Activity of Fungistatic Compounds from Soil

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ABSTRACT

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Propagules of many fungi do not germinate in soil unless provided with nutrients. According to the nutrient deprivation hypothesis, soil microflora prevent germination by acting as a sink for nutrients that leak from fungal propagules. Alternatively, soil microflora may produce germination inhibitors. In this study, fungistatic activity diffused from soil into agarose blocks and was quantified by percentage of germination of *Cochliobolus victoriae* conidia incubated on the blocks. The blocks were kept sterile and separate from soil by placement either on polycarbonate membranes with 0.2-μm pores (diffusion assay) or on glass (volatile assay). Conidia on membranes also were exposed to soil without agarose (direct assay). Four test soils were fungistatic and gave similar results in all assays. Agarose blocks became more fungistatic with increasing time on soil. Thin (2.5 mm) blocks became fungistatic more quickly than thick (7.5 mm) blocks, and blocks became fungistatic more quickly in the diffusion assay than in the volatile assay. Activity persisted in the blocks after removal from soil, and persisted longer when blocks were incubated in still rather than moving air. The results of this study do not support the nutrient deprivation hypothesis. Furthermore, the results indicate the occurrence of water-soluble, volatile, and possibly nonvolatile, fungistatic compound(s) in a variety of soils.

Additional keywords: *Bipolaris*, *Helminthosporium*, mycostasis.

Soil inhibits germination of many fungal propagules (17,35). The phenomenon, termed "fungistasis" (6), occurs in many soils (31,35). In most soils, fungistasis is a result of microbial activity; it is annulled by sterilization (17) and re-established by any of a variety of bacteria (10). Fungistasis is alleviated by addition of nutrients, especially organic carbon (1,6) or root exudates (18,34). Sensitivity to soil fungistasis may increase the long-term survival of fungal propagules in soil (6). For example, fungistasis-sensitive isolates of *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur persist in soil longer than fungistasis-insensitive isolates (3).

Elucidation of the mechanism of soil fungistasis has implications for soil microbiology generally and for control of soilborne plant pathogenic fungi specifically. Two mechanisms of fungistasis have been proposed and debated (24,25,28,40). The nutrient deprivation hypothesis (24) posits that germination is regulated by loss of nutrients from the fungal propagules to a microbial sink in soil. However, nutrient loss from conidia is not always correlated with suppression of germination (8,9). Alternatively, the existence of compounds that inhibit germination has been proposed with considerable supporting evidence (12,30,31,37), especially in alkaline soils (13–16,20). However, the compounds either were not identified conclusively or were not found in sufficient concentrations to account for fungistasis in most soils (14,28,38).

Volatile compounds from soil inhibited germination of *Trichoderma viride*, *Zygorhynchus* sp., *Penicillium chrysogenum*, and *Aspergillus flavus* conidia (13). The activity was greater in alkaline than in acid soils. Germination of *C. sativus* conidia was inhibited by volatile compounds in soils from pH 3.8 to 8.8 (31). Compounds diffused from soil through either a water film or a gas space into agar blocks. However, the results were quite variable and depended on soil type, soil pH, and duration of time during which the soils were incubated between wetting the soil and adding the conidia for the bioassay. Furthermore, suppression of germination in the fungistatic treatments was not complete; germination typically was 20–40% of the controls. Finally, no data were presented on viability of the conidia subjected to fungistatic conditions. Thus, whether the conidia were dead, nongerminating, or simply asynchronous in germination rate is impossible to determine.

Our objective in this study was to test the two hypotheses for soil fungistasis: germination inhibitors and nutrient deprivation. The test conditions allowed distinguishing between death, dormancy, and asynchronous germination rates of the fungal propagules.

MATERIALS AND METHODS

Soils. A Panoche clay loam (Table 1) was used in all assays. Where indicated, other soils were also used. The soils differed in classification, pH, organic carbon content, cation exchange capacity, vegetation history, and geographic origin. Before use, soil was sieved through a 0.5-mm-pore screen, air-dried, and stored at approximately 20 C in the dark for up to 2 yr.

Fungal cultures. Spores of four species were chosen for their differing nutrient requirements for germination and differing sensitivities to soil fungistasis (2,17,23). Conidia of both *Cochliobolus victoriae* R. R. Nelson (anamorph *Bipolaris victoriae* (F. Meehan & Murphy) Shoemaker, = *Helminthosporium victoriae* F. Meehan & Murphy) and *C. sativus* are nutrient-independent for germination (i.e., in an axenic environment they germinate without exogenous nutrients). Conidia of both species also are sensitive to soil fungistasis. Activated ascospores of *Neurospora tetrasperma* Shear & Dodge are nutrient-independent for germination and insensitive to fungistasis. *Verticillium dahliae* Kleb. conidia are nutrient-dependent for germination.

Cultures of *C. victoriae*, *C. sativus*, and *V. dahliae* were stored as suspensions of conidia and hyphae in an aqueous solution of 25% (w/w) glycerol at −80 C. *N. tetrasperma* ascospores were stored in water at 4 C. *N. tetrasperma* was obtained originally from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City). The culture is a mixture of strains 1270 and 1271, which are opposite mating types and form mature perithecia when co-cultured.

C. victoriae, *C. sativus*, and *V. dahliae* were cultured on V8 agar containing 200 ml of V8 juice (Campbell Soup Co., Camden, NJ), 4.0 g of $CaCO_3$, 17.5 g of agar, and water to a total volume of 1 L. *N. tetrasperma* was cultured on N medium (39). All cultures

were grown at 20 C, with 12 h of fluorescent light-12 h of darkness. Conidia of *C. victoriae*, *C. sativus*, and *V. dahliae* were harvested after 10 days; *N. tetrasperma* ascospores were harvested 10 days after the appearance of perithecia.

Spore preparation. To harvest spores, culture plates were flooded with an aqueous solution of 0.1 M 2-(4-morpholino)-ethane sulfonic acid, pH 6.1, and 0.05% Tween 20 (MEST). Harvested spores were washed three times by centrifugation at 1,610 *g* in 50 ml of MEST at 4 C. Washed spores were resuspended in approximately 5 ml of MEST, and spore density was adjusted to 4×10^4 conidia ml^{-1}. Ascospores of *N. tetrasperma* were diluted to 10^4 spores ml^{-1}, and then activated by incubation at 60 C for 30 min (29).

Spores were vacuum-filtered onto polycarbonate membranes (Nuclepore Corporation, Pleasanton, CA) at a density of 10^3 conidia cm^{-2}. Membranes were 0.8 × 0.8 cm, with 0.2-μm pores. Water used in all experiments was deionized (Nanopure II, Barnstead, Dubuque, IA).

Fungistasis assays. Assays were performed in petri dishes (15 × 100 mm) that contained 20 g of soil (air-dry weight) wetted to approximately −5 kPa Ψ (8–12 g of water, depending on the soil type). After the soil surface was smoothed, the soil was incubated in a moist chamber at 20 C. Soil was wetted 5–7 days before the start of each assay.

Fungistatic activity was measured using three assays: direct, diffusion, and volatile (Fig. 1), which were modifications of those used by Romine and Baker (31). In all three assays, the soil surface was covered with a large (2 × 2 cm) polycarbonate membrane. In the direct assay, spores on small (0.8 × 0.8 cm) polycarbonate membranes were placed on the larger membranes. For the diffusion assay, an agarose block (1% w/w; ultraPure electrophoresis grade, BRL, Gaithersburg, MD) was placed on the larger membrane. Unless otherwise indicated, blocks were 0.25 high × 1 × 1 cm. Spores on a small membrane were laid on top of the agarose block. The volatile assay was identical to the diffusion assay except that a glass cover slip was placed between the agarose block and the soil, so that all contact between the agarose and the soil occurred via the gas space in the petri dish. Controls without soil were in all other respects identical to treatments.

In most experiments, agarose blocks were incubated on soil for 24 h before the start of the bioassay. Then spores were added and dishes were incubated in moist chambers in the dark. Incubations were at 20 C unless indicated otherwise. Germination time was 2–4 h for *C. victoriae*, 6 h for *C. sativus*, 12 h for *N. tetrasperma*, and 15 h for *V. dahliae*. *V. dahliae* conidia on membranes were difficult to see under the microscope; therefore, these conidia were deposited directly onto the agarose blocks in the diffusion and volatile assays, and the direct assay was not done. In most experiments, only *C. victoriae* was used.

In all experiments, sterility of membranes and blocks was checked periodically. Membranes and blocks were transferred to V8 or nutrient (32) agar, incubated at 20 C, and examined for fungal and bacterial growth. Whether work was done on a clean laboratory bench or in a transfer hood, >75% of the blocks remained sterile in each experiment. When blocks were nonsterile, colonies of contaminating organisms were detected only after incubating for several days on media, suggesting that few contaminants were present. The data from contaminated and noncontaminated replicates did not differ (data not shown).

After incubation in each of the three assays, membranes with spores were fixed and stained in an aqueous solution of 10% (w/w) lactic acid, 0.05% (w/w) Cotton Blue, and 25% (w/w) glycerol. Membranes were mounted in glycerol (80% [w/w] in water), and spores were examined at ×200 on a Zeiss light microscope with epi-illumination provided by a Series 180 high-intensity illuminator and fiber optic light ring (Dolan-Jenner Industries, Inc., Woburn, MA). Spores with germ tubes longer than one-half the width of the spore were considered germinated. For each replication, at least 100 spores were counted.

Experiments were done as completely randomized designs and analyzed with an analysis of variance (ANOVA). All experiments were repeated at least three times. When data from several independent trials were pooled, results were analyzed as a randomized complete block design, with trial dates as blocks. Values reported are mean ± one standard error of the mean (SEM).

Effect of washing *C. victoriae* conidia on sensitivity to soil fungistasis. Conidia were collected with a sterile brush from a culture plate and deposited dry onto a membrane. Wet- and dry-harvested conidia were compared for germination on nonfungistatic agarose blocks and for sensitivity to fungistasis in the direct, diffusion, and volatile bioassays.

Fungistatic activity of four soils. The fungistatic activity of four soils (Table 1) was estimated using the germination of *C. victoriae* conidia in the three bioassays (Fig. 1). Nonsterile and autoclaved soil was used. Assay materials (large membranes, agarose blocks, and glass cover slips) were incubated on soil for 24 h. Then conidia were applied, incubated 4 h, and examined microscopically to assess the percentage of germination. To determine if nongerminated conidia on fungistatic soil were still viable, a subsample of conidia was transferred from soil to agarose blocks that were not previously exposed to soil and incubated an additional 4 h before assessing the percentage of germination.

To determine the effect of soil type on rates at which agarose blocks become fungistatic, agarose blocks were exposed to each of the four soils. For the diffusion and volatile bioassays, blocks were exposed to soil for intervals from 0 to 6 h and 0 to 24 h, respectively, before adding the conidia. *C. victoriae* conidia were added to the blocks, incubated for 3 h, and then assessed for

TABLE 1. Soil characteristics

Soil series	Soil classification	pH[y]	Organic C[z] (%)	CEC[z]	Vegetation	Location
Boyer sandy loam	Typic Haploudalf	6.2	1.0	4.0	Corn-fallow rotation	Rose Lake Wildlife Area, Shiawassee County, MI
Holdrege silt loam	Typic Argiustoll	6.3	2.0	15.0	Mixed grass prairie	Loup City, Sherman County, NE
Holland sandy loam	Ultic Haploxeralf	5.7	5.1	14.8	Mixed conifer forest	University of California Blodgett Forest Research Station, El Dorado, CA
Panoche clay loam	Typic Torriorthent	7.7	0.5	23.2	Cotton	University of California West Side Field Station, Five Points, CA

[y] pH values are means of duplicate samples. Soil and water were mixed as a 1:2.5 (w/v) slurry, stirred for 10 min and then measured with a pH electrode.

[z] Organic carbon (C) and cation exchange capacity (CEC) data were obtained from the following sources: Boyer (11); Holdrege (R. Amundson, *personal communication*); Holland, Organic C (27) and CEC (P. Zinke, *personal communication*); and Panoche (7).

percentage of germination. As a control, agarose blocks were incubated without soil for intervals from 0 to 24 h before adding conidia.

Effect of temperature on fungistasis. To determine the effect of temperature on rates at which agarose blocks become fungistatic, agarose blocks were incubated on Panoche clay loam at 18, 23, or 28 C. For the diffusion and volatile assays, blocks were exposed to soil for intervals from 0 to 24 h and 0 to 48 h, respectively. Then *C. victoriae* conidia were added to blocks, incubated at the indicated temperature for 3 h, and assessed for percentage of germination. As a control, blocks were incubated without soil for intervals from 0 to 48 h.

To distinguish the effect of temperature on fungistatic activity of soil from an effect on the rate of conidial germination, the germination rate of conidia was measured at the three temperatures. *C. victoriae* conidia were incubated on agarose blocks on plastic at 18, 23, and 28 C. After intervals from 0 to 6 h, the percentage of germination was assessed.

Effect of agarose block thickness on the diffusion assay. To determine the effect of agarose block thickness on the rate at which the blocks become fungistatic, blocks 2.5–7.5 mm thick were exposed to Panoche clay loam. Blocks were incubated for intervals from 0 to 8 h on either soil or plastic. Conidia of *C. victoriae* were added to the blocks, incubated for 3 h, and then assessed for percentage of germination.

Persistence of fungistatic activity in agarose blocks. Because agarose blocks became fungistatic when exposed to soil, we determined if aeration of the blocks caused a loss of the fungistatic activity. Blocks were made fungistatic by exposure to soil for 24 h as in the diffusion assay and then were removed from soil and assayed for fungistatic activity. The experiment was done in two ways. In the first procedure, blocks were incubated on soil and then transferred to sterile plastic. Conidia were added, incubated for intervals from 0 to 24 h, and assessed for germination. As controls, blocks were transferred from soil to fresh soil or from plastic to plastic. Work was done on a clean laboratory bench protected from drafts. In the second procedure, blocks were incubated on soil, transferred to sterile plastic, and incubated for an additional 0–24 h. Then conidia were added, incubated for 3 h, and percentage of germination was assessed. As above, control blocks were transferred from plastic to plastic or from soil to soil. Because of the need to maintain sterility during the long incubations, all work was done in a laminar flow transfer hood.

In another aeration experiment, blocks were made fungistatic by exposure to Panoche clay loam for 24 h as in the diffusion and volatile assays. Blocks were then removed from the substratum and exposed for 5 min to sterile air moving at 21 m min^{-1} in a laminar flow transfer hood. Conidia of *C. victoriae* were added to the blocks, incubated for 2 h, and then assessed for percentage of germination. As controls, blocks were transferred from plastic to plastic, or from soil to soil.

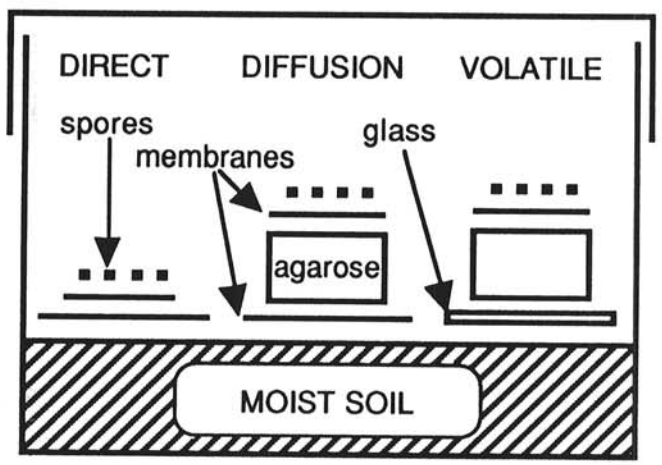

Fig. 1. Schematic drawing of the direct, diffusion, and volatile bioassays for soil fungistasis (not drawn to scale). Polycarbonate membranes have 0.2-μm pores.

RESULTS

Germination of four fungi in response to selected nutrients and on soil. Conidia of *C. victoriae* and *C. sativus* germinated on sterile agarose, but not when exposed to soil (Table 2); thus, they were nutrient-independent for germination and sensitive to soil fungistasis. Ascospores of *N. tetrasperma* germinated in all test conditions and therefore were nutrient-independent for germination and insensitive to soil fungistasis. Conidia of *V. dahliae* germinated on agarose only if nutrients were added (i.e., they were nutrient-dependent for germination).

On soil, percentage of germination of *C. victoriae*, *C. sativus*, and *N. tetrasperma* spores was similar in both the direct and diffusion assays. *C. victoriae* conidia did not germinate in either the direct or the diffusion assay. With *C. sativus* conidia, germination was only slightly higher in the diffusion assay than in the direct assay. Activated ascospores of *N. tetrasperma* germinated at a high percentage (95 and 99%) in both soil assays.

Effect of washing on sensitivity of *C. victoriae* conidia to soil fungistasis. Percentage of germination of *C. victoriae* conidia was similar regardless of wet or dry harvest. Conidia did not germinate when exposed to soil in either the direct or diffusion assay (data not shown). Conidia harvested by either method had greater than 90% germination after 3 h on agarose.

Fungistatic activity of four soils. *C. victoriae* conidia did not germinate in either the direct or the diffusion assay on any of the four soils (Table 3). The four soils were fungistatic, not

TABLE 2. Germination of four fungi on soil and in response to selected nutrients[v]

Treatment	Germination (%)[w]			
	Cochliobolus victoriae	*C. sativus*	*Neurospora tetrasperma*	*Verticillium dahliae*
Agarose[x]	95 a	86 b	98 a	0.5 c
Agarose with 100 μM glucose[x]	ND[y]	ND	ND	78 a
V8 agar[x]	98 a	99 a	96 a	83 a
Direct fungistasis assay[z]	0 b	1 d	99 a	ND
Diffusion fungistasis assay[z]	0.3 b	15 c	95 a	36 b
Treatment effect (P value)	<0.001	<0.001	0.11	<0.001

[v] Spores were incubated on the indicated substratum at 20°C in the dark, and then assessed microscopically for percentage of germination. Incubation times were 4 h for *C. victoriae*, 6 h for *C. sativus*, 12 h for *N. tetrasperma*, and 15 h for *V. dahliae*.
[w] Values are means of four replications from a single trial. Within a column, values followed by the same letter are not statistically different according to Student-Newman-Keuls test ($\alpha = 0.05$). Treatment effect was determined by an F test on a one-way ANOVA.
[x] Spores incubated in an axenic environment without soil. See Materials and Methods for details.
[y] Not determined.
[z] Spores incubated on Panoche clay loam soil. See Materials and Methods for details.

fungicidal; nongerminated conidia on soil remained viable, as indicated by the high percentage of germination (89–97%) when transferred from soil to agarose for the viability check. None of the soils were fungistatic after autoclaving, which suggests fungistasis in these soils is of microbial origin.

Agarose blocks became fungistatic more quickly when exposed to soil in the diffusion assay than in the volatile assay (Fig. 2). In the diffusion assay, blocks became fungistatic (0% germination) within 3 h. In the volatile assay, blocks never became fully fungistatic, but germination was reduced the longer the blocks were exposed to soil; after exposing agarose blocks to soil for 24 h, 9–17% of the conidia, depending on the soil, germinated after a 3-h incubation. Results were similar for all four soils. Agarose blocks incubated on sterile plastic as a control never became fungistatic (91% germination).

Effect of temperature on fungistasis. Agarose blocks became fungistatic when exposed to soil in either the volatile or the diffusion assay at 18, 23, or 28 C (Fig. 3A). In the diffusion assay, blocks became fungistatic (0–4.5% germination) within 3 h at all temperatures. In the volatile assay, the percentage of germination was higher than for equivalent time points with the diffusion assay, but blocks still became fungistatic over time at all three temperatures. After blocks were preincubated on soil for 48 h, germination was 28, 21, and 7% at 18, 23, and 28 C, respectively. Results were similar for all three temperatures. Agarose blocks incubated on sterile plastic as a control never became fungistatic; after 48 h preincubation, 82% of the conidia germinated.

When incubated on agarose without soil, conidia germinated at similar rates at all three temperatures (Fig. 3B). After 3 h at 18, 23 or 28 C, 81, 81, and 84%, respectively, of conidia germinated. Thus, temperatures between 18 and 28 C did not affect the rate at which agarose blocks became fungistatic and had little or no affect on the rate at which conidia germinated in the absence of soil.

Effect of agarose block thickness on the diffusion assay. Thickness of the agarose block affected the rates at which the blocks became fungistatic when exposed to soil (Fig. 4). When no agarose block was present (i.e., the direct bioassay), soil was immediately fungistatic (0% germination at 0 h exposure). Blocks 2.5 mm thick became fully fungistatic (0% germination) after 2 h of exposure to soil. Blocks 5 mm thick required 4 h to become fully fungistatic, and blocks 7.5 mm thick required 8 h. Agarose blocks incubated on sterile plastic as a control did not become fungistatic (92% germination) within the 8-h test period.

Persistence of fungistatic activity in agarose blocks. After agarose blocks were exposed to soil for 24 h, the germination rate of freshly applied conidia was retarded (Fig. 5A). After 12 h, only 38 ± 12% of these conidia had germinated, and the percentage of germination did not increase even after 24 h of incubation. In contrast, 85 ± 4% of conidia germinated after 3 h when incubated on agarose blocks that were never exposed to soil. Control blocks that were continuously on soil were fully fungistatic (0% germination) at all times.

Agarose blocks made fungistatic by a 24-h exposure to soil became less fungistatic after removal from soil (Fig. 5B). Conidia applied to blocks immediately after blocks were removed from soil germinated at 51 ± 2%. Fungistatic activity diminished as the amount of time blocks were off of soil increased; 83 ± 6% of conidia germinated on blocks that had been removed from soil for 24 h. Control blocks, incubated continuously on soil, remained fully fungistatic (0% germination) at all times. Additional control blocks that were never on soil permitted germination (86–91%) at all times.

Results for the zero hour time points indicated a difference between the two procedures described above; fungistatic activity in agarose blocks appeared to be lower when the experiment was done in a laminar flow transfer hood (i.e., the second of the two procedures). Further experimentation indicated that ventilation significantly reduced the fungistatic activity of agarose blocks that were removed from soil (Table 4). With agarose blocks made fungistatic by exposure to soil in the diffusion assay, nonventilated blocks were more fungistatic (17 ± 4% germination) than ventilated blocks (51 ± 4%). Ventilation did not affect the fungistatic activity of blocks that remained in contact with soil; these blocks were fungistatic in both ventilated (6 ± 4% germination) and nonventilated (4 ± 3%) treatments. Ventilation also did not affect percentage of germination in nonfungistatic conditions; blocks that were not exposed to soil were conducive to germination in both ventilated (70 ± 7%) and nonventilated (67 ± 5%) treatments. Results with the volatile assay were similar, except that blocks that were removed from soil were not as fungistatic (31 ± 2% germination) as their counterparts in the diffusion assay (17 ± 4%). Similarly, ventilated blocks that remained on soil were slightly less fungistatic (15 ± 3% germination) than their counterparts in the diffusion assay (6 ± 4%).

DISCUSSION

Conidia of *C. victoriae* and *C. sativus* did not germinate when incubated either on membranes on soil (direct assay) or on agarose blocks separated from soil by membranes (diffusion assay). Thin blocks became fungistatic more quickly than thick blocks. Thus, fungistatic activity diffused from soil into the agarose blocks. The agarose became fungistatic when in contact with the soil water (diffusion assay) or when exposed only to the gas space above the soil (volatile assay). Activity persisted in the agarose blocks after removal from soil and persisted longer when blocks were incubated in still rather than moving air. The results suggest that soil contains water-soluble, volatile, and possibly nonvolatile, fungistatic compounds. Furthermore, these compounds are pres-

TABLE 3. Fungistatic activity of four soils, as measured by germination of *Cochliobolus victoriae* conidia[x]

		Germination (%)[y]			
		Soil type			
Soil treatment	Assay method	Boyer	Holdrege	Holland	Panoche
Nonautoclaved	Direct a[z]	0	0	0	0
	Direct/viability b	94	95	89	92
	Diffusion a	0	0	0	0
	Diffusion/viability b	92	92	95	97
Autoclaved	Direct b	92	92	95	91
	Diffusion b	96	95	95	95
	Volatile b	96	91	95	93

[x] Assay materials were incubated on soil for 24 h before adding conidia. Conidia were incubated 4 h. To determine viability, conidia were transferred from soil to sterile agarose and incubated an additional 4 h. Conidia were examined microscopically to assess percentage of germination. On agarose blocks incubated on sterile plastic, 92 ± 1% of the conidia germinated.

[y] Values are means of two trials, each with three replications. An F test performed on a two-way ANOVA (soil type × assay treatment) indicated no significant differences between soils ($P = 0.94$), highly significant differences between treatments ($P < 0.0001$), and no significant soil × treatment interaction ($P = 0.51$).

[z] Treatments followed by the same letter do not differ from each other according to Fisher's protected LSD $\alpha = 0.05$.

ent apparently at concentrations sufficient to account for soil fungistasis.

Four test soils, differing in texture, classification, pH, organic carbon content, cation exchange capacity, vegetation history, and geographic origin, gave similar results in the three assays. This indicates fungistatic compounds in soil are common and are not restricted to only a few soils. Temperatures between 18 and 28 C had little effect on rates at which agarose became fungistatic or on rates at which conidia germinated, which suggests that fungistasis in these soils occurs over a range of temperatures typical of field conditions.

Fungistatic compounds in soil have been reported previously, and the literature has been reviewed (24,26). Fungitoxic levels of Al ion were found in acid soils, and fungistatic volatiles were found in alkaline soils (20,21). In neither case was the activity associated with microbial activity. This fungistatic activity, termed "residual fungistasis" (5), is inorganic rather than biological in origin and has been found in only a few soils. In most soils, including the ones used in the present study, fungistasis appears to be of microbial origin. Volatile fungistatic factors of biological origin were found in alkaline soils (13) but were not definitively identified (28). Volatile and possibly nonvolatile fungistatic activity, presumably of biological origin, also was found in both acidic and basic soils (31).

The present study clarifies points suggested in earlier work (13,28,31). In the present study, variation both between and within soils was low. Germination in fungistatic conditions was near zero, typically <3% in 24 h. The viability of the population of nongerminating spores was confirmed and high. In addition, the germination rates in nonfungistatic conditions were rapid and synchronous, typically >80% in 3 h. Thus, nongerminating spores were viable and sensitive to soil fungistasis, not asynchronous for germination. Furthermore, the present study demonstrates for the first time the rates at which agarose blocks gain and lose fungistatic activity under various soil types, assay types, temperatures, and amounts of time separated from soil. These data strongly suggest that compounds that inhibit germination diffuse from soil into the agarose blocks. Moreover, nutrient-independent spores were used, in contrast to several other studies (13,36). The use of nutrient-dependent spores in experiments on the mechanism of soil fungistasis has been criticised, because it is not possible to determine if the spores are not germinating because inhibitory compounds are present, as presumed by some authors, or if the substrate contains insufficient nutrients to support germination (24). Germination of *V. dahliae* conidia, which are nutrient-dependent, and activated ascospores of *N. tetrasperma*, which are nutrient-independent and fungistasis-insensitive, also was tested to confirm that the agarose in the diffusion and volatile assays served only as an inert matrix. To facilitate the comparison of our results with earlier work, we used *C. victoriae*, which has been the subject of much research on fungistasis and the nutrient deprivation hypothesis (2,8–11,17,19,23). In addition, we included one soil (Boyer sandy loam), which has been used in other studies on the nutrient deprivation hypothesis (11).

The nutrient deprivation hypothesis is not consistent with the data from this study. First, agarose blocks became fungistatic when exposed only to the atmosphere over the soil surface. Soil could not exert a diffusive or nutrient stress (24,25) under these test conditions (31). Second, when placed on soil, thin agarose blocks became fungistatic more quickly than thick blocks. This is consistent with diffusion of germination inhibitors from soil into the agarose blocks. Soil was not a sink for nutrients from the agarose blocks because agarose has very little nutritional carbon. Furthermore, the spores cannot be the source of the

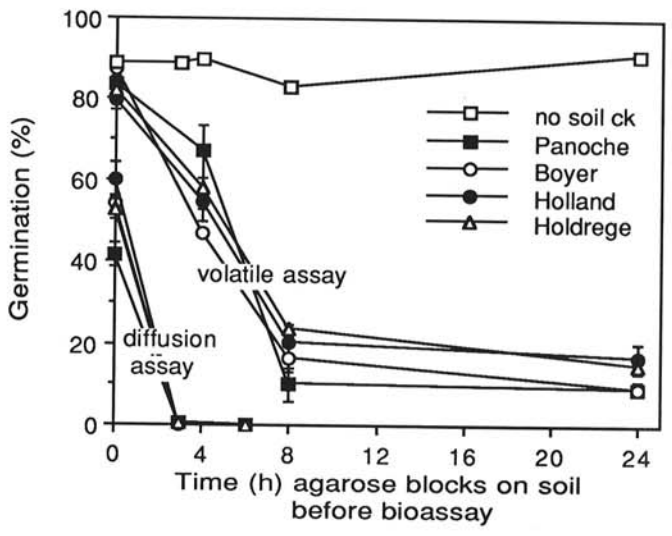

Fig. 2. Effect of soil type on rates at which agarose blocks become fungistatic, as measured by percentage of germination of *Cochliobolus victoriae* conidia in the volatile and diffusion bioassays. Values are means ± one SEM of three trials, each with three or four replications.

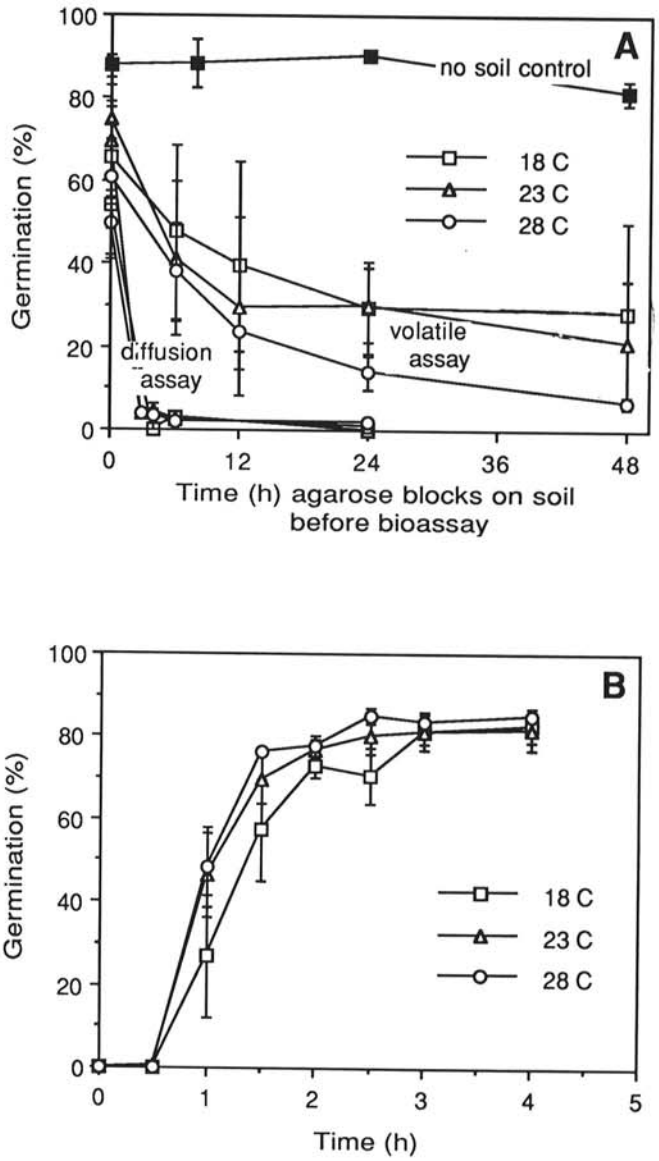

Fig. 3. Effect of temperature on fungistasis. Values are means ± one SEM of three trials, each with three or four replications. **A,** rates at which agarose blocks become fungistatic, as measured by percentage of germination of *Cochliobolus victoriae* conidia in the volatile and diffusion bioassays on Panoche clay loam. In the controls without soil, there were no statistically significant differences for the three temperatures; therefore the data were pooled for all temperatures. **B,** germination rates of *C. victoriae* conidia on sterile agarose.

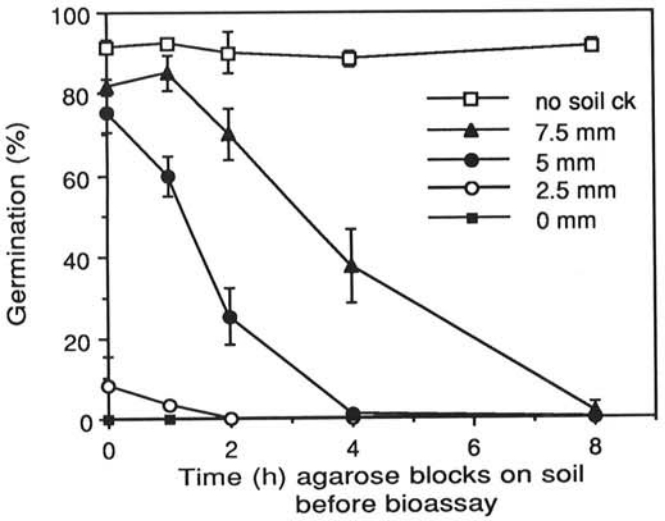

Fig. 4. Effect of agarose block thickness on rates at which blocks become fungistatic, as measured by percentage of germination of *Cochliobolus victoriae* conidia in the diffusion bioassay. Values are means ± one SEM of a single trial with two replications. In the controls without soil, there was no significant effect ($P > 0.5$) of block thickness on percentage of germination; therefore, these data were pooled for all block thicknesses.

nutrients, as required by the nutrient deprivation hypothesis, because they were not added until after the blocks were exposed to soil. Third, in controls without soil, the size of the agarose blocks had no effect on percentage of germination. If the agarose blocks were sinks for nutrients or other germination-promoting compounds from the spores, the spores would have had lower percentages of germination on the large than on the small agarose blocks. This was not the case; therefore, the agarose itself was not a sink for compounds from the spores. Fourth, fungistatic activity was found in blocks that were removed from soil. Conidia cannot be the source of a nutrient gradient in these blocks, as required by the nutrient deprivation hypothesis, because no spores were placed on the blocks until after the blocks were removed from soil. Although nutrient deprivation may be a factor in soil fungistasis, there was no evidence in this study that nutrient deprivation was involved in the suppression of germination of *C. victoriae* conidia on soil.

The identity of the fungistatic compound(s) remains unknown; the assay methods used in this study may be useful in further work on characterization of the compound(s). Most previous work has concerned highly volatile compounds with low molecular weight. Ethylene has been proposed as the compound responsible for regulating fungistasis in soil (37), but others have rejected this hypothesis (4,28,38). Acetone and formaldehyde were rejected as causes of fungistasis in natural soil (28). Ammonia was considered a prime candidate for a volatile inhibitor, but only in

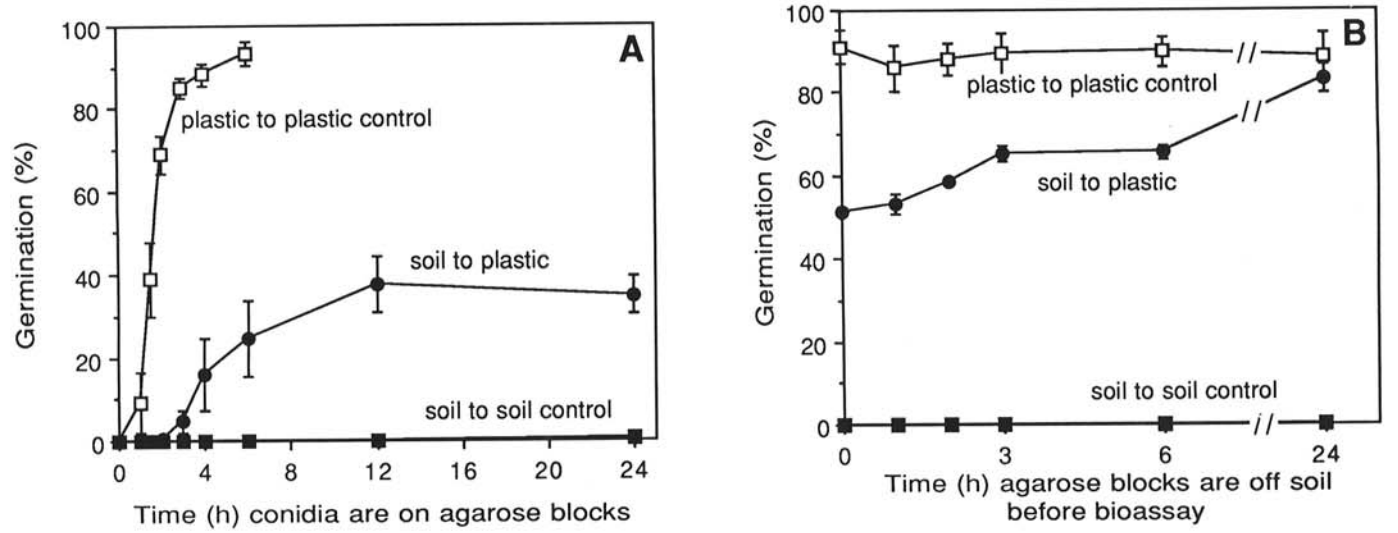

Fig. 5. Persistence of fungistatic activity in agarose blocks that were removed from soil. Activity was measured in a bioassay as percentage of germination of *Cochliobolus victoriae* conidia. Values are means ± one SEM of four trials, each with three replications. **A,** germination rates of conidia on soil. Agarose blocks were first incubated for 24 h on soil or on sterile plastic and then were transferred to sterile plastic. Conidia were added immediately, and their percentage of germination was followed over time. **B,** loss of fungistatic activity over time. Blocks were incubated on soil for 24 h, then transferred to plastic, and incubated an additional 0–24 h. Conidia were added, incubated 3 h, and their percentage of germination was assessed.

TABLE 4. Effect of ventilation on fungistatic activity in agarose blocks, as measured by percentage of germination of *Cochliobolus victoriae* conidia in two bioassays on Panoche clay loam[x]

Fungistasis assay method[z]	First substratum	Second substratum	Germination (%) ± SEM[y]	
			Ventilated	Nonventilated
Diffusion assay	Plastic	Plastic	70 ± 7	67 ± 5
	Soil	Plastic	51 ± 4	17 ± 4
	Soil	Soil	6 ± 4	4 ± 3
Volatile assay	Plastic	Plastic	73 ± 2	74 ± 2
	Soil	Plastic	56 ± 4	31 ± 2
	Soil	Soil	15 ± 3	5 ± 2

[x] Agarose blocks were incubated on the first substratum for 24 h, and then transferred to the second substratum. Immediately, one-half the blocks were ventilated by a 5-min exposure to sterile air moving at 0.35 m sec^{-1} in a laminar flow transfer hood. Nonventilated blocks were protected from the airflow. Conidia were added to the blocks and incubated 2 h before microscopic examination to assess percentage of germination.

[y] Values are means of three or four trials, each with three replications. An F test performed on a two-way ANOVA (substratum × ventilation) indicates highly significant ($P < 0.002$) effects for both ventilation and substratum, and for interaction between treatments.

[z] Experiments with the agarose assay and the volatile assay were performed and analyzed separately.

soils with high pH. Other work (22,33) also indicates a role of ammonia in causing fungistasis in alkaline soils. The results of the present study indicate that the fungistatic compounds, including volatile compounds, are present in both acidic and basic soils. Therefore, a compound that is less pH-sensitive than ammonia would be a more likely candidate than ammonia for a common fungistatic compound. The similarity of the rates at which agarose blocks became fungistatic on the four test soils suggests that the fungistatic compounds may be the same in all four soils. The compounds diffuse into the agarose blocks and therefore apparently are water soluble. However, whether the water-soluble and volatile components are the same or distinct compounds is unclear.

LITERATURE CITED

1. Boosalis, M. G. 1962. Precocious sporulation and longevity of conidia of *Helminthosporium sativum* in soil. Phytopathology 52:1172-1177.
2. Bristow, P. R., and Lockwood, J. L. 1975. Soil fungistasis: Role of spore exudates in the inhibition of nutrient-independent propagules. J. Gen. Microbiol. 90:140-146.
3. Chinn, S. H. F., and Tinline, R. D. 1964. Inherent germinability and survival of spores of *Cochliobolus sativus*. Phytopathology 54:349-352.
4. Cornforth, I. S. 1975. The persistence of ethylene in aerobic soils. Plant Soil 42:85-96.
5. Dobbs, C. G., and Gash, M. J. 1965. Microbial and residual mycostasis in soils. Nature (London) 207:1354-1356.
6. Dobbs, C. G., and Hinson, W. H. 1953. A widespread fungistasis in soils. Nature (London) 172:197-199.
7. Doner, H. E., Amundson, R. G., and Lilieholm, B. 1990. A comparison of Se and As concentrations in soils of the Western San Joaquin Valley, California, 1946-1985. Arid Soil Res. Rehabil. 3:315-325.
8. Epstein, L., and Lockwood, J. L. 1983. The role of exudation in the germination of *Cochliobolus victoriae* conidia. J. Gen. Microbiol. 129:3629-3635.
9. Epstein, L., and Lockwood, J. L. 1984a. Suppression of conidial germination of *Helminthosporium victoriae* in soil and in model fungistatic systems. Phytopathology 74:90-94.
10. Epstein, L., and Lockwood, J. L. 1984b. Effect of soil microbiota on germination of *Bipolaris victoriae* conidia. Trans. Brit. Mycol. Soc. 82:63-69.
11. Filonow, A. B., and Lockwood, J. L. 1983. Mycostasis in relation to the microbial nutrient sinks of five soils. Soil Biol. Biochem. 15:557-565.
12. Herrington, P. R., Craig, J. T., and Sheridan, J. E. 1987. Methyl vinyl ketone: A volatile fungistatic inhibitor from *Streptomyces griseoruber*. Soil Biol. Biochem. 19:509-512.
13. Hora, T. S., and Baker, R. 1970. Volatile factor in soil fungistasis. Nature (London) 225:1071-1072.
14. Hora, T. S., and Baker, R. 1972. Extraction of a volatile factor from soil inducing fungistasis. Phytopathology 62:1475-1476.
15. Hora, T. S., and Baker, R. 1974a. Abiotic generation of a volatile fungistatic factor in soil by liming. Phytopathology 64:624-629.
16. Hora, T. S., and Baker, R. 1974b. Influence of a volatile inhibitor in natural or limed soil of fungal spore and seed germination. Soil Biol. Biochem. 6:257-261.
17. Hsu, S. C., and Lockwood, J. L. 1973. Soil fungistasis: Behavior of nutrient-independent spores and sclerotia in a model system. Phytopathology 63:334-337.
18. Jackson, R. M. 1957. Fungistasis as a factor in the rhizosphere phenomenon. Nature (London) 180:96-97.
19. Jasalavich, C. A., Hyakumachi, M. and Lockwood, J. L. 1990. Loss of endogenous carbon by conidia of *Cochliobolus victoriae* exposed to soil and its effect on conidial germination and pathogenic aggressiveness. Soil Biol. Biochem. 22:761-767.
20. Ko, W. -H., and Hora, F. K. 1972a. The nature of a volatile inhibitor from certain alkaline soils. Phytopathology 62:573-575.
21. Ko, W. -H., and Hora, F. K. 1972b. Identification of an Al ion as a soil fungitoxin. Soil Sci. 113:42-45.
22. Ko, W. -H., Hora, F. K., and Herlicska, E. 1974. Isolation and identification of a volatile fungistatic substance from alkaline soil. Phytopathology 64:1398-1400.
23. Ko, W. -H., and Lockwood, J. L. 1967. Soil fungistasis: Relation to fungal spore germination. Phytopathology 57:894-901.
24. Lockwood, J. L. 1977. Fungistasis in soils. Biol. Rev. 52:1-43.
25. Lockwood, J. L. 1988. Evolution of concepts associated with soilborne plant pathogens. Annu. Rev. Phytopathol. 26:93-121.
26. Lockwood, J. L., and Filonow, A. B. 1981. Responses of fungi to nutrient-limiting conditions and to inhibitory substances in natural habitats. Adv. Microb. Ecol. 5:1-61.
27. Norton, J. M., Smith, J. L., and Firestone, M. K. 1990. Carbon flow in the rhizosphere of Ponderosa pine seedlings. Soil Biol. Biochem. 22:449-455.
28. Pavlica, D. A., Hora, T. S., Bradshaw, J. J., Skogerboe, R. K., and Baker, R. 1978. Volatiles from soil influencing activities of soil fungi. Phytopathology 68:758-765.
29. Perkins, D. D. 1986. Hints and precautions for the care, feeding and breeding of *Neurospora*. Fungal Genet. Newsl. 33:35-41.
30. Romine, M., and Baker, R. 1972. Properties of a volatile fungistatic factor in soil. Phytopathology 62:602-605.
31. Romine, M., and Baker, R. 1973. Soil fungistasis: Evidence for an inhibitory factor. Phytopathology 63:756-759.
32. Schaad, N. W., ed. 1980. Laboratory Guide for Identification of Plant Pathogenic Bacteria. The American Phytopathological Society, St. Paul, MN. 72 pp.
33. Schippers, B., and Palm, L. C. 1973. Ammonia, a fungistatic volatile in chitin-amended soil. Neth. J. Plant Pathol. 79:279-281.
34. Schreiber, L. R., and Green, R. J., Jr. 1963. Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. Phytopathology 53:260-264.
35. Schüepp, H., and Frei, E. 1969. Soil fungistasis with respect to pH and profile. Can. J. Microbiol. 15:1273-1279.
36. Schüepp, H., and Green, R. J., Jr. 1968. Indirect assay methods to investigate soil fungistasis with special consideration of soil pH. Phytopathol. Z. 61:1-28.
37. Smith, A. M. 1973. Ethylene as a cause of soil fungistasis. Nature (London) 246:311-313.
38. Smith, K. A. 1978. Ineffectiveness of ethylene as a regulator of soil microbial activity. Soil Biol. Biochem. 10:269-272.
39. Vogel, H. J. 1964. Lysine pathways among fungi. Am. Nat. 98:435-446.
40. Watson, A. G., and Ford, E. J. 1972. Soil fungistasis—A reappraisal. Annu. Rev. Phytopathol. 10:327-348.