Partial Characterization of Volatile Fungistatic Compound(s) from Soil

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ABSTRACT

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Many soils contain volatile, water-soluble compound(s) that inhibit germination of *Cochliobolus victoriae* conidia in the absence of a carbon source. The volatile fungistatic compound(s) from soil were separated into a cell-free extract. Loss of fungistatic activity from the extract was time- and temperature-dependent; all activity was lost within 5 min at 90 C, 48 h at 21 C, and 5 days at −70 C. Much of the fungistatic activity was lost after the soil extract was diluted by 10%, incubated in an uncapped vial, or transferred to a new vial via a gas-tight syringe. Fungistatic activity was not detected in material collected from soil into a liquid N_2 cold trap. Agarose blocks adjusted to pH 5.5–8.0 became fungistatic when incubated on soil, suggesting that the fungistatic compound(s) were relatively unaffected by hydrogen ion concentrations in this range. Carbon monoxide (CO), carbon dioxide (CO_2), nitric oxide (NO), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), ammonia (NH_3), ethylene (C_2H_4), and reduced concentrations of oxygen (O_2) apparently were not responsible for fungistasis of *C. victoriae* conidia in soil because these compounds were not fungistatic at concentrations detected in soil.

Additional keywords: *Bipolaris, Helminthosporium,* mycostasis.

Soil inhibits germination of many fungal propagules, a phenomenon termed "fungistasis" (4,12). Bacteria in the soil are necessary for the phenomenon; however, the mechanism of suppression of germination is controversial (6,21,25,30,32,33). Recently, volatile, water-soluble compound(s) that inhibit germination of fungal spores were detected in four soils with different characteristics (18). The compound(s) inhibited spore germination at concentrations found in soil.

The identification of a naturally occurring, bacterially produced (7) volatile compound that regulates soil fungistasis might suggest new materials and approaches for bioremediation and plant-disease control. Therefore, our objective was to extract the compound(s) from soil and to characterize some of their properties. A preliminary report was published (19).

MATERIALS AND METHODS

Soils. Panoche clay loam, Holdrege silt loam, Boyer sandy loam, and Holland sandy loam were described previously (18). Yolo sandy loam was collected from the University of California Armstrong Experiment Station, Solano County near Davis, CA.

Culture and preparation of fungi. Conidial suspensions of *Cochliobolus victoriae* R.R. Nelson (anamorph *Helminthosporium victoriae* F. Meehan & Murphy, =*Bipolaris victoriae* (F. Meehan & Murphy) Shoemaker) were stored in an aqueous solution of 25% (w/w) glycerol at −70 C (18). The fungus was cultured on V8 agar for 7–10 days at 21 C with cycles of 8 h of fluorescent light and 16 h of dark. Conidia were harvested from culture plates in 0.02% Tween 20, washed three times by centrifugation at 1,000 × *g* for 3 min in 50 ml of 0.02% Tween 20 at 4 C, and vacuum-filtered onto 6- × 6-mm polycarbonate membranes (Nuclepore Corporation, Pleasanton, CA). Membranes had 0.2-μm pores, and spores were deposited at a density of 10^3 conidia per square centimeter.

Fungistasis bioassay. Germination of *C. victoriae* conidia was used to quantify fungistasis (18, reviewed in 21); fungistasis and germination are inversely proportional. We used three bioassay methods: direct, diffusion, and volatile (18). For bioassays with soil, air-dried Panoche clay loam (20 g) was placed in a 15- × 100-mm-diameter plastic petri dish, wetted to a matric potential (ψ_m) of approximately −5 kPa (18), and incubated for 5–7 days in a moist chamber at 21 C. In the direct and diffusion assays on soil, a 2- × 2-cm polycarbonate membrane with 2-μm pores was placed on the soil. In the volatile assay on soil, a glass coverslip was placed on the soil surface. In the diffusion and volatile assays, a block (1 × 1 × 0.5 cm high) of 1% (w/w) agarose (electrophoresis grade, GIBCO BRL, Gaithersburg, MD) was placed on the membrane or coverslip, respectively. The agarose was incubated in a covered petri dish at 21 C for 12–18 h. For the direct assays on soil, conidia, borne on polycarbonate membranes as described above, were placed on top of the large membrane in a soil dish. For the direct assay on soil extract, conidia on membranes were floated on an aqueous extract in vials. For the diffusion and volatile assays, spores on the small membranes were placed on top of the agarose. After incubation for 3 h at 21 C in the dark, the spores were fixed and stained in an aqueous solution (w/w) of 10% lactic acid, 0.05% Cotton Blue, and 25% glycerol and examined at 200× on a light microscope with epi-illumination (18). Spores with germ tubes longer than one-half the width of the spore were considered germinated.

Extraction of soil. Soil columns were prepared in 30-ml plastic syringes (2 cm internal diameter; Becton-Dickinson and Co., Franklin Lakes, NJ). A 5-mm-diameter hole was drilled 5 cm from the syringe tip and two glass-fiber filter disks (G6; Fisher Scientific, Pittsburgh) were placed inside the barrel over the syringe tip. After 8 g of air-dried soil was placed in each syringe, the soil was wetted with approximately 10 ml of water and allowed to drain. The syringe plunger was positioned 3 cm above the soil, so moisture loss by evaporation was limited; the drilled hole permitted gas exchange and insertion of the syringe piston without an increase in soil pressure. Soil columns were incubated for 7–10 days before use in experiments.

To sterilize the soil extract, a 4-mm-diameter nylon-membrane filter, with 0.2-μm pores and a 5-μl dead volume (Nalgene; Nalge Co., Rochester, NY), was attached to the tip of the syringe. After the syringe was placed in a specially constructed vise, the soil extract, consisting of gas and liquid, was expressed from the syringe; the first 200 μl was discarded to avoid assaying material from dead volume. To determine if the soil extract was microbe-

free, 10-μl aliquots were spotted onto nutrient agar, and the bacterial population was calculated by a most-probable-number method (9).

For the fungistasis bioassay, soil extract was expressed from the syringes into glass (Shorty; Wheaton, Millville, NJ) or polypropylene (Sigma Chemical Co., St. Louis) vials. The 2-ml borosilicate glass vials had open-hole screw caps and Teflon-faced silicone septa. The 750-μl polypropylene vials had tapered bottoms and screw caps with O-rings. After adding 500 μl of soil extract, vials were capped immediately. Unless otherwise indicated, within 10 min after collection of the soil extract, the bioassay was started by uncapping the vials for 5–10 s and placing the spores, borne on floatable membranes, on the extracts. To ensure that spore viability was unaffected by the various procedures, a control with 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 6.1, was included.

Also, soil extract was expressed and assayed in a continuously closed vial. For these experiments, spores on membranes were placed on the bottom of glass vials. After the vials were capped and evacuated, the soil extract was expressed from the syringe through a needle (25 G, 1.6 cm long) and into the vial, so the membrane was floating on the extract.

When indicated, we transferred the fungistatic extract from one continuously closed vial to another continuously closed vial. Vials were evacuated with a 5-ml glass syringe (B-D GlasPak; Becton-Dickinson, Lincoln Park, NJ). Next, 500 μl of liquid soil extract plus an unknown volume of gaseous soil extract was expressed directly from a soil column, through a filter and needle, and into an evacuated vial. The liquid extract and gas in the head space then were transferred to a new evacuated vial with a 2.5-ml glass gas-tight syringe fitted with a MicroValve Teflon lock (Spectrum, Los Angeles). The second evacuated vial contained spores borne on a membrane.

Experimental design and statistical analysis. Individual experiments had completely randomized designs with two or three replications per treatment and two or three determinations per replication; each determination was obtained from a membrane on which at least 100 spores were evaluated for germination. Each experiment was performed at least two times with similar results. For statistical analysis of percent data, values were transformed to $\sin^{-1}[(x/100) + 0.5]^{0.5}$; we report detransformed means ± one standard error of the mean.

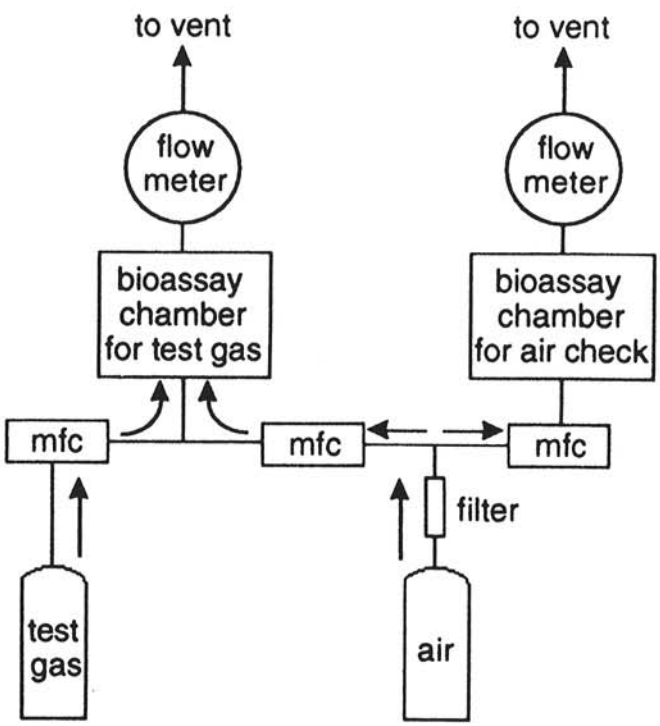

Fig. 1. Schematic of apparatus used to test the sensitivity of *Cochliobolus victoriae* conidia to selected gases. mfc = mass flow controller.

Effect of dilution on fungistatic activity of soil extract. An "inactive" soil extract was prepared by incubating soil extract in an open vial at 24 C for 1 h. To determine the dilution end point for fungistatic activity in soil extract, various volumes of inactivated soil extract were placed in plastic vials, and soil extract was added for a total volume of 500 μl. After spores on membranes were introduced, the standard bioassay was performed.

Effect of temperature on fungistatic activity of soil extract. After soil extract was placed in vials as indicated above, the vials were incubated for varying time periods in a freezer at −70 C, an incubator at 21 C, or water baths at 90 or 100 C. After the indicated time period, the vials were brought rapidly to 21 C in a water bath, the caps were opened, and spores on membranes were added to the vials for the bioassay.

Effect of pH on fungistatic activity in agarose blocks. The pH of 100 mM MES or *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) buffer was adjusted with 2 N KOH. Agarose blocks (1% w/w) containing 100 mM buffer were prepared for the diffusion assay as described above. Control treatments included agarose blocks incubated in dishes without soil. The pH of the agarose was measured with a flat-bottom electrode (Fisher Scientific, Pittsburgh) before incubation on soil and after the bioassay. To determine the pH of unbuffered agarose, agarose was amended with 100 mM NaCl.

Attempt to collect fungistatic compound(s) in a cold trap. Moist soil (60 g of air-dried soil per 30 g of water) in a large (15 × 150 mm) petri dish was incubated for 7–10 days at 21 C, then placed in a 4-L glass dessicator jar. The dessicator jar was connected to a glass cold finger, which was chilled in liquid nitrogen, and to a vacuum pump (model D-150, Precision Scientific, Chicago; maximum 13.3 mPa). Condensed material was collected from the cold trap either every 20 min for a 2-h period or after a single 3-h period, at which time the soil was dry. The material was chipped out of the cold finger and packed into 10-ml glass vials with open-hole screw caps and Teflon-faced silicone septa. After the material was warmed to room temperature, 250-μl aliquots were transferred to glass vials with a glass gas-tight syringe fitted with a Teflon valve and bioassayed for fungistatic activity.

Sensitivity of *C. victoriae* to selected compounds. We determined whether selected volatile compounds inhibited germination of conidia. Bioassay chambers were constructed from 125-ml canning jars. For all experiments, we used a soil control, an air control, and gas-air mixtures (Fig. 1). Soil controls consisted of sealed jars containing 50 g of moist soil. For the other treatments, the jar lids had two holes plugged with Hungate septa; each septum had a 3-mm hole through which steel tubing (3 mm outside diameter) was inserted. Compressed air was filtered through a hydrocarbon trap (Scott Specialty Gases, Inc., Plumsteadville, PA). Gas tanks with certified concentrations were mixed with air using mass flow controllers (FC-260; Tylan Corp., Torrance, CA). Air and gas-air mixtures were passed through the steel tubing into the inlet port of the bioassay chamber. Gases in the chamber were mixed with a Teflon-coated magnetic stir bar. The outlet port from the chamber was connected to a flow meter.

Agarose blocks (1 × 1 × 0.5 cm high) were placed in the test chamber and incubated for 12–18 h with either gas or air flowing at 4.8 ml/min. After spores on membranes were placed on top of the agarose blocks, gas or air was passed through the chamber at 14.4 ml/min. These flow rates assured a 90% turnover of the gas volume every 60 and 20 min for the preincubation period and bioassay, respectively.

The following potentially fungistatic gases were selected for bioassays: NO (1,24), SO_2 (23), NH_3 (22,25,28), C_2H_2 (13–15, 22,30,31), and CO (3,5,16,29). For SO_2 and NH_3, agarose blocks were buffered with 100 mM MES adjusted to pH 6.1. The pH of the agarose blocks was measured at the conclusion of the experiment; after exposure to the test gases, only minimal changes (<0.4 pH units) were detected.

After moist soil was incubated in the bioassay jars for 12 h, i.e., sufficient time for agarose blocks in the head space to become

fungistatic, 1-ml gas samples from the head space were used to determine the concentrations of selected gases. Concentrations of NO and NO_2 were determined with a NO-to-NO_2 converter and NO_2 chemiluminescence detector (LNC-3 and LMA-3, respectively; Scintrex Corp., Concord, ON, Canada). Concentrations of O_2 and CO_2 were determined with a gas chromatograph (AGC Series 100, Carle, Loveland, CO) fitted with Molecular Sieve 5A and Porapak (Carle; 80% N, 20% Q) columns, respectively.

We also tested the effect of isoamyl alcohol (=isopentyl alcohol) on germination (34). For the bioassay, membranes bearing spores were floated on 500 μl of 1 nM to 100 mM aqueous isoamyl alcohol in capped, glass vials.

RESULTS

Bacterial populations in soil extract. Nonfiltered soil extract contained $1.1 \times 10^6 \pm 0.5 \times 10^6$ bacterial colony-forming units (cfu) per milliliter. In contrast, extracts passed through a 0.2-μm filter contained 0 cfu/ml. Thus, the filtered soil extract used in the following experiments was free of bacteria and fungi.

Fungistatic activity of soil extract. Panoche clay loam soil was highly fungistatic, i.e., only 1% of conidia germinated when incubated directly on soil (Table 1). Soil extract had some fungistatic activity, i.e., only 18-19% of the conidia germinated when incubated on the extract in a closed vial. However, soil extract lost fungistatic activity rapidly in vials without caps. When vials were left open during the 3-h bioassay period, 73% of the conidia germinated. Similarly, when soil extract was incubated for 1 h in an open vial before spores were added and the vials were capped for a bioassay, 86% of spores germinated. For spores incubated on MES, a nonnutritive buffer (8), 76% germinated in both closed and open vials.

Although the fungistatic activity was lost from a continuously open vial, no difference in activity was detected between vials that were closed during both the deposition of the soil extract and the bioassay and vials that were opened briefly when the soil extract was deposited and spores were placed on the extract (Table 1). For ease, we opened the vials to deposit the spores, unless otherwise indicated. No significant difference ($P = 0.26$) in fungistatic activity of soil extract was detected between glass and plastic vials.

Transferring fungistatic activity. Some of the fungistatic activity was lost when soil extract was transferred between two vials. Whereas only 19% of the conidia germinated when incubated on nontransferred soil extract, 43% germinated when incubated on transferred soil extract (Table 1). Moreover, fungistatic activity was retained only when transfers were made from one closed vial into a gas-tight syringe and into another closed vial. Little or no fungistatic activity was detected in soil extracts that were transferred between open vials with pipettes or between closed vials with non-gas-tight glass syringes (data not shown). Control treatments with spores incubated on buffer had a high percentage of germination regardless of whether the buffer was transferred (79%) or not transferred (73%).

Effect of dilution on fungistatic activity of soil extract. Soil extract could be diluted only very slightly before fungistatic activity was lost (Fig. 2). Whereas only 23% ± 6% of the conidia germinated when incubated on 100% active soil extract, 41% ± 11% germinated when incubated on 98% active soil extract. A solution with 90% active soil extract was not fungistatic (84% ± 4% germination), compared to controls incubated on 100% inactive soil extract (86% ± 4% germination).

Effect of temperature on fungistatic activity of soil extract. The fungistatic activity of soil extract was affected greatly by the preincubation temperature. At a given temperature, loss of activity was time-dependent. At 21 C, soil extract lost much of its fungistatic activity within 24 h and nearly all of its fungistatic activity within 48 h (Table 2). At 90 or 100 C, fungistatic activity was lost within 5 min. At −70 C, fungistatic activity was lost within the 5-day experimental period but at a slower rate than at 21 C.

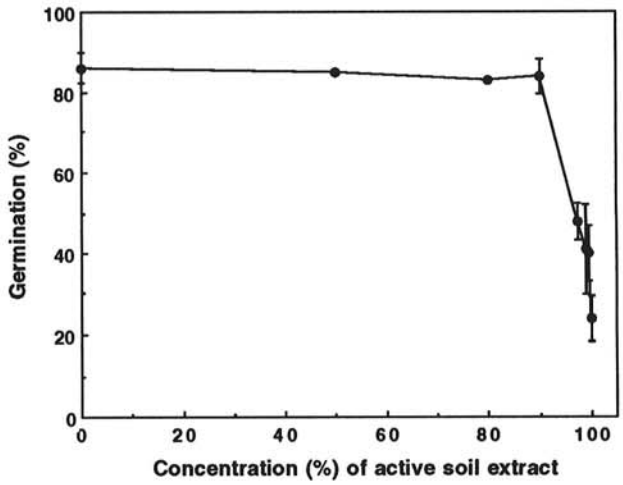

Fig. 2. Effect of dilution of soil extract on fungistatic activity, as measured by percent germination of *Cochliobolus victoriae* conidia. Values are means ± one SEM of three trials, with three replications per trial.

TABLE 1. Fungistatic activity of Panoche clay loam soil and a cell-free soil extract, as measured by percent germination of *Cochliobolus victoriae* conidia

Fungistasis assay method	Bioassay chamber: During introduction of soil extract	Bioassay chamber: During bioassay	Germination (%)[y]
Directly on soil	Not extracted	Covered petri dish	1 a
Soil extract[z]	Closed vial	Closed vial	19 b
	Open vial	Closed vial	18 b
	Closed vial with transfer	Closed vial	43 c
	Open vial	Open vial	73 d

[y] Data from five trials with two or three replicates each were analyzed by an analysis of variance (ANOVA) as a completely randomized design. Treatment effect was highly significant ($P < 0.0001$). Means followed by the same letter are not significantly different according to Fisher's protected LSD ($\alpha = 0.05$).

[z] For each of the soil-extract treatments, a control treatment with 10 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 6.1, also was bioassayed. An ANOVA of all treatments with buffer indicated no significant differences ($P = 0.47$) and a mean germination of 76% ± 2%.

TABLE 2. Effect of temperature and storage time on fungistatic activity in a cell-free extract from Panoche clay loam soil[y]

Storage conditions: Temp. (C)	Storage conditions: Duration (h)	Germination[z] (% ± SEM)
21	0.08	18 ± 7
	3	24 ± 8
	6	28 ± 8
	24	55 ± 7
	48	63 ± 4
	168	71 ± 2
90	0.08	65 ± 9
100	0.08	78 ± 6
−70	24	41 ± 10
	48	38 ± 12
	120	70 ± 10

[y] Soil extract or 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 6.1, was incubated in closed vials at the indicated temperature. After the indicated period, the vials were brought to 21 C and used in a fungistasis bioassay that measured germination of *Cochliobolus victoriae* conidia. In controls with 10 mM MES, pH 6.1, conidia germinated 73% ± 8%.

[z] Data are from four trials with two to four replications per trial.

Effect of pH on fungistatic activity in agarose blocks. In the absence of soil, spore germination was high on agarose blocks adjusted to pH 5.5–7.0 (Table 3). Percent germination was less at pH $\geq$ 7.5 than at a lower pH. When agarose blocks were incubated on soil for 12 h and then bioassayed for fungistatic activity (i.e., in the diffusion bioassay), the percentage of spore germination was low (<2.5%) at all acidities. The pH of the agarose blocks changed only slightly during incubation on soil. These data suggest pH values from 5.5 to 8 have little effect on the activity of the soil fungistatic compound.

Effect of soil type on fungistatic activity in soil extract. In the direct soil assay, all five soils were fungistatic, i.e., <3% of the *C. victoriae* conidia germinated (data not shown). In contrast, soil extracts were fungistatic from only Panoche clay loam and Yolo sandy loam (Table 4).

Attempt to collect fungistatic compound in a cold trap. No fungistatic activity was detected in extracts from soil collected under vacuum into cold traps with either ethanol/dry ice (−78 C) or liquid nitrogen (−195 C). With the liquid nitrogen trap, neither a single bulk fraction nor fractions collected over 20-min intervals were fungistatic. In all of these treatments, spore germination was >77%.

Sensitivity of *C. victoriae* to selected compounds. In bioassays with *C. victoriae* incubated on agarose blocks in soilless chambers, the following atmospheres were not fungistatic, i.e., germination was >85%: NO at 10 and 500 nl/L; ethylene at 1 and 10 μl/L; CO at 1 μl/L; ammonia at 10, 100, and 257 μl/L; sulfur dioxide at 0.1, 1, and 10 μl/L; a combination of 20% CO_2 and 1% O_2; and a tank air control. Gas bioassay chambers (125-ml jars) containing 50 g of moist Panoche clay loam were sealed and incubated for 12–18 h. Spores in these chambers had low germination when incubated directly on soil (2% ± 2%) or on agarose blocks on soil either in the soil diffusion (8% ± 3%) or soil volatile (23% ± 5%) bioassays. The head space in these chambers contained 6% ± 2% CO_2, 15% ± 3% O_2, and NO at 11.2 ± 1.7 nl/L. Nitrogen dioxide was not detected (limit of detection 0.1 nl/L).

Isoamyl alcohol was not fungistatic at concentrations from 1 nM to 1 mM. Solutions containing 10 mM isoamyl alcohol were slightly inhibitory (40% ± 5% germination), and 100 mM solutions were completely inhibitory (0% ± 0% germination).

DISCUSSION

We prepared a filter-sterilized extract of liquid and gas from soil. The unconcentrated extract was fungistatic in bioassays with *C. victoriae* conidia. We believe this is an important step in the purification and identification of a water-soluble, volatile fungistatic compound(s) present in a variety of soils (18). The separation of volatile fungistatic compound(s) from soil supports the hypothesis that soil fungistasis is regulated by germination inhibitors produced by common soil bacteria (7,18).

Previous attempts to extract fungistatic compounds from soil have had limited success. When soil extracts were prepared by passing organic solvents or water through soil and then concentrating the extracts by evaporation under vacuum, no fungistatic activity was detected (20); however, any volatile compounds would have been removed. Volatile fungistatic compounds emanating from many soils have been detected in agar (10,26,27) or agarose (18) incubated over soil. A fungistatic volatile compound that inhibited germination of *C. victoriae* was detected in acidified aqueous extracts from soil in another study, but the effect of acidity on the spores was not considered, and fungistatic activity was detected only if spores were added to the extract during the extraction process (11). In another study, volatile compounds from soil were collected in activated charcoal cooled with liquid nitrogen, and the concentration of these compounds required to inhibit fungal spore germination was determined (25). Of the compounds tested, ammonia was probably the fungistatic compound, but only in alkaline soils. However, in that study, the material in the cold trap was not bioassayed. In our study, material collected in a cold trap was not fungistatic.

In several of our experiments, loss of fungistatic activity probably was due primarily to loss of the volatile compound(s). For example, in the temperature experiments, when the extract was incubated at varying temperatures and then bioassayed, volatiles could have been lost when the vials were opened briefly to deposit the spores. However, loss of activity was not due entirely to gas loss. For example, a significant amount of activity was lost when the material was transferred with a gas-tight glass syringe. This may be a result of nonspecific adsorption onto both glass and plastic. Our data also suggest that the soil extract contains the fungistatic compound(s) in minimum effective concentration. After passage through an ultrafilter, the soil extract was fungistatic only when the filter had a small surface area and small void volume (data not shown). In addition, the material could not be diluted to less than 90% purity without losing all fungistatic activity.

The stability of fungistatic activity in soil extract was not altered by conducting the experiment in a photographic dark room with a red safety light rather than in the laboratory with sunlight and fluorescent lighting (data not shown). Thus, there is no evidence the compound is light-unstable.

Soil extract was fungistatic only in the two most alkaline soils. We do not know if soil acidity is causally or only coincidentally related to fungistatic activity of soil extract. However, all five of the soils we tested were fungistatic in the direct, diffusion, and volatile assays on soil (18), and volatile fungistatic compounds were produced in soils at pH 3.8–8.8 (18,27). In the experiments reported here, fungistatic activity apparently was not affected by

TABLE 3. Effect of pH on fungistatic activity in agarose blocks incubated on Panoche clay loam soil[x]

Buffer in agarose[y] (100 mM)	pH of agarose[z]		Germination (%) ± SEM	
	Before	After	On soil	No soil
MES	5.5	5.9 ± 0.04	2 ± 0.6	81 ± 2
	6.0	6.2 ± 0.03	1 ± 0.7	79 ± 4
	6.5	6.7 ± 0.06	1 ± 0.4	71 ± 4
	7.0	7.1 ± 0.03	0 ± 0	69 ± 4
HEPES	6.5	6.8 ± 0.07	0 ± 0.5	77 ± 2
	7.0	7.0 ± 0.01	0 ± 0	76 ± 5
	7.5	7.4 ± 0.03	0 ± 0.2	61 ± 5
	8.0	7.8 ± 0.01	0 ± 0	45 ± 2
None	5.8	7.1 ± 0.1	1 ± 0.6	91 ± 2

[x] Buffered 1% (w/w) agarose blocks (1 × 1 × 0.5 cm high) were incubated for 18 h either on soil in the diffusion assay or in no-soil control dishes. *Cochliobolus victoriae* conidia were then placed on the blocks for a bioassay of fungistatic activity. Data are the means ± SEM of two trials with four replications per trial.

[y] MES = 2-[*N*-morpholino]ethanesulfonic acid; HEPES = *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid].

[z] Before and after incubation on soil.

TABLE 4. Fungistatic activity of extracts from five loam soils, as measured by germination of *Cochliobolus victoriae* conidia[w]

Loam	Soil pH[x]	Germination[y] (% ± SEM)
Panoche clay	7.7	18 a
Yolo sandy	7.1	34 a
Holdrege silt	6.3	80 b
Boyer sandy	6.2	72 b
Holland sandy	5.7	82 b
No soil[z]	6.1	75 b

[w] Soil extract was expressed into open vials, which were immediately capped and bioassayed.

[x] Except for the Yolo loam, all soil pH values were reported previously (18).

[y] Data from four trials, each with one to three replicates were analyzed by an analysis of variance as a randomized complete block design. Block effect was not significant ($P = 0.42$), effect of soil type was highly significant ($P < 0.0001$), and block-soil interaction was not significant ($P = 0.57$). Means followed by the same letter are not significantly different according to Fisher's protected LSD ($\alpha = 0.05$).

[z] Ten millimolar 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer.

the pH of the agarose blocks in the diffusion assay. However, our experimental design did not allow us to distinguish between effects of pH directly on the spores versus effects on the fungistatic compound.

A review of the literature on soil fungistasis (21) suggests soil fungistatic compound(s): are produced in essentially all agricultural soils (18,27); are produced by a variety of bacteria (7); cause a reversible inhibition of germination of conidia of *C. victoriae* and other fungistasis-sensitive fungi; are water-soluble and volatile (18); and are fungistatic at concentrations commonly found in soil and not fungistatic at concentrations commonly found in ambient air. Based on these criteria, we selected gases to bioassay for fungistatic activity. In contrast to some previous studies in which fungistatic activity of particular soil gases was tested (25,28), we conducted the bioassay on nutrient-free agarose and, when necessary, buffered the agarose.

Previously, relatively few gases were evaluated as the potential soil fungistatic compound. Smith (30) proposed that ethylene caused soil fungistasis and regulated soil respiration (31), but others have disputed this claim (reviewed in 22). Although Smith (30) reported that the head space over moist soil typically contained ethylene at 10–30 μl/L, others reported that flooded soil contained ethylene at only 0.5-16 μl/L (0.5–1.8 Pa) (15). Similarly, Ioannou et al (14) reported mean maximum soil ethylene concentrations of 6.5 μl/L in flooded soil and lower concentrations (1–3 μl/L) in soils that were not flooded. Although Smith (30) reported that as little as 1 μl/L inhibited germination of *C. sativus* conidia and *Sclerotium rolfsii* sclerotia, in our tests, ethylene at 1 and 10 μl/L did not inhibit germination of *C. victoriae* conidia. Similarly, germination of *Verticillium dahliae* microsclerotia was not affected by ethylene concentrations from 0 to 35 μl/L (13).

Ammonia was proposed as a volatile soil fungistatic compound, especially in alkaline soils (reviewed in 22). Concentrations of ammonia at 34–94 μl/L were measured in head space over soils and were correlated with reduced germination of *Gonatobotrys simplex* and *Penicillium chrysogenum* conidia (25). Schippers et al (28) reported ammonia at only 2–17 μl/L in the atmosphere above an alkaline soil; concentrations as low as 2 μl/L inhibited germination of conidia of *Botrytis cinerea* and *P. nigricans*. However, in both papers, the authors did not use a buffer, and, consequently, the effects of high pH were not separated from ammonia per se. In addition, the authors do not appear to have accounted for the high solubility of ammonia in water. In our tests, atmospheres containing ammonia at 10, 100, and 257 μl/L were not fungistatic to spores on buffered agarose blocks, indicating *C. victoriae* conidia are relatively insensitive to ammonia.

In conclusion, soil fungistatic compound(s) can be trapped in agarose or extracted from soil in water. Both of these methods yield a sterile but complex mixture of chemicals. The mixture is difficult to purify because the fungistatic material is volatile and apparently present in our preparation of soil extract at concentrations near the limit of its biological activity. Our data indicate that none of the following gases are solely responsible for soil fungistasis: CO, CO_2, NO, NO_2, SO_2, NH_3, C_2H_4, and low concentrations of O_2. However, we cannot preclude the possibility that fungistasis is caused by a combination of gases, which might act synergistically. Currently, we are bioassaying other potential fungistatic gases in soil, especially gaseous sulfur-containing compounds produced by soil bacteria (2,17). Once we identify the compound(s), we can determine if other nutrient-independent, fungistasis-sensitive fungi are regulated by the same compound(s).

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