# Copper-Induced Fungistasis of Microsclerotia of Verticillium albo-atrum and Its Influence On Infection of Cotton in the Field

L. J. Ashworth, Jr., O. C. Huisman, R. G. Grogan, and D. M. Harper

Departments of Plant Pathology, University of California, Berkeley 94720 and Davis 95616.

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ABSTRACT

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We describe a fungistasis of microsclerotia of *Verticillium albo-atrum* (*V. dahliae*) in soils of the San Joaquin Valley of California, which was absent or of low intensity between summer, 1971 and fall, 1972. It developed after unusually early and heavy rainfall during the fall of 1972 and continued throughout 1973 and 1974. Results of tests reported here indicate that the effect principally was caused by $Cu^{++}$ in the soil base exchange system and that it can be relieved by treatment of sieved soil residues with NaOCl. Fungistasis appeared to reduce inoculum efficiency; in two growing seasons prior to its onset slopes of inoculum density-infection curves were about 0.7, whereas they were about 0.4 in the two years following the onset of fungistasis.

*Additional key words:* mycostasis, metal toxicity, fungi, dormancy, inoculum density.

A procedure for quantitatively estimating numbers of microsclerotia (MS) of *Verticillium albo-atrum* R. & B. (*V. dahliae* Kleb.) in field soils (3) was developed in 1971. This technique was used to monitor the fate of MS in variously cropped fields in the San Joaquin Valley of California beginning in 1971. The procedure worked well until November 1972, when apparent inoculum densities in eight fields dropped from a range of 1.1 - 43 MS/g soil to a maximum of 0.6 MS/g soil. We report here results of research which indicate development of a fungistasis not recognized earlier, observations on the nature of the fungistasis, and its effect on efficiency of inoculum for disease production. We also relate these findings to reports of inhibition of bacterial growth in soils (17) and the influence of $Al^{+++}$ and $Fe^{+++}$ (6) upon fungal activity in fungistasis tests.

## MATERIALS AND METHODS

All soils were neutral to slightly alkaline loams to clay loams from commercial cotton fields in the San Joaquin Valley of California. Soil samples were collected in a horseshoe pattern. Ten soil cores taken 0-30 cm in depth and 10 steps apart were combined from each 100-meter segment of the sampling pattern. Each bulk sample was assayed in triplicate and the data were expressed as the grand mean of three bulk samples from each field. Assays for MS were by our standard procedure (2) or the sucrose flotation method (9). Media containing pectate (8) was used routinely, but sugar-free Czapek's agar overlaid with cellophane (3) also was used in some tests. In other assays, MS were separated individually with a fine platinum wire from other soil particles that floated on 65% sucrose solution (9).



Inoculum densities in field soils were compared with percentages of infected cotton plants at the end of the growing season, as indicated by xylem discoloration near the crown of plants (2), in 1971, 1972, 1973, and 1974.

Residues of wet-sieved soils, in some tests, were treated while still on the sieve with a 10-second rinse of 0.5% aqueous NaOCl or with 0.01 M $KMnO_4$. A 10-second rinse of residues with these agents reduced fungistasis as effectively as a 20-second rinse, but 25 or more seconds of either of the treatments reduced MS germination. The NaOCl or $KMnO_4$ rinses always were followed by a 25-second rinse with running deionized water. Residues routinely were cultured on 15 plates of pectate agar. Solutions of NaOCl were prepared just prior to use, otherwise the results were quite variable.

The inhibitory effect of untreated soil residues on MS in NaOCl-treated residues was determined in 1:1 (v/v) mixtures made just prior to culturing on 30 pectate agar plates instead of the 15 plates used for routine assays. Only 15 plates were used, however, in dilution end-point tests, in which untreated and treated residues were diluted in deionized water to provide the proportion of untreated to NaOCl-treated residues indicated. These tests in triplicate were repeated three or more times.

Metal concentrations were determined in 500-g soil samples. Duplicate samples were extracted with 500 ml of deionized water, 0.5% aqueous NaOCl, or 0.01 M KOH for 45 minutes, 3 hours, or 24 hours at room temperature. Three hundred milliliters of extract was concentrated to 20 ml with a flash evaporator, then determinations for Cu, Fe, Mn, and Zn were made with a Perkin-Elmer Model 290-B atomic absorption spectrophotometer. Results are expressed as $\mu g/g$ (ppm) of metal in soils at about field capacity. This value was used because concentrations would be unrealistically high, with regard to biological activity, if expressed on a dry weight basis. A precipitate only moderately soluble in strong acids

formed in all concentrates of NaOCl extracts upon standing. Precipitates were collected by centrifugation, ashed, diluted with dilute HCl, and the metal concentrations were determined as described above. The amounts of metals detected in precipitates were added to amounts detected in the clear part of extracts.

Suspensions of MS, obtained from field-grown tomatoes or produced on cellophane-covered Czapek's agar plates were used to determine the inhibitory capacity of metals. Microsclerotia were treated on Nuclepore filter pads in a glass 100-ml Millipore filter apparatus with water or with 0.001 M solutions of metal salts, singly or as mixtures. The metals were added to the filter apparatus in approximately 50 ml of test solution for the desired time and then were washed with 200 ml of deionized water. The MS-bearing pad was transferred to pectate agar (8) plates and percentage germination of MS from three replicates for each treatment was determined after 24 hours at 26 C.

Nuclepore filter pads were used in all tests because, unlike Millipore filter pads, they did not adsorb $Cu^{++}$ ions. This was determined by soaking six pads in a 0.1 M $Cu^{++}$ solution after which they were rinsed with water, dried, then dissolved in chloroform. The solvent was extracted with water in a separatory funnel and the extract was reduced to 10 ml and analyzed by atomic absorption spectrophotometry. Tests for $Cu^{++}$ adsorbed to Nucleopore pads were negative but $Cu^{++}$ adsorption to Millipore filter pads was visually obvious.

## RESULTS

**Procedures examined.**—In routine quantitative assays the apparent inoculum densities from eight sampled fields declined sharply between 5 October and 10 November 1972. Data for four of the fields are in Fig. 1. Assays with an alternative sucrose flotation procedure (9) were compared with those of the standard procedure, using both the original culture medium (3) and a pectate substrate (8). Regardless of assay procedure or substrate, soils collected before 10 November 1972 had relatively high apparent inoculum densities and those collected afterwards had undetectable or very low apparent inoculum densities.

**Germination of microsclerotia isolated individually from soil residues.**—Microsclerotia were isolated with a fine platinum needle from soil residues obtained by the sucrose flotation procedure (9) from two samples collected from one field. One collection was made on 18 October 1972 before assay failure was observed, and the other on 23 January 1973, after assay failure was observed. The efficacy of a low-energy substrate, sugar-free Czapek's - cellophane agar, was compared with a high-energy substrate, potato-dextrose agar (PDA). There were three replications of 50 MS for each soil collection and for each substrate. *Verticillium albo-atrum* colonies that developed from individually cultured MS and associated microorganisms were determined after 10

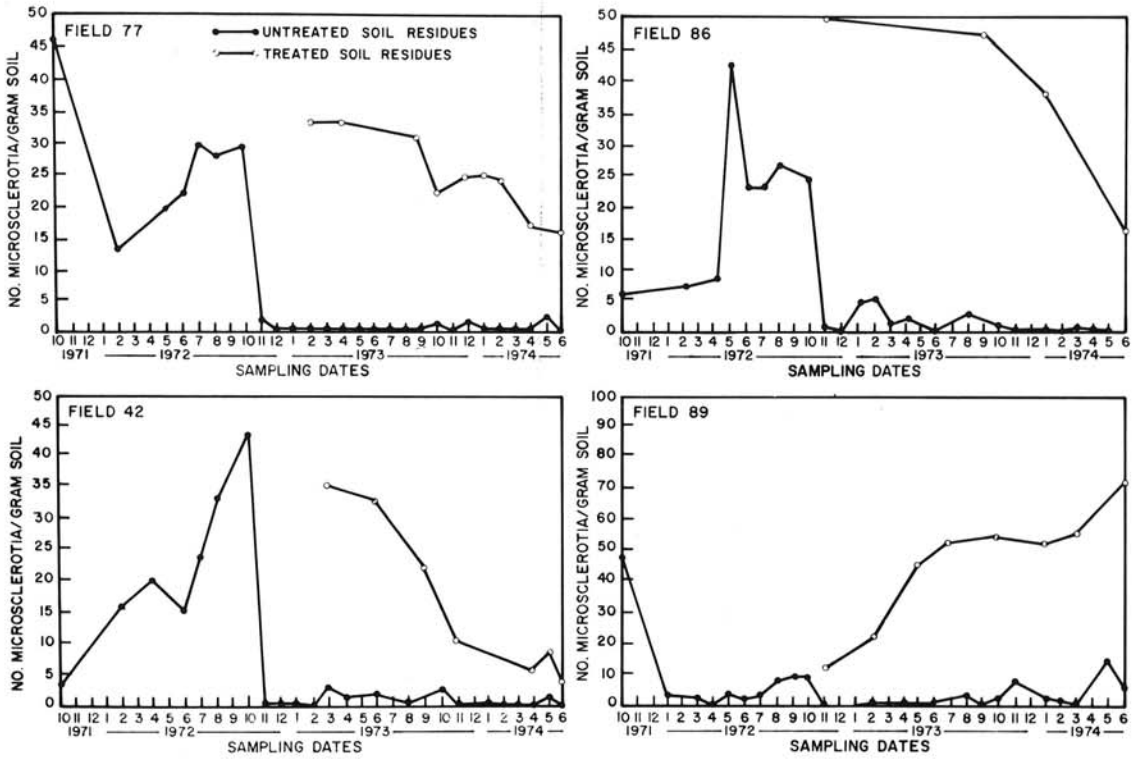


**Fig. 1.** Inoculum densities of *Verticillium albo-atrum* in four commercial fields in the San Joaquin Valley of California. Results are for untreated wet-sieved residues and residues treated with NaOCl or treated first with NaOCl then with KOH. Field 77 was planted with cotton in 1971, with alfalfa in 1972 and 1973, and with cotton in 1974; Field 86 was planted with cotton in 1971 and thereafter with corn; Field 42 was planted with cotton in 1971, with corn in 1972, with sugar beets in 1973, and with alfalfa in 1974; Field 89 was planted continuously with cotton.

days. For the October collection, 47% of MS produced colonies of the fungus on both substrates. Somewhat more MS produced colonies on PDA (45%) than on sugar-free Czapek's - cellophane agar (37%) in the January collection. Most MS (87-91%) appeared to be free of other microorganisms.

These results indicate that MS from residues obtained from soils collected before and after 10 November 1972, were equally germinable. Germination percentages in these tests agree with results of tests reported earlier (9). The data indicate that a change had occurred in soils that resulted in inhibition of germination of most MS in routine assay procedures and that the inhibitory factor(s) was (were) associated with residues and not the MS per se. There was no evidence of parasitism of MS.

**Influence of NaOCl and $KMnO_4$ on apparent Verticillium albo-atrum content of soils.**—NaOCl was used in an initial experiment to determine if treatment of sieved soil residues would improve recovery of *V. albo-atrum* by differential removal of competitors or antagonists. A soil collected in October 1972 had an apparent inoculum density of 27.5 MS/g but in January 1973, the same soil yielded only 0.04 MS/g. When residues from the soil collected in January were rinsed 1, 2, and 3 times with NaOCl, they yielded 22.5, 34.4, and 26.5 MS/g, respectively. $KMnO_4$ was as effective as NaOCl in other tests. The means for three comparisons were 0.6, 7.0, and 8.1 MS/g soil for untreated, NaOCl-treated, and $KMnO_4$-treated residues, respectively. NaOCl, because of convenience, was used in all other experiments.

The influence of untreated-soil residues on growth of MS in NaOCl-treated residues was determined. We found that apparent inoculum densities resulting from assays of 1:1 mixtures of treated and nontreated soil residues usually were similar to those of nontreated-soil residues (Table 1). Most MS in treated portions of mixtures apparently were inhibited by the untreated portions of mixtures.

TABLE 1. The effect of untreated sieved soil residues on germination of *Verticillium albo-atrum* microsclerotia (MS) when mixed with sodium hypochlorite-treated residues

| Soil number | Apparent inoculum density (MS/g air-dry soil) | | |
|---|---|---|---|
| | Soil residue treatments | | |
| | None (A)[a] | Sodium hypochlorite (B)[b] | 1:1 Mixture A and B |
| 1 | 0.2 | 5.9 | 2.1 |
| 2 | 2.0 | 27.3 | 1.5 |
| 3 | 0.4 | 29.4 | 0.6 |
| 4 | 0.1 | 0.5 | 0.2 |
| 5 | 1.0 | 42.5 | 1.8 |
| 6 | 0.2 | 29.3 | 5.3 |
| 7 | 0.5 | 15.4 | 0.6 |
| 8 | 0.3 | 28.4 | 0.2 |
| 9 | 0.4 | 8.0 | 0.6 |
| 10 | 8.6 | 41.6 | 12.8 |
| 11 | 7.4 | 19.4 | 4.4 |
| 12 | ND[c] | 8.1 | 0.7 |

[a]Sieved soil residues washed only with water.
[b]Sieved soil residues rinsed with 0.5% sodium hypochlorite.
[c]*Verticillium albo-atrum* not detected.

In experiments to determine the dilution end-points of the dormancy inducing factor(s) in soils, MS in treated residues of three soils were inhibited from growing when mixed 1:1 (v/v) prior to plating with untreated residues but differences between the soils also were observed at greater dilutions. Residues of one of the soils were inhibitory only in 1:1 mixtures, a second soil residue retained about 60% of initial inhibitory capacity at a dilution of $10^{-6}$, whereas a third soil residue was inhibitory at a $10^{-1}$ dilution (Table 2).

Inhibitory capacity of water supernatants of untreated soil residues following transfer of these residues from sieves to test tubes was determined at 1:5 dilutions. Refraction of light passed through tubes that contained supernatant water indicated that it contained much colloid-sized material. In one test, apparent inoculum densities of nontreated- and NaOCl-treated control residues were, respectively, 0.6 and 5.4 MS/g soil, whereas that of a NaOCl-treated residue transferred to culture plates in a 1:5 dilution of supernatant from an untreated residue was 0.4 MS/g soil. Apparent inoculum densities for the same treatments in a second test were 1.3, 5.6, and 3.3 MS/g soil, respectively.

Several observations suggested that fungistasis of MS was abiotic. In some cases, the factor was active at a very high dilution end-point (Table 2). Other data suggested that fine particles in soils might be involved in fungistasis of MS since even water supernatants of residues, containing abundant fine particles were inhibitory. Furthermore, many MS germinated when mechanically separated from residues. Possible abiotic origin of fungistasis also was suggested by the almost instantaneous effects of NaOCl and $KMnO_4$ treatments, although the possibility remained that the effect of these compounds was biocidal to competitors or antagonists in residues.

**The potential of metals in soils as the cause for fungistasis of microsclerotia.**—The relative effectiveness of the metal chelating agent, disodium EDTA, NaOCl, and monovalent desorbing agents (17) for relief of

TABLE 2. Variation in dilution end-points of inhibitory capacity of untreated soil residues on growth of microsclerotia (MS) of *Verticillium albo-atrum* evidenced by changes in apparent inoculum densities of NaOCl-treated residues blended with various dilutions of untreated residues

| Sieved soil residues | | Apparent inoculum densities of soils | | |
|---|---|---|---|---|
| Treatments | Dilution | Soil-1 (MS/g) | Soil-2 (MS/g) | Soil-3 (MS/g) |
| Untreated | ... | 0.2 | 3.8 | 24.1 |
| NaOCl-treated | ... | 16.8 | 21.2 | 39.8 |
| NaOCl-treated plus untreated | 1:1 | 3.2 | 1.6 | 20.3 |
| | $10^{-1}$ | 3.1 | 7.3 | ... |
| | $10^{-2}$ | 2.2 | ... | ... |
| | $10^{-3}$ | 2.6 | 20.5 | 34.8 |
| | $10^{-4}$ | 8.8 | ... | ... |
| | $10^{-5}$ | 8.3 | ... | ... |
| | $10^{-6}$ | 10.9 | 20.2 | 34.9 |
| LSD ($P = 0.05$) | | 3.1 | 4.9 | 7.0 |

fungistasis of MS was determined. Soil residues were treated 10 seconds with the substances, in 0.01 N solutions, then rinsed with water before culturing. Treatments with EDTA and several monovalent cations were as effective as NaOCl for two of three soils, but were less effective in a third soil (Table 3). Other comparisons between NaOCl, KOH, and NaCl indicate that soil-3 (Table 3) was more typical for San Joaquin Valley soils than soils-1 or -2 during 1974. However, EDTA, and ions involved in exchange reactions in soils can effectively relieve fungistasis of MS in cultures of wet-sieved residues from some soils (Table 3).

**Metals in soils.**—The comparative effectiveness of water, 0.01 M KOH, and 0.5% NaOCl for extraction of the four most common metals in basic soils (Fe, Cu, Mn, and Zn) was determined. A 45-minute extraction period was as effective as 3- or 24-hour extraction periods. Therefore, data (Table 4) are means of five tests. More Fe consistently was removed by water or KOH than by NaOCl (Table 4). But, Cu, Mn, and Zn were most effectively removed by NaOCl. Significant additional amounts of all four metals were extracted by KOH treatments of residues first extracted by NaOCl. More Cu always was extracted with NaOCl than with other solvents. The amounts of the metals in nine other soils collected in the San Joaquin Valley were determined by NaOCl extraction (Table 5).

**Inhibition of microsclerotial germination by metals.**—Copper was the only metal that inhibited germination of laboratory-produced MS at the concentrations extractable from soils (Tables 4, 5) with solutions that relieved fungistasis (Table 3). In fact, Mn, as $MnCl_2$, Zn, as $ZnSO_4$, and Fe as $FeCl_3$ or $FeSO_4$, were not inhibitory at concentrations up to 250 $\mu$g/ml (ppm) metal ion. Mixtures of Cu with other metals, in the concentrations observed in soils, were less effective than Cu alone, which agreed with the literature reports on ion antagonism (13). Our attention, therefore, was drawn to Cu. A 1-minute treatment of MS, whether with 3 or 10 $\mu$g/ml Cu-acetate was ineffective (Table 6). But germination was significantly reduced after 5 minutes of treatment with $\geqslant 5$ $\mu$g/ml Cu. In no case, however, was germination completely inhibited by Cu on pure culture agar plates.

Microsclerotia from tomato stems were more sensitive to Cu than were laboratory-produced MS. Treatment time was for 5 minutes. Germination after 24 hours when treated for 5 minutes with 0, 5, 10, and 15 $\mu$g/ml Cu was, respectively, 42, 8, 5, and 6% for native MS and 71, 43, 29, and 21% for laboratory-produced MS.

**Fungistasis of laboratory-produced microsclerotia and relief of copper-induced fungistasis of microsclerotia in soil residues by NaOCl.**—Microsclerotia in NaOCl-treated residues usually failed to germinate and produce colonies when mixed 1:1 with untreated soil residues (Table 1). Similarly, laboratory-produced MS also failed to grow when added to untreated residues but grew when added to NaOCl-treated residues (Table 7). Untreated MS grew whether residue-MS mixtures were treated with NaOCl before or after mixing. On the other hand, MS did not grow when treated for 5 minutes with 5 $\mu$g/ml Cu, then rinsed with water, before being added to either untreated or NaOCl-treated residues. The addition of Cu-treated MS also prevented germination of the MS in the NaOCl-treated residues (Table 7) which mimicked the effect of adding untreated residues to treated residues (Table 1). The latter result, which was observed in four such tests, suggested that competition from other microorganisms prevented the slower germinating Cu-treated MS in mixed culture from germinating (Table 7) whereas many did germinate and grow in pure culture tests (Table 6). Microsclerotia also grew when mechanically separated from soil residues as discussed earlier in this report. Both the fungistatic effect of soil residues and that caused by copper treatment of added MS were relieved by NaOCl treatment of residue-MS mixtures before culturing (Table 7, last entry).

**The influence of fungistasis on inoculum efficiency in the field.**—The log-log relationship between numbers of MS in soils and percentage of cotton plants infected with *V. albo-atrum* at harvest in 1971 (2) was verified in 1972. The slopes of inoculum-infection curves for both years were similar, about 0.7 (Fig. 2). Slopes of the curves for 1973 and 1974, following onset of fungistasis, were similar

TABLE 3. Comparative effectiveness of 0.01 N solutions of NaOCl and common salts, bases, and disodium EDTA for relieving fungistasis of microsclerotia (MS) of *Verticillium albo-atrum* in field soils

| Residue treatments | Apparent inoculum densities | | |
|---|---|---|---|
| | Soil-1 (MS/g) | Soil-2 (MS/g) | Soil-3 (MS/g) |
| None | 2.5 | 0.7 | 0.7 |
| EDTA | 7.4 | 4.0 | 0.3 |
| NaOCl | 6.9 | 5.1 | 4.7 |
| NaCl | 5.2 | 3.0 | 1.3 |
| NaOH | 1.6 | 3.4 | 0.8 |
| NaBr | 5.1 | 1.7 | 1.3 |
| KOH | 3.7 | 4.8 | 0.9 |
| $KNO_3$ | 4.0 | 6.0 | 1.0 |
| LiCl | 4.2 | 8.4 | 1.1 |

TABLE 4. Amounts of metals extracted from a San Joaquin Valley soil with water, NaOCl alone, and NaOCl extraction followed by KOH extraction

| Extracting agent | Amounts of metals in soil ($\mu$g/g)[a] | | | |
|---|---|---|---|---|
| | Cu | Fe | Mn | Zn |
| Deionized water | 0.2 ± 0.2 | 43.4 ± 33 | 0.8 ± 0.3 | 0.5 ± 0.4 |
| KOH | 0.5 ± 0.4 | 69.3 ± 35 | 1.0 ± 0.6 | 0.6 ± 0.4 |
| NaOCl | 3.7 ± 1.2 | 17.8 ± 2.0 | 4.8 ± 1.9 | 1.4 ± 0.6 |
| NaOCl-KOH[b] | 1.9 ± 1.1 | 26.5 ± 2.0 | 1.6 ± 1.6 | 0.5 ± 0.1 |

[a]Mean values based on five tests; two for 45 minutes, two for 3 hours, and one for 24 hours.
[b]Values show amounts extracted by KOH after extraction with NaOCl.

to each other but they were lower than in 1971 and 1972 (Fig. 2). Correlation coefficients favored a log-log relationship between inoculum density and infection for 1971, 1972, 1973, and 1974 for which they were, respectively, 0.941, 0.658, 0.896, and 0.734. The data suggested that inoculum efficiency was reduced in 1973 and 1974, probably because of fungistasis, since temperature conditions favored infection for a month or more before harvest in all 4 years. This conclusion also is supported by observations that as little as 10% infection occurred where 10 or more MS/g of soil were indicated by assays during 1973 and 1974. This amount indicated by assays in 1971 and 1972 was more than enough to induce essentially 100% infection (Fig. 2).

TABLE 5. Amounts of metals in San Joaquin Valley soils extracted with NaOCl

| Field identification | Amounts of metals in soils (μg/g) | | | |
|---|---|---|---|---|
| | Cu | Fe | Mn | Zn |
| 88 | 4.0 | 18.4 | 1.7 | 1.1 |
| 89 | 3.4 | 55.0 | 2.9 | 1.4 |
| 42 | 4.6 | 88.0 | 2.0 | 1.3 |
| 42-a | 4.4 | 13.6 | 1.1 | 2.5 |
| 77 | 2.8 | 17.6 | 3.0 | 1.5 |
| 79 | 6.0 | 30.4 | 4.2 | 6.8 |
| 86 | 6.2 | 22.0 | 1.0 | 1.6 |
| KHFS | 5.4 | 16.5 | 5.7 | 1.9 |
| WSFS | 4.2 | 6.4 | 0.3 | 1.3 |

TABLE 6. The influence of short-term treatments of microsclerotia (MS) of *Verticillium albo-atrum* with copper of copper acetate, on germination after 24 hours in pure culture tests

| Treatment time, minutes | Germination of copper-treated MS after 24 hours (% of control)[a] | | | |
|---|---|---|---|---|
| | 3 μg/ml | 5 μg/ml | 7 μg/ml | 10 μg/ml |
| 1 | 93 | 93 | 82 | 85 |
| 5 | 87 | 72 | 70 | 51 |
| 10 | 77 | 56 | 54 | 33 |
| 15 | 69 | 50 | 37 | 21 |

[a]Mean values for four tests with laboratory-produced MS; water-treated MS had 90-96% germination after 24 hours.

**Real and apparent inoculum densities.**—We routinely collect soils from fields, return them to the laboratory in ice chests, air-dry them for 48 hours at 21-24 C, and assay them immediately. At times, however, soils may be stored up to 1 week at 4 C before being assayed. Assay results, nevertheless, have been similar whether samples were assayed after 48 hours drying or were stored an additional week at 4 C after drying (1). This, however, was not true for soils stored for longer periods of time. The following data illustrate this point.

Three soils collected during the summer of 1972 were air-dried, broken up with a soil shredder, and stored in boxes at room temperature. Soil fungistasis at that time was not recognized. Triplicate samples of each soil were assayed six times over a period of 100 days. Little difference in apparent inoculum densities of water-washed sieved residues were observed over a period of 1 month. Thereafter, however, apparent inoculum densities declined sharply within 60 days and the fungus was not detectable after 100 days (Fig. 3). One year later, following discovery of the effect of NaOCl treatment, the soils were reassayed. Apparent inoculum densities of soils-1, -2, and -3 were, respectively, 0.7, 1.3, and 1.5 MS/g soil when residues were washed only with water, and were 20.1, 17.6, and 10.3 MS/g soil when residues received NaOCl treatment prior to culturing. These values are in general agreement with those at zero time (Fig. 3).

Studies on the long-term fate of MS in eight variously cropped fields were begun in 1971 (10). Soils from the fields were assayed at about 1-month intervals through the summer of 1974 and residues of most samples were stored at 4 C following assay. When the effect of NaOCl treatment on fungistasis was recognized, monthly collections thereafter were assayed both with and without NaOCl treatment. During the past year, there was excellent agreement between month-to-month assay results, using NaOCl. This is illustrated by the results from three fields planted with cotton in 1971 in which inoculum densities declined during 3 years of immune crops and from one field in which inoculum densities increased during 3 years of cotton production. The upper curves (Fig. 1) are considered to represent real inoculum densities; the lower curves represent apparent inoculum densities which are low because of fungistasis.

TABLE 7. Germination of background microsclerotia (MS) and of untreated and copper-treated MS added to untreated and sodium hypochlorite (NaOCl)-treated wet-sieved soil residues, without and with NaOCl treatment following addition of MS

| Soil residue and microsclerotia treatments | | | Apparent inoculum density (MS/g soil) |
|---|---|---|---|
| Treatment of residues prior to addition of microsclerotia | Treatment of added microsclerotia[a] | Final NaOCl treatment | |
| None | No MS added | No | 0.1 |
| NaOCl | No MS added | Yes | 3.0 |
| None | Untreated MS | No | 0.3 |
| NaOCl | Untreated MS | No | 6.9 |
| None | Untreated MS | Yes | 6.1 |
| None | Cu-treated MS | No | 0.3 |
| NaOCl | Cu-treated MS | No | 0.5 |
| None | Cu-treated MS | Yes | 5.5 |

[a]Microsclerotia produced on Czapek's agar covered with cellophane were added at rates of about three or four MS per gram soil.

Portions of the upper curves (Fig. 1) were developed from soil samples stored two or more years at 4 C. These samples responded variously to NaOCl treatment. That is, some results agreed with both pre- and post-fungistasis data whereas NaOCl treatment was only moderately effective on some samples. Extended treatment times with NaOCl were precluded due to its fungitoxicity. We found, however, that a 10-second NaOCl treatment followed by further desorption with 0.001 M KOH for 0.25-3 hours, depending upon particular samples, dependably relieved fungistasis. This procedure, although laborious, allowed completion of studies on long-term fate of MS in response to cropping sequences (Fig. 1) and (10).

The lower curves (Fig. 1), at least in part, may be artifacts in that the number of germinable MS may be decreased when MS and Cu are concentrated during sieving. Data for two soils illustrate this point. The amount of Cu in duplicate 500-g soil samples and sieved residues of 500-g samples was determined. One soil had 5.4 mg/kg (ppm) Cu and sieved residues of other samples had 8.1 mg/kg Cu. Another soil had 6.2 mg/kg Cu and sieved residues had 11.8 mg/kg Cu.

## DISCUSSION

The observations we report here for *V. albo-atrum* are similar to observations made on soil bacteria in New York soils about 40 years ago by Peele (17). He found that two species of soil bacteria were differentially sorbed with soil particles both larger and smaller in size than bacterial

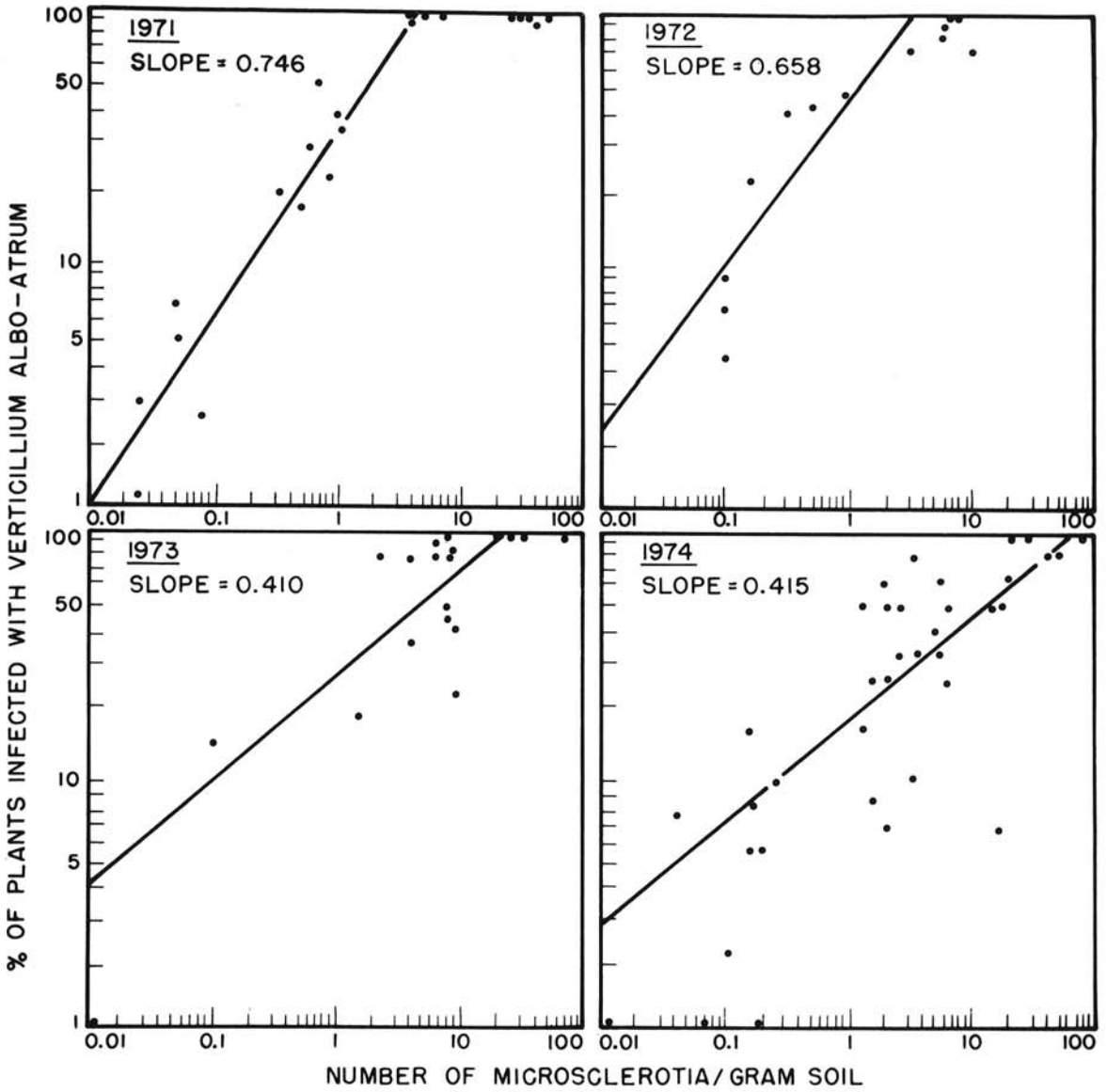


**Fig. 2.** Inoculum densities of *Verticillium albo-atrum* and amounts of infection of cotton by the fungus in commercial plantings of the San Joaquin Valley of California over a period of 4 years. In 1971 and 1972, residues were not treated with NaOCl; it was used in 1973 and 1974.

cells. Sorption could be complete in soils with exchange systems saturated with $Al^{+++}$ or $Fe^{+++}$; that is, the bacterial cells could not be washed from soils with water. Furthermore, respiration in such soils was nil. In contrast, soils saturated with $NH_4^+$ or $Na^+$ had respiration rates similar to comparable numbers of bacteria in parallel pure cultures. Likewise, bacteria were not sorbed with suspensions of soils saturated with $NH_4^+$ or $Na^+$ or other monovalent cations. Lastly, he showed that bacteria sorbed by $Fe^{+++}$- or $Al^{+++}$-saturated soils could be washed from soils with solutions of monovalent cations, including $Li^+$, $Na^+$, $K^+$, and $NH_4^+$. Peele suggested that sorption of legume nodule bacteria with soil might explain their lack of spread in soil when added as inoculum, and that spread of bacteria in soil might be altered by changing the predominating base in the soil exchange complex.

Peele's results (17) appear to be confirmatory but more definitive than earlier reports. Cutler (5) concluded that protozoa cells were sorbed in soil particles since they settled with soil when soil was added to shaken water suspensions of the cells. Somewhat similar results by several Russian workers also were summarized by Peele (17). They observed that different soil types differentially sorbed bacterial cells; life processes of some species were decreased but others were increased in the sorbed state; and dead bacterial cells were sorbed in the same way as live cells, but Marshall (14) reported that dead cells were not always sorbed as were live cells.

The principles of sorption of cells with soil particles were recently summarized by Marshall (14). It occurs between oppositely charged sites of cells and particles, mostly of colloidal size. Marshall suggested that most microorganisms are sorbed in soil, but there are important intra- and inter-generic exceptions to this. Peele (17) showed that 96% of cells of *Bacillus ceireus* var. *mycoides*, 41% of cells of *B. mesentericus*, and 12% of *Escherichia coli* cells were sorbed into the same soil.

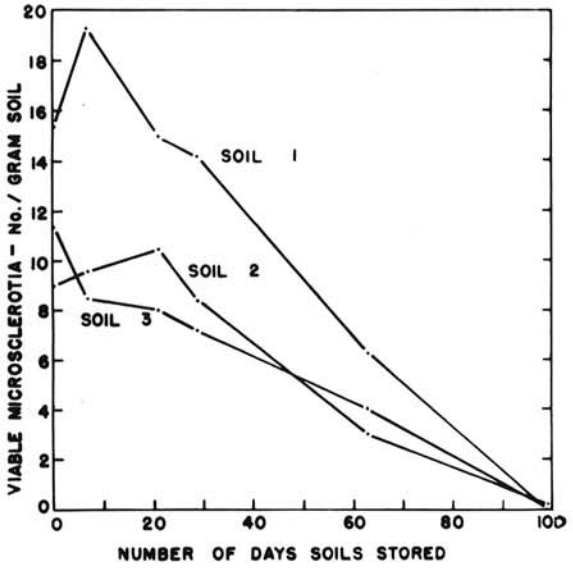


**Fig. 3.** Apparent attrition rates without NaOCl treatment for microsclerotia of *Verticillium albo-atrum* in three soils stored air dry at room temperature. After 1 year, NaOCl treatment of sieved residues restored apparent microsclerotia numbers to about original levels.

Very little apparently is known about the influence of the sorption phenomenon upon the activities of microorganisms other than bacteria, although Zvyagintsev (19, 20) observed, using fluorescent microscopy, sorption between fungi and actinomycetes and soil particles. He primarily was interested in methods for dispersion of organisms in soil suspensions to increase accuracy of assays. He and others (14) reported that desorption of microorganisms held in aggregates of soil particles greatly influence apparent populations based upon colony counts.

The increased or decreased growth rates of bacteria attributable to sorption were explored in recent literature. Some bacteria benefit nutritionally since soil colloids, due to their great exchange capacity (14), concentrate, from solution, substances at their surfaces. *Escherichia coli* grew in a dilute medium containing glass beads on the surfaces of which nutrients were concentrated, but failed to grow in the medium without beads (7). Similarly Conn and Conn (4) reported stimulation of growth of certain bacteria in the presence of montmorillonite clay. Contrary to this, however, are observations that clay envelopes sorbed around cells may reduce or prevent diffusion of nutrients and water into cells (14). Likewise, the catalytic activity of extracellular enzymes are decreased by sorption, as discussed by Skujins (18) and by McLaren and Petersen (15).

Inhibition of growth of bacteria due to sorption between cells and soil particles bound with $Fe^{+++}$ and $Al^{+++}$ (17) appears to be similar to the stasis we observed. Peele (17) did not, however, determine whether lack of growth was directly attributable to $Fe^{+++}$, $Al^{+++}$ or other cations. Dobbs and Gash (6) reported on a similar phenomenon which they called residual mycostasis; e.g., one not relieved by autoclaving, by leaching with water, or by addition of glucose. The stasis was eliminated by leaching soil first with dilute HCl then neutralizing it by washing well with water. The HCl leachate contained $5 \times 10^3$ to $1.3 \times 10^4$ $\mu$g/ml Fe. Solutions of inorganic Fe compounds had a very low pH, when used in these amounts, so they tested unbuffered (pH 5-5.9) and buffered (pH 8.5) Fe-EDTA for inhibition of germination of spore of *Mucor ramannianus*. Unbuffered Fe-EDTA induced slight inhibition at $5 \times 10^3$ $\mu$g/ml and about 50% inhibition at $1.5 \times 10^4$ $\mu$g/ml. At pH 8.5, where Fe is liberated from EDTA, germination again was reduced only slightly at $5 \times 10^3$ $\mu$g/ml, about 60% at $7.5 \times 10^3$ $\mu$g/ml, and was nil at $1.5 \times 10^4$ $\mu$g/ml. They did not determine whether affected spores recovered from the stasis if retreated with Na-EDTA or other desorbing agents. Ko and Hora (12) made similar observations with $Al^{+++}$ in Hawaii, where considerable amounts of soluble Al occurs in acid soils. Neither soil extracts nor $Al^{+++}$ were inhibitory to spores of *Neurospora tetrasperma* at pH 7.0, but both the extract and $Al^{+++}$ at 0.65 $\mu$g/ml (about the same amount as in the extract) completely inhibited spores at pH 4.8. They concluded that $Al^{+++}$ was fungicidal, not fungistatic, since spores did not recover when washed with water. We tested Fe-EDTA (0.1 M solution) for inhibition of germination of *V. albo-atrum* MS in tests like those reported above for Cu. Fe-EDTA completely inhibited germination of MS, but inhibition was relieved by brief treatment with a

desorbing agent, NaCl in this case. It is not known whether $Al^{+++}$ would be desorbed in the same manner.

Results of observations reported here indicate that metal ions in soils are important fungistatic agents. In California soils, copper appears to be important in the ecology of *V. albo-atrum,* decreasing the efficiency of its inoculum, but also probably contributing to its survival. It appears, however, to be present in sufficient quantities in some soils, such as in a peat swamp, to limit the ranges of fungal species (11). Other toxic ions than those discussed here may be important depending on native underlying rock structures which are the source of exchangeable mineral substances in soils (16). But, as pointed out above, some microorganisms may be favored while others are inhibited by fungistasis due to sorption with an inhibitory substance.

The fungistasis described here occurred after early and unusually heavy fall rains in 1972. We have not, however, experimentally reproduced this situation. Neither do we know the precise mechanism of action of Cu. We can postulate, however, at least three modes of binding: (i) MS-Cu-inorganic soil particles, (ii) MS-Cu-organic particle-inorganic particle, or (iii) coordination complexes such as -Cu-MS-Cu. NaOCl, because it is both a desorbing agent and a strong oxidizing agent, has the potential of breaking all of the bonds described above.

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