The Nature of a Volatile Inhibitor from Certain Alkaline Soils

W. H. Ko and Frances K. Hora

Assistant Professor and Technician, respectively, Department of Plant Pathology, University of Hawaii, Hawaii Agricultural Experiment Station, Hilo 96720.

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ABSTRACT

A volatile fungistatic substance released from remoistened, air-dried alkaline soils was inhibitory to conidia of fungi as well as to ascospores of *Neurospora tetrasperma* which are not sensitive to widespread soil fungistasis. The volatile inhibitor existed in autoclaved and gas-sterilized soils, but disappeared when soil was treated with HCl. Its inhibitory effect also was not affected by addition of nutrients. Therefore, the nature of the volatile inhibitor in certain alkaline soils is different from that of widespread soil fungistasis.

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Dobbs & Hinson (2) originally observed the inability of most fungal spores to germinate in soil. This widespread phenomenon, termed soil fungistasis or mycostasis, is nullified by sterilization of soil or addition of nutrients (2, 9). Soil fungistasis frequently has been attributed to the presence of diffusible inhibitory substances in soil, but despite numerous attempts, no conclusive evidence has been obtained to support this assumption (9). On the other hand, Ko & Lockwood (7) provided evidence which suggests that soil fungistasis is a consequence of unavailability in soil of nutrients required for spore germination. Recently, both direct (3) and indirect (1) evidence for the existence of a volatile inhibitor in certain remoistened air-dried alkaline soils has been reported. In this paper, we report the characteristics of inhibition of spore germination due to the volatile inhibitor in soil and the dissimilarity of this phenomenon to soil fungistasis.

MATERIALS AND METHODS.—A Red Desert soil (sandy loam, pH 8.2) and an uncultivated soil of Dark Magnesium Clay (clay, pH 7.6) were collected from the Islands of Hawaii and Oahu, respectively. A clay loam soil (pH 8.3) used by Hora & Baker (3) to demonstrate the presence of a volatile inhibitor was obtained from R. Baker, Colorado State University. Soil was stored air-dried until used. Carbonate content of soil was determined by the method of gravimetric loss of CO_2 (10), and expressed as $CaCO_3$ equivalent in per cent.

Conidia of *Trichoderma viride* Fr., *Mucor ramannianus* Möller, *Aspergillus fumigatus* Fresenius, and *Penicillium frequentans* Westling were obtained from cultures grown on potato-dextrose agar (PDA). Ascospores of *Neurospora tetrasperma* Shear & Dodge were collected from cultures grown on an agar medium containing 10 g maltose, 4 g yeast extract, 4 g glucose, and 20 g agar/liter distilled water. They were separated from conidia by sedimentation in a column of distilled water and heat-activated at 58 C for 20 min before use. The volatile inhibitor was trapped in agar discs (20 × 3.5 mm, Noble agar, Difco Laboratories) placed on the inside surface of a sterile cover of a petri plate (100 × 20 mm) which contained 50 g air-dried soil remoistened to 70% water-holding capacity, unless stated otherwise. Agar discs were about 8 to 10 mm above the soil surface. Soils in petri plates were sterilized by autoclaving for 15 min or by exposing overnight to propylene oxide in a desiccator (4). Sterility of soils was checked by transferring about 0.5 g treated soils to PDA plates. After incubation for 7 days at 28 C, no microbial growth was observed on the plates. Sterilized soil was moistened with sterile distilled water under aseptic conditions. After 24-hr exposure of discs above the soil surface at 28 C, spores in suspensions were transferred with a disposable pipette onto the discs, and incubated for 9 to 16 hr for conidia and 4 hr for ascospores. Agar discs exposed above distilled water in a petri plate were used as controls. Sterility of agar discs was checked by the method described previously. The agar discs remained sterile on PDA plates after incubation for 7 days at 28 C. The method of Lingappa & Lockwood (8) was used to recover spores directly from soil in studies of inhibition of spore germination on soil. The per cent spore germination was based on 200 spores counted for each treatment. All experiments were done in duplicate, and repeated at least once. Results presented are averages of experiments.

RESULTS.—*Inhibitory effects of the volatile inhibitor.*—Two alkaline soils from the State of Hawaii were used to compare with the Colorado soil for their production of the volatile inhibitor, using *T. viride* as the test fungus. Conidia of *T. viride* germinated 0, 20, and 66% on agar discs exposed to Colorado, Oahu, and Hawaii soils, respectively. Germination was 96% on control discs. Since agar discs were not in direct contact with soil, our results confirmed those of Hora & Baker (3) in demonstrating that a volatile inhibitor was released from remoistened air-dried alkaline soils. Among the

TABLE 1. Germination of fungal spores directly on natural, autoclaved, or nutrient-amended Colorado soil, or on agar discs exposed to the atmosphere directly above treated and untreated soils

	Germination (%)									
	T. viride[a] (Conidia)		*M. ramannianus* (Conidia)		*A. fumigatus* (Conidia)		*P. frequentans* (Conidia)		*N. tetrasperma* (Ascospores)	
Material	Soil	Agar	Soil	Agar	Soil	Agar	Soil	Agar	Soil	Agar
Natural soil	0	0	0	0	0	2	0	0	0	0
Autoclaved soil	0	25	0	0	8	13	0	29	0	0
Nutrient-amended soil[b]	0	1	0	0	0	10	0	0		
Distilled water, control		93		95		71		94		94

[a]*Trichoderma viride, Mucor ramannianus, Aspergillus fumigatus, Penicillium frequentans, Neurospora tetrasperma.*
[b]Soil was amended to contain 0.1% glucose and 0.1% peptone.

three soils tested, the volatile inhibitor released from Colorado soil was most inhibitory. Therefore, it was selected for further studies. In some experiments, Oahu soil also was included for comparison.

The inhibition spectrum of the volatile inhibitor was studied, using spores either sensitive or insensitive to soil fungistasis. Conidia of *T. viride, M. ramannianus, A. fumigatus,* and *P. frequentans,* which are sensitive to soil fungistasis (7, 9), did not germinate on agar discs exposed to Colorado soil or on soil directly, whereas 71 to 95% germinated on control discs (Table 1). However, ascospores of *N. tetrasperma*, which are not sensitive to soil fungistasis (5, 7, 8), were also inhibited on the activated agar discs and on soil directly. This indicates that the inhibition spectrum of the volatile inhibitor is different from that of widespread soil fungistasis.

The inhibition of *N. tetrasperma* ascospores by the volatile inhibitor was only temporary. After 4 more hr of exposure to Colorado soil, germination of ascospores increased from 0 to 98%. Germination of conidia of the test fungi on activated agar discs also increased from 0 to 90-100% when discs were transferred to PDA plates and further incubated for 24 hr. Therefore, in similar fashion to soil fungistasis, the volatile inhibitor is fungistatic rather than fungicidal.

Effect of sterilization on the volatile inhibitor.—One of the important characteristics of soil fungistasis is its absence in sterilized soil (2, 9). Apparently, the inhibitory effects of soil and the volatile inhibitor released from it were not destroyed by sterilization. Conidia of *T. viride, M. ramannianus, A. fumigatus,* and *P. frequentans* germinated 25, 0, 13, and 29%, respectively, on agar discs exposed to autoclaved Colorado soil (Table 1). None of them germinated on autoclaved soil directly. Ascospores of *N. tetrasperma* did not germinate on agar discs exposed to autoclaved Colorado soil or on autoclaved soil directly. Similar results were obtained with propylene oxide-sterilized Colorado soil. When Oahu soil was used, conidia of *T. viride* germinated 38% on agar discs exposed to autoclaved soil and 26% directly on autoclaved soil.

Effect of nutrient amendment on the volatile inhibitor.—The other important characteristic of soil fungistasis is its annulment by addition of nutrients (2, 9). Conidia of the test fungi germinated 0 to 13% in distilled water, and 85 to 99% in a nutrient solution containing 0.1% glucose and 0.1% peptone. However, no conidia germinated on Colorado soil amended to contain 0.1% glucose and 0.1% peptone, and only 0 to 10% germinated on agar discs exposed to nutrient-amended soil (Table 1). Ascospores of *N. tetrasperma* were not tested because they do not require nutrients for germination (7). When nutrient-amended Oahu soil was used, conidia of *T. viride* did not germinate on soil directly, nor did they germinate on agar discs exposed to soil. These data demonstrated that nutrients neither nullified the inhibitory effects of these soils nor prevented the release of the volatile inhibitor from them.

Effect of HCl treatment on release of the volatile inhibitor.—The carbonate contents of Colorado, Oahu, and Hawaii soils were 11.0, 21.7, and 10.5% $CaCO_3$ equivalent, respectively. To determine the relationship between carbonate content and the presence of the volatile inhibitor in soil, 50 g Colorado soil in 30 ml water was deprived of carbonates by slow addition of 20 ml of 5 N HCl (10). After 24-hr reaction, the soil suspension, which was pH 3.9, was air-dried with an electric fan for 24 hr. The air-dried soil was ground with a mortar, remoistened with 20 ml water, and assayed for the presence of the volatile inhibitor. Soil similarly treated with water was used as a control. No volatile inhibitor was released from HCl-treated soil. Conidia

TABLE 2. Germination of conidia on agar discs exposed to Colorado soil with or without HCl treatments or to distilled water control

	Germination (%)	
Material	*P. frequentans*[a]	*T. viride*
Soil treated with		
H_2O[b]	0	13
HCl[c]	99	98
Distilled water, control	99	95

[a]*Penicillium frequentans, Trichoderma viride.*
[b]Fifty g soil was suspended in 50 ml water overnight.
[c]Fifty g soil suspended in 30 ml water was treated with 20 ml of 5 N HCl overnight; pH of the soil suspension was about 3.9 after treatment.

of *P. frequentans* and *T. viride* germinated completely on agar discs exposed to HCl-treated soil, but did not germinate or germinated poorly on agar discs exposed to the control soil (Table 2). Oahu soil also failed to release the volatile inhibitor after it was treated with 2 times more HCl than Colorado soil.

DISCUSSION.—A volatile inhibitor was suspected previously as the possible cause of soil fungistasis; however, sufficient evidence could not be obtained to substantiate this assumption (9). Recently, Hora & Baker (3) obtained direct evidence for the presence of a volatile inhibitor in certain air-dried alkaline soils remoistened with water. Our results show that the volatile inhibitor, or the soil producing it, is inhibitory to ascospores of *N. tetrasperma* which are not sensitive to widespread soil fungistasis (5, 7, 8). Moreover, unlike soil fungistasis, the volatile inhibitor is very heat-stable and is present in sterile soils. Annulment by nutrients is the other characteristic of soil fungistasis (2, 9). However, inhibition of spore germination by the volatile inhibitor is not affected by addition of nutrients. The only similarity between soil fungistasis and inhibition due to the volatile inhibitor is that both are fungistatic. Therefore, it is suggested that the inhibition of spore germination due to the volatile inhibitor is different from that of widespread soil fungistasis.

The volatile substance has not been identified. Although there was no correlation between amount of the volatile inhibitor released and the concentration of carbonates in soils tested, no volatile inhibitor was detected after the carbonate content of soil was removed by HCl treatment. This suggests that the volatile inhibitor either was inactivated or escaped from soil along with CO_2 as a result of HCl treatment.

Recently a heat-stable, nonvolatile fungitoxin was detected in certain acid soils in Hawaii (5), and was subsequently shown to be an Al ion (6). Several features of the soil fungitoxin are similar to those of the volatile inhibitor. The soil fungitoxin is not widespread in soils, nor associated with microbial activity. Nutrients also only partially counteract the inhibitory effect of this soil fungitoxin. However, unlike the volatile inhibitor, the soil fungitoxin is fungicidal and is not active at pH 7.

Ko & Lockwood (7) provided evidence that the widespread soil fungistasis is a result of nutrient deprivation imposed by microbial activity in soil. Apparently, in certain acid and alkaline soils the inhibitory substance may be an additional factor responsible for failure of spores to germinate.

LITERATURE CITED

1. BALIS, C., & V. KOUYEAS. 1968. Volatile inhibitors involved in soil mycostasis. Ann. Inst. Phytopathol. Benaki, N.S. 8:145-149.
2. DOBBS, C. G., & W. H. HINSON. 1953. A widespread fungistasis in soils. Nature 172:197-199.
3. HORA, T. S., & R. BAKER. 1970. Volatile factor in soil fungistasis. Nature 225:1071-1072.
4. KLARMAN, W. L., & J. CRAIG. 1960. Sterilization of agar media with propylene oxide. Phytopathology 50:868.
5. KO, W. H., & F. K. HORA. 1971. Fungitoxicity in certain Hawaiian soils. Soil Sci. 112:276-279.
6. KO, W. H., & F. K. HORA. 1972. Identification of an Al ion as a soil fungitoxin. Soil Sci. 113:42-45.
7. KO, W. H., & J. L. LOCKWOOD. 1967. Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57:894-901.
8. LINGAPPA, B. T., & J. L. LOCKWOOD. 1963. Direct assay of soils for fungistasis. Phytopathology 53:529-531.
9. LOCKWOOD, J. L. 1964. Soil fungistasis. Annu. Rev. Phytopathol. 2:341-362.
10. RICHARDS, L. A. (ed.). 1954. Diagnosis and improvement of saline and alkaline soils. USDA Agr. Handbook No. 60, p. 105.