Isolation and Identification of a Volatile Fungistatic Substance from Alkaline Soil

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ABSTRACT

A volatile fungistatic substance was extracted with water from alkaline soil. The inhibitor in the extract volatilized at pH 7.6 but not at pH 2. Crystals were obtained when acidified distillate containing the inhibitor was evaporated to dryness. The unknown crystals were identified as ammonium chloride by infrared spectroscopy and X-ray powder diffraction. The biological spectra of the volatile inhibitor from soil, soil extract and ammonium chloride solution were identical. The results indicate that the fungistatic substance volatilized from alkaline soil is ammonia.

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Most fungi exist in soil as spores (12) and the ability of spores to rest in soil without germination, a phenomenon commonly called soil fungistasis, is widespread in nature (4, 10). The fungistatic effect disappears when soil is sterilized or when nutrients are added. Soil fungistasis has been attributed to unavailability to spores of the exogenous and endogenous nutrients required for germination in soil, due to microbial competition (9).

Recently a volatile inhibitor has been detected in some alkaline soils (5). The nature of the volatile inhibitor is different from that of widespread soil fungistasis. The volatile inhibitor is inhibitory to ascospores of *Neurospora tetrasperma* which are not sensitive to soil fungistasis in most soils, and is present in sterilized soil as well as nutrient-amended soil (7). The inhibitor was shown to be inorganic in nature (8). Despite differences in inhibition characteristics, the volatile inhibitor may also contribute to failure of fungal spores to germinate in these soils.

We report herein the isolation and identification of a volatile inhibitor from alkaline soil.

MATERIALS AND METHODS.—The following bacteria were maintained on a nutrient agar (per liter: 10 g of maltose, 4 g of yeast extract, 4 g of dextrose, 20 g of agar): *Agrobacterium tumefaciens* (Smith & Townsend) Conn, *A. radiobacter* (Beijerinck & van Delden) Conn, *Bacillus megaterium* de Bary, *B. subtilis* Cohn emend Prazmowki, *Escherichia coli* (Migula) Castellani & Chalmers, and *Xanthomonas campestris* (Pam.) Dows. These bacterial cultures were supplied by I. W. Buddenhagen. Fungal spores were obtained from cultures grown on V-8 juice agar: Conidia of *Aspergillus fumigatus* Fresenius, *Calonectria crotalariae* (Loos) Bell & Sobers, *Mucor ramannianus* Möller, and *Penicillium frequentans* Westling; and macroconidia of *Fusarium solani* (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans. Ascospores of *Neurospora tetrasperma* Shear & Dodge were obtained and used as previously described (7).

Dark magnesium clay (clay, pH 7.6) collected from the Island of Oahu and Colorado swale soil (clay loam, pH 8.3) were used (8). The volatile inhibitor was extracted by submerging 100 g of soil in 200 ml of distilled water for 3 days. The extracts obtained by decantation were centrifuged at 27,000 *g* for 15 min, and the supernatant filtered through Whatman No. 1 filter paper. Soil extracts thus prepared were dialyzed against distilled water or treated with ion-exchange resins as previously described (6) to study the effects of these treatments on volatile inhibitors in the extracts.

A volatile inhibitor was also trapped in moistened filter papers incubated approximately 15 mm above the Colorado soil in a large petri dish (150 × 20 mm). Ten sheets of Whatman No. 1 filter paper (150 mm diam) moistened with 40 ml of distilled water were placed on the inside surface of a petri dish cover. The bottom of the petri dish contained 100 g of soil moistened with 65 ml of distilled water. After a 3-day incubation at room temp, filter papers from 10 plates were soaked in one liter of distilled water. The inhibitor in water was squeezed out from the filter papers, and the solution was steam distilled. The 10-ml fractions which contained the inhibitor were pooled. The inhibitory distillate was adjusted to pH 2.0 with 5 N HCl, and evaporated to dryness at 90 C. The resulting crystals of the volatile inhibitor were analyzed by infrared spectroscopy using the KBr pellet technique and X-ray powder diffraction method. Analysis by X-ray powder diffraction was done by the Interpretive Analytical Services, Dow Chemical Co., Midland, Mich., U.S.A.

For assaying the volatile inhibitor, water agar disks (Noble agar, Difco Laboratories) placed on the inside surface of a small petri dish cover (50 × 15 mm) were incubated approximately 5 mm above the solution containing the inhibitor in the bottom of the petri dish. After 12-h incubation at 24 C, a conidial suspension of *Penicillium frequentans* was pipetted onto the disks and was exposed to the same solution for 12 h before germination was counted. Spore germination on agar disks exposed to distilled water was used as a control. To quantify the volatile inhibitor, various amounts of inhibitory solution were included in each experiment. One unit was defined as the amount of inhibitor volatilized from the solution which was capable of reducing spore germination to 50% as determined from a dosage-response curve.

TABLE 1. Effects of different treatments of soil extracts on the activity of a volatile fungal inhibitor

Treatment	Germination (%)[a]	
	Colorado soil	Oahu soil
No treatment	0	0
Cation exchange resins	95	97
Anion exchange resins	0	0
Dialysis	99	94
Adjusted to pH 2[b]	93	95
Adjusted back to pH 7.6[b]	0	0
Water, control	96	95

[a]Conidia of *Penicillium frequentans* placed on pre-incubated water agar disks were incubated above extracts for 16 h before germination was determined.

[b]Soil extracts were adjusted from pH 7.6 to 2.0. After 12 h, half of them were adjusted back to pH 7.6 again.

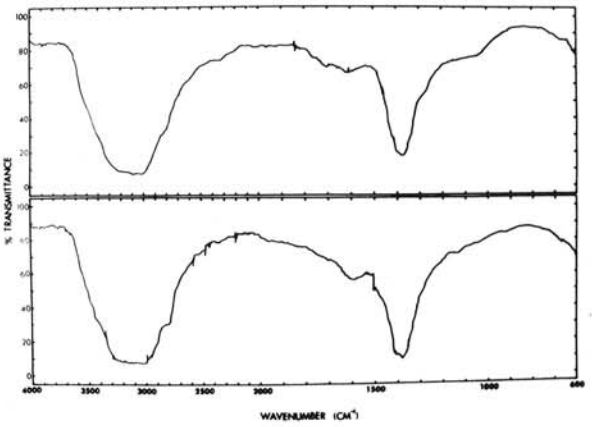

Fig. 1. Infrared spectrograms of unknown crystals (upper curve) originated from the Colorado soil and NH_4Cl (lower curve) taken in KBr pellets.

Soil, soil extract, and NH_4Cl solution containing 20 units of the volatile inhibitor were used to determine the similarity of the biological spectrum of the volatile inhibitor from each source. The effect of the volatile inhibitor on bacteria was determined by comparing the growth visually on a disk of nutrient agar (per liter: 2.4 g Bacto dehydrated potato-dextrose broth, 20 g agar) with and without exposure to the inhibitor. Spore-germination tests were used to study the effect of the volatile inhibitor on fungi.

RESULTS.—*Isolation and characterization of the volatile inhibitor.*—Aqueous extracts of Colorado and Oahu soils contained, respectively, about 8.4 and 0.5 units of the volatile inhibitor per ml. No volatile inhibitor was detected in the extract from a separate soil which did not contain the inhibitor. Extracts from both Colorado and Oahu soils did not release the volatile inhibitor when pH was decreased from 7.6 to 2.0 (Table 1). The volatile inhibitor was released from the extracts again when their pH was adjusted back to 7.6 after 24 h. The extracts released the volatile inhibitor following treatment with anion exchange resins (Table 1). However, no inhibitor was detected in the extracts after dialysis or treatment with cation exchange resins. This indicated that the inhibitor existed in aqueous solution as a small cationic molecule and that it was able to volatilize from solution only at high pH but not at low pH.

Identification of the volatile inhibitor.—The infrared absorption spectra of the unknown crystals and NH_4Cl taken in KBr pellets were identical at every major point of reference (Fig. 1). Analysis of the unknown crystals by X-ray powder diffraction also showed that the major constituent was NH_4Cl and the minor constituent ($<5\%$) was NaCl. From the data presented, it seems justifiable to conclude that the unknown volatile inhibitory substance is NH_4Cl.

The biological spectra of the volatile inhibitor from soil, soil extract and NH_4Cl solution were identical (Table 2). The volatile substance was inhibitory to spore germination of *N. tetrasperma, M. ramannianus, A. fumigatus,* and *P. frequentans,* but stimulatory to the growth of *B. subtilis, B. megaterium, A. radiobacter, A.*

TABLE 2. Biological spectra of the volatile fungal inhibitor from soil, soil extract, and NH_4Cl solution[a]

Microorganism[b]	Soil	Soil extract	NH_4Cl solution
Fungi:			
Caloncectria crotalariae (conidia)	0[c]	0	0
Fusarium solani f. sp. *phaseoli* (macroconidia)	0	0	0
Neurospora tetrasperma (ascospore)	−	−	−
Mucor ramannianus (conidia)	−	−	−
Aspergillus fumigatus (conidia)	−	−	−
Penicillium frequentans (conidia)	−	−	−
Bacteria:			
Escherichia coli	0	0	0
Bacillus subtilis	+	+	+
B. megaterium	+	+	+
Agrobacterium radiobacter	+	+	+
A. tumefaciens	+	+	+
Xanthomonas campestris	+	+	+

[a]The activity of the volatile inhibitor from each source was 20 units (0.5 g of the Colorado soil, 10 ml of the Colorado-soil extract, and 4 ml of 100 μg/ml NH_4Cl solution).

[b]Spore germination was used for fungi and visual colony growth was used for bacteria.

[c]0 = no effect; − = inhibition; + = stimulation.

tumefaciens, and *X. campestris.* It had no effect on growth of *E. coli,* or spore germination of *C. crotalariae* and *F. solani* f. sp. *phaseoli.*

Aqueous solutions containing 100 ppm of isolated crystals, NH_4Cl, and $(NH_4)_2SO_4$ released the volatile inhibitor at pH 7.6 but not at pH 2. The activity of these three compounds was 60, 70, and 55 units of volatile inhibitor per mg, respectively. None of them released the volatile inhibitor in crystal form. NaCl at the same concn did not release volatile inhibitor at either pH. These results suggest that the substance responsible for inhibition of spore germination is ammonia.

DISCUSSION.—Ammonia and most ammonium salts are very water-soluble (3). However, it was difficult to extract the volatile inhibitor with water from soil. Even when soil was submerged in water for several days, only a small portion of the volatile inhibitor was extracted from the soil. The volatile inhibitor still could be detected in the soil after several extractions. Evidently, ammonia or ammonium is fixed in soil in a relatively nonchangeable form (1).

Despite the complex nature of soil, there have been very few reports of isolation of microbial inhibitors from nonamended natural soil. In addition to the present communication, Ko and Hora (6) also reported the isolation of a nonvolatile fungitoxin from certain acid soils. The toxic substance was subsequently identified as an Al ion.

Natural soils, even under dry conditions, are capable of absorbing ammonia from the atmosphere (11). Almost no ammonia volatilizes from alkaline soils under natural conditions because air-dried soils do not release ammonia (8) and alkaline soils are characteristic of arid and semi-arid regions (2). This may account for the continuous existence of the volatile inhibitor in these soils.

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