benomyl, carbendazim (methyl-2-benzimidazolecarbamate), thiophanate M [1,2-*bis*-(3-methoxycarbonyl-1-thioureido benzene)], and folcidin. All were wettable powders and were prepared as suspensions before application to plant tissue by dipping or spraying. A water-soluble formulation of carbendazim was prepared with hydrochloric acid (MBC·HCl) (5, 6). Dosages are expressed as active ingredient and, when different chemicals were compared in an experiment, the weights were equilibrated with that of benomyl on a molecular-weight basis. A paraffinic oil [Orchex 795®, Esso Research and Engineering Co., Baytown, TX 77520 (essentially the same as Savol® and Super Savol®, Thompson-Hayward Co., Kansas City, KS 66110)], was used to augment the penetration of fungicides into plant tissue (13, 29).

Microsclerotia were formed in infected plant tissue after incubation for various periods of time, usually 7 days or longer in either moist, nonsterilized sandy-loam soil in the greenhouse (21 C night and 28-30 C day) or in a polyethylene bag that contained a wet paper towel (moist chamber).

Plant tissue was soaked in 95% ethanol to remove chlorophyll, and examined qualitatively for the presence of microsclerotia by observation under a stereoscope. Microsclerotia in plant tissue were evaluated quantitatively by blending ethanol-cleared plant tissues and counting microsclerotia using a stereoscope (26). A dilution assay modified from the method of Thomas and Huisman (25) also was employed in one experiment for the estimation of numbers of viable units of *V. dahliae* in the plant tissue.

Infected leaves, petioles, and stems from artificially inoculated plants were dipped for various periods of time in suspension of benomyl and of folcidin (2,500 μg/ml). This was done to determine the effects of chemicals applied externally to plant tissue on production of microsclerotia.

The adjuvant, Span-20, sorbitan monolaureate (ICI United States, Wilmington, DE 19897), was added (7,500 μg/ml) to the fungicides since some previous work indicated Span-20 augmented uptake of benomyl in plant tissue. To prevent the test fungicides from penetrating the stems or petioles through the excised ends, the end surfaces were coated with paraffin wax prior to the fungicidal treatment. After treatment, the plant tissue was incubated in moist nonsterilized sandy-loam soil (5 cm in depth) for about 2 mo to allow normal production of microsclerotia. Each treatment was replicated four times.

The effect of drying plant tissue on production of *V. dahliae* propagules was studied by drying infected leaves, which had been cut into small (1 × 1 cm) squares for various periods of time. The amount of water loss was recorded. A 1-g sample of leaves at each drying regime was blended in a Sorvall Omni-mixer for three 20-sec intervals in 200 ml of sterile distilled water. A 1-ml sample of a 1:10 dilution was pipetted on the surface of sodium polypectate agar (19) in petri dishes and incubated at room temperature for 10 days after which *V. dahliae* colonies were counted.

In another experiment infected cotton stems, petioles, and leaves were soaked in a suspension of benomyl (0, 100, 1,000, and 2,000 μg/ml) in a 10% paraffinic oil emulsion (Orchex 795). A nontreated control was included. After treatment, the plant material was air dried in the greenhouse (20-27 C) for 0-, 3-, and 7-day increments of time, after which it was incubated for 45 days in a flat of moist nonsterilized sandy-loam soil incubated in a lath house in which temperature varied from 15-30 C. There were four replicate treatments of leaves and petioles and two of stems.

The plant material was incubated in soil, recovered,

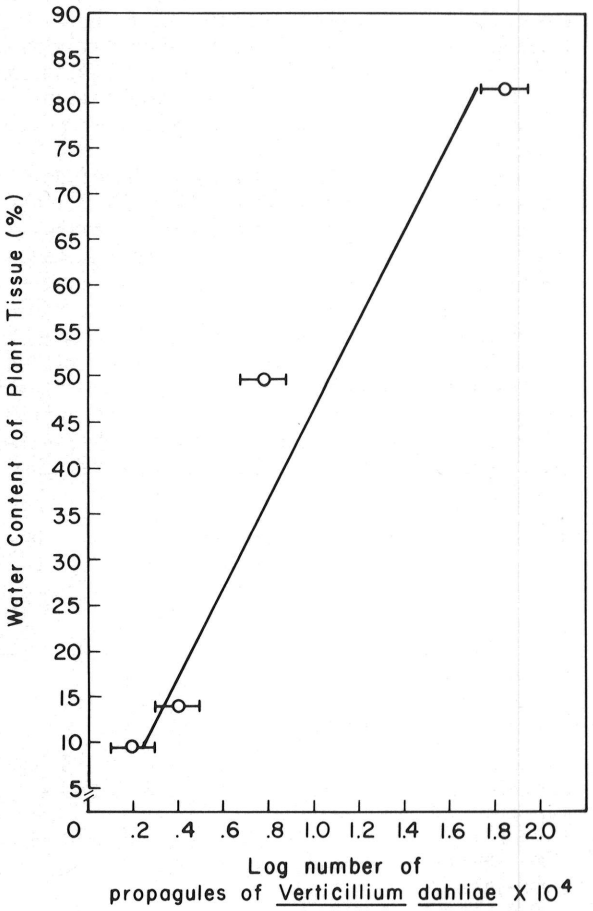

Fig. 1. Effect of drying leaf sections (1 cm^2) of cotton plants infected with *Verticillium dahliae* on the number of viable propagules per gram of tissue (dry weight) in a blended homogenate detectable by growth on sodium-polypectate agar. Propagules consisted of both conidia and mycelia but not microsclerotia.

and weighed. Chlorophyll was removed by extraction in 95% ethanol, and blended in a Sorvall Omni-mixer. Microsclerotia were recovered from this material and counted in a Hawksley eel-worm counter (26). The experiment was repeated.

RESULTS

Effect of drying plant tissue on the production of microsclerotia in infected cotton tissue.—During August and September of 1973, fallen leaves from infected cotton plants were collected at random from the Tulare County farm. At time of collection, only one of 300 petioles examined contained any microsclerotia. However, after leaves from this collection were incubated in a moist chamber at 21-24 C, microsclerotia ranging from 5-50/cm^2 of tissue had formed after about 7 days. This indicated that microsclerotia were formed predominantly in moribund plant tissue.

To determine whether drying might affect formation of microsclerotia, leaves from infected plants were collected and allowed to dry in a lath house for various periods of time from 1 to 8 wk at temperatures that varied from about 21 C at night to 28 C in the day. After drying for the different times, 20 leaves were incubated for 9 days on a moist paper towel in a plastic bag. Microsclerotia were abundant in the nondried leaves after incubation for 30 days but only a few were present in leaves that were dried longer than 1 wk.

Effect of drying infected plant tissue on the viability of propagules of Verticillium dahliae.—*Verticillium dahliae* probably exists in newly infected tissue as conidia and mycelium, both of which are sensitive to desiccation (9). When the pieces of infected leaves were dried to different moisture levels, blended, diluted, and plated on sodium polypectate agar, the numbers of propagules (probably conidia and mycelium) detected were lowest from the dried tissue and highest from the nondried tissue (Fig. 1). Even though desiccation caused a decrease in the number of propagules in plant tissue, this environmental stress did not completely eliminate all of the propagules of *V. dahliae* from leaves. Even when leaves were dried to 9.4% water, 1.66×10^4 propagules/g were detected.

Suppression of microsclerotia in infected cotton plant tissue by benomyl, thiophanate M, carbendazole, and folcidin.—Diseased leaves (five replicates) from 38-day-old infected plants grown in the greenhouse were dipped in suspensions of benomyl, thiophanate M, and carbendazole. After treatment, the leaves were allowed to

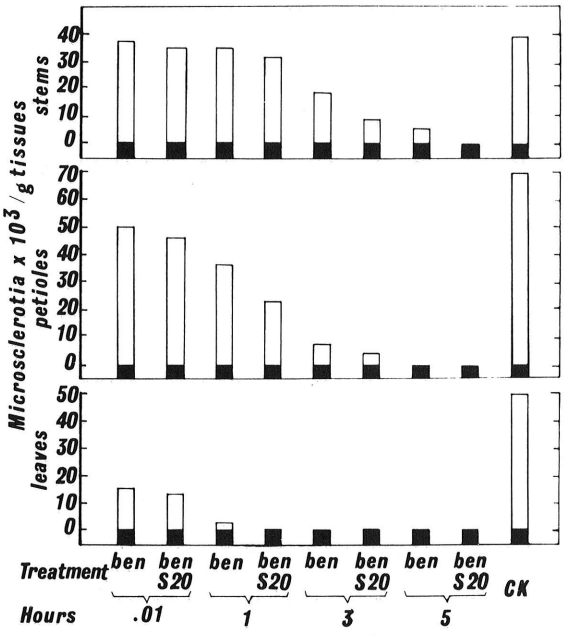

Fig. 2. Effect of benomyl on production of microsclerotia in infected cotton leaves, petioles, and stems soaked in a benomyl suspension at 2,500 μg/ml with and without Span-20 (7,500 μliter/ml) for different periods of time before being incubated in moist nonsterilized sandy-loam soil. Abbreviations: Ben = benomyl, S20 = Span-20, and Ck = check nontreated. Similar responses were obtained by use of folcidin.

TABLE 1. The effect on production of microsclerotia of application of benomyl and thiophanate M (with and without oil additives) to cotton infected with *Verticillium dahliae*

Fungicide[a]	Concentration (μg/ml)	Microsclerotia[b]	
		Leaves	Petioles
Benomyl	25	+	++
Benomyl plus oil	25	0	+
Benomyl plus kerosene	25	+	++
Benomyl	100	0	+
Benomyl plus oil	100	0	+
Benomyl plus kerosene	100	0	+
Thiophanate M	25	+	++
Thiophanate M plus oil	25	0	+
Thiophanate M plus kerosene	25	0	++
Thiophanate M	100	0	+
Thiophanate M plus oil	100	0	0
Thiophanate M plus kerosene	100	0	++
Oil	0	+	++
Kerosene	0	+	++
No treatment	0	+	++

[a]Paraffinic oil, (Orchex 795), and kerosene were used at 20% (v/v).
[b]Symbols: 0 indicates no microsclerotia, + = sparse (1-15/cm^2), and ++ = abundant production (16-50/cm^2) of microsclerotia.

dry at room temperature for about 24 hr after which they were incubated in a moist chamber at room temperature (24 C) for 9 days. At dosages of 100, 500, and 1,000 μg/ml of fungicides, formation of microsclerotia was suppressed by all chemicals in leaves but not in petioles.

Diseased leaves from 30-day-old plants were dipped in suspensions of benomyl and thiophanate-M at 25 μg/ml and 100 μg/ml. Leaves were incubated in soil for 14 days and observed for microsclerotia. Thiophanate M at 100 μg/ml plus 20% Orchex 795 (v/v) supressed microsclerotia formation in both leaves and petioles. Benomyl suppressed formation of microsclerotia in leaf tissue but not in petioles. Orchex 795 oil increased the effectiveness of the fungicides at 25 μg/ml but not at 100 μg/ml. Kerosene did not increase the effectiveness of either chemical (Table 1).

In another experiment, diseased leaves and petioles from the Tulare field were dipped in suspensions of benomyl (100 μg/ml) emulsified with oil (20% v/v) and allowed to dry for 3 days before incubating in nonsterilized soil for 45 days. Microsclerotia were observed in petioles and leaves of nontreated plants, but not in treated leaves. In benomyl-treated petioles there was a marked reduction (>50%) in microsclerotia formation but not complete suppression.

The effect of systemic fungicides on the reduction of

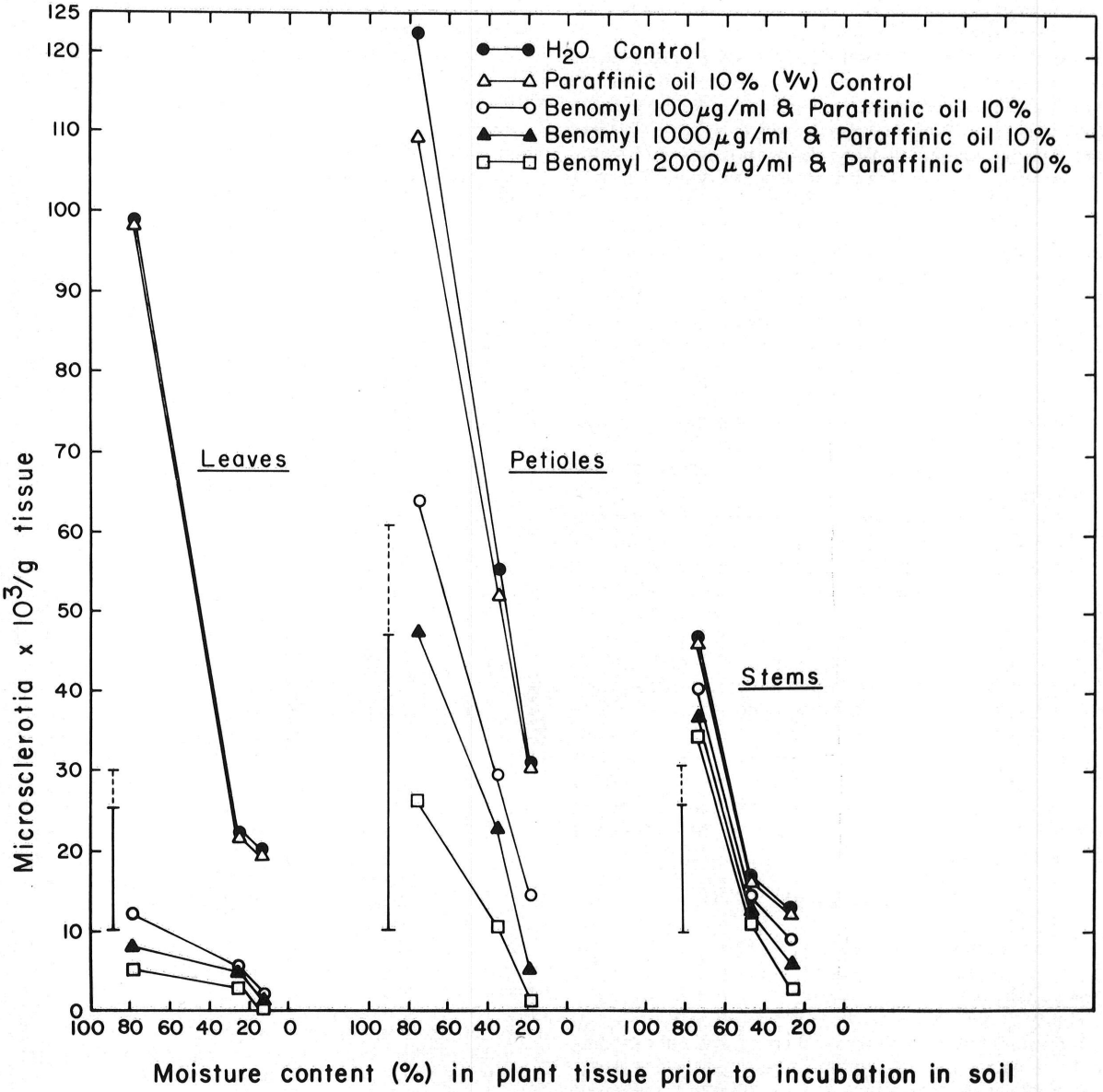

Fig. 3. Effect of benomyl applied to plant tissue infected with *Verticillium dahliae* and of subsequent drying of cotton plant tissue on the production of microsclerotia in moist nonsterilized sandy-loam soil (incubated 45 days). The vertical lines indicated confidence limits at $P = 0.05$ (solid line) and at $P = 0.01$ (solid plus dotted line).

microsclerotia was determined. Infected leaves, petioles and stems were treated with benomyl and folcidin by soaking for 0.01-, 1-, 3-, or 5-hr periods of time after which the nondried infected tissue was incubated in moist nonsterilized sandy-loam soil for over 1 mo. Since the effect of benomyl was so similar to that of folcidin, only those data are shown. The numbers of microsclerotia/g tissue for untreated petioles, leaves, and stems were 70×10^3, 50×10^3, and 40×10^3, respectively; for the 0.1 hr benomyl treatment without Span-20, the numbers were 50×10^3, 15×10^3, and 38×10^3/g, respectively. In the 1 hr benomyl treatment the numbers were 31×10^3, 3×10^3, and 35×10^3/g, respectively. Microsclerotia in petioles and stems, however, were not greatly suppressed except when soaked for 3- and 5-hr periods. The adjuvant had little effect in enhancing penetration of benomyl or folcidin into plant tissue (Fig. 2).

Combined effect of benomyl and drying on formation of microsclerotia.—Formation of microsclerotia in previous experiments was suppressed in infected leaves that had been dried before incubation in soil. This experiment compared treating leaves, petioles, and stems from infected plants with benomyl with drying. Benomyl was applied at three concentrations as an oil emulsion (10% v/v Orchex 795 paraffinic oil) to infected stems, petioles, and leaves. Samples from each treatment were dried to different degrees of dryness after treatment and before the plant tissue was incubated in soil. The numbers of microsclerotia were reduced to nearly as great an extent by drying as by treatment with benomyl (Fig. 3).

The degree of suppression of microsclerotia in leaves was marked, especially when the effect of benomyl was added to drying. However, the degree of suppression was much less in petioles and stems. This was probably due to the difficulty in obtaining penetration by benomyl into the internal xylem tissue of petioles and stems.

Effect of spraying cotton in the field with benomyl and MBC·HCl.—To study the effect of applying benomyl to infected cotton foliage on microsclerotia production in the field, cotton foliage was sprayed with benomyl and with MBC·HCl on 4 October at a rate of 561 liters/ha (60 gal/A) at 1,000 μg/ml, with a machine sprayer at the West Side Field Station at Five Points, California. Both fungicides were amended with Savol oil (10% v/v). Leaves collected on 25 October were incubated in soil for 30 days and the number of microsclerotia/g of plant tissue was determined by observation in a Hawksley eelworm counting chamber with a stereoscope. The numbers of microsclerotia were significantly reduced in benomyl or MBC treatments to about 31% of the nontreated control (Fig. 4).

DISCUSSION

Even though *Verticillium dahliae* persists as microsclerotia for long periods of time in the absence of a host, Ashworth and Huisman (1) reported that the viability of microsclerotia was reduced 76% by air drying for 2-3 mo at 30 C. Storage of microsclerotia at different relative humidities (0 to 90%) resulted in increased germination percentages as storage humidity increased (1). Schreiber and Green (24) reported that conidia and mycelium of *Verticillium* were sensitive to desiccation. The finding that desiccation of plant tissue also suppressed microsclerotia formation almost as much as benomyl may indicate that physical measures might be useful for control. Ioannou et al (20) reported that microsclerotia production was increased 25-fold by drying infected plant tissue from a matric-potential of −5.8 bars to −18.7 bars, but that further drying to matric-potential values of less than −18.7 bars, resulted in a marked decrease of microsclerotia. At −86 bars, microsclerotia production was negligible. Although use of benomyl and other benzimidazole fungicides and drying of plant material resulted in a reduction in the numbers of microsclerotia formed, it would appear that of the two methods, drying would be the more culturally feasible. For control in the field, all infected tissue (roots, stems, petioles, and leaves) would have to be dried to reduce the inoculum that is formed in infected plant tissue returned to the soil.

Brinkerhoff (4) reported that the microsclerotial stage of *V. albo-atrum* (*V. dahliae*) developed rapidly at 18-30 C in abscised dry cotton leaves incubated in soil at the

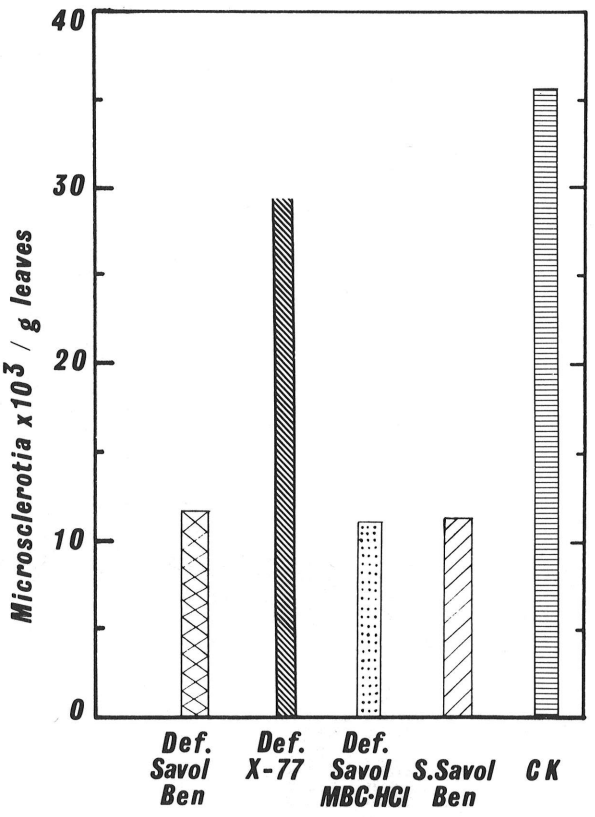

Fig. 4. Effect of benomyl (ben) and methyl-2-benzimidazole-carbamate HCl (MBC·HCl) applied to *Verticillium dahliae*-infected cotton foliage at the Westside Field Station (WSFS), Five Points, California on subsequent production of microsclerotia in leaves. Microsclerotia were produced in infected leaves incubated in moist nonsterilized soil for 30 days at 21-28 C. DEF indicates the combination of the commercial defoliant, S,S,S-tributyl phosphorotrithioate, used at 2.4 liters/ha (2 pints/A) and the desiccant, paraquat, used at 0.6 liters/ha (0.5 pints/A). X-77 is a sticker spreader. Savol and S. Savol (Super Savol) are paraffinic oil products.

water holding capacity. If decomposition of infested cotton plant debris could take place at 32 C or above, microsclerotia would be inhibited. He also stated that, "Relatively few microsclerotia developed in nonsterile soil at the lower moisture level, whereas at field capacity large numbers of microsclerotia were found in both leaf laminae and petioles."

The numbers of microsclerotia (about 35×10^3/g) formed in cotton stem tissue (Fig. 4) were somewhat higher than reported by Thomas and Huisman (25). Suppression of the primary microsclerotia inoculum produced by *V. dahliae* in infected cotton residue, could be important in controlling Verticillium wilt, but the practical effectiveness of this approach would depend on suppression of microsclerotia formation in large infected stems. Figure 2 and 3 indicate that the degree of control by fungicides was much less in stems than in petioles or leaves. However, drying before incubation in soil markedly reduced microsclerotia formation even in stems.

Although formation of microsclerotia in leaves and petioles was reduced significantly following a field spray treatment with benomyl-oil, it was probably not great enough for effective control of Verticillium wilt. Ashworth et al (2) reported that inoculum densities of 0.3 to 1.0 microsclerotium/g of soil, induced disease incidences (indicated by vascular discoloration) of 20-50% and, when the inoculum density exceeded 3.5/g soil, 100% incidence of disease occurred. This indicates that the efficacy of any control practice that involves reduction of inoculum levels must be judged in relation to the concentration of inoculum already present in the soil. Use of any method for decreasing numbers of microsclerotia inoculum will be successful for any single year, but only if the residual inoculum in the soil is at or below a certain level which as yet is undefined except for the data by Ashworth et al (2) and by Butterfield and DeVay (8, 9). Despite the importance of inoculum density, factors other than inoculum density also can affect severity of the disease (2). Some of these include soil and air temperatures and nutrition of the host. However, the inoculum density in soil is an important primary factor, and if inoculum can be reduced below a minimal level, the incidence of disease should be reduced. If so, one might expect that further field research on reduction of the inoculum returned to the soil in infected plant tissue should be a sound research goal.

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