Soil Fungistasis: Evidence for an Inhibitory Factor

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ABSTRACT

Bioassay techniques previously used to detect germination inhibitors from soil were modified to increase their sensitivity. Nutrients, which tend to mask fungistatic inhibitors, were removed from Difco purified water agar discs by washing in running tap water for 90 min. Conidia of *Helminthosporium sativum* and *Fusarium solani* f. sp. *phaseoli*, not requiring exogenous nutrients for germination, were placed on these washed discs. Reduction of germination due to volatiles was similar to that observed for conidia in direct contact with seven soils of various textures and hydrogen ion concentrations collected from different locations. Inhibition due to volatiles was not detected in nonwashed discs. These results suggest that the phenomenon of soil fungistasis is due to an inhibitory factor which can be annulled with appropriate nutrients.

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Some of the properties of a volatile fungistatic factor (VFF) in soil recently described (5) correlate well with those of the widespread phenomenon of soil fungistasis originally reported by Dobbs & Hinson (2). Both are found in many soils; however, apparent activity is greater in alkaline than in acid soils (5, 6, 14). The VFF is absorbed on activated charcoal and becomes less active with increased soil depths (13). It is apparently biological in origin (6). Although the VFF may be extracted and is evidently soluble in water, it is quite ephemeral (7).

The role and significance of the VFF relative to soil fungistasis have not been established. Volatiles inhibitory to fungal spores have been detected most consistently from neutral or alkaline soils; activity in acid soils has been low or nondetectable (5, 6). Immediately after wetting dry soil, bioassays for inhibition due to volatiles have indicated high fungistatic activity which declines with time (5, 6, 13). In contrast, conidium inhibition is initially low after wetting in the phenomenon of soil fungistasis (2). The reason for these discrepancies may be: (i) the VFF in soil is only present under special circumstances (e.g., immediately after wetting alkaline soils) and thus has limited ecological impact (8), or (ii) the VFF is consistently present but in some soils may not be detectable with previous bioassay techniques. Evidence is presented in this paper supporting the latter hypothesis.

MATERIALS AND METHODS.–Soil fungistasis was detected by the direct assay (D) using conidia-impregnated membrane filters covered with soil and the sterile cellophane agar diffusion (SCAD) assay with agar discs inoculated with conidia placed on membrane filters on soil, as described previously (5). These assays were modified by substituting the nutritionally more inert Nuclepore® (General Electric Co.), membrane filters (General Electric, Inc.) for the Millipore® (Millipore Corp.) filters in the direct method and the cellophane in the SCAD method. Hereafter, the former modification will be described as the sterile Nuclepore agar diffusion (SNAD) assay. In some treatments, agar discs without conidia were incubated on Nuclepore filters on soil for 24 hr. The discs were removed from the soil, inoculated with a drop of aqueous conidial suspension, and placed in a moist chamber in the dark at 22 C for germination. This procedure will be referred to as SNAD, postgermination.

The soil emanation agar (SEA) assay for detection of the VFF, which consists of the suspension of conidia-inoculated agar discs over soil for germination (13), was modified to improve sensitivity. The nutrient content of the medium supporting the germinating conidia is known to have profound influences on soil fungistatic activity (3). Even 2% Difco purified agar discs supported germination of nutrient dependent conidia of *Aspergillus flavus* Link (12). Accordingly, such agar discs were washed in running tap water for various periods of time and attempts were made to germinate conidia of *A. flavus* (15 hr incubation) on their surfaces. Discs washed to 90 min did not support germination of this fungus (Fig. 1). Conidia of *Helminthosporium sativum* Pam., King & Bakke, which do not require exogenous nutrients (9, 11), did germinate (12-hr incubation). The same results were observed when discs were washed in distilled water indicating that the observed response was not due to toxic materials in the tap water. Thus purified water agar discs washed for 90 min were used in subsequent experiments in both SEA and SNAD methods to eliminate as much as possible the masking effect of nutrients on the activity of soil fungistasis.

Controls in all cases consisted of incubation of washed agar discs with conidial suspensions (for the SEA and SNAD methods) or conidia-impregnated, Nuclepore filters (for the direct method) without soil

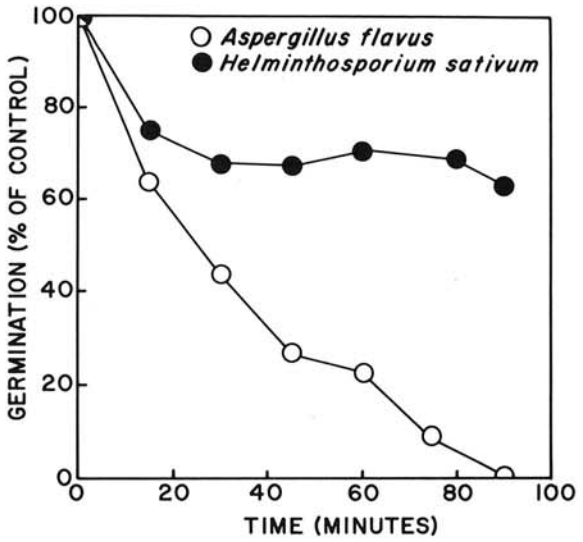

Fig. 1. Germination of *Aspergillus flavus* and *Helminthosporium sativum* on 2% Difco purified water agar discs washed for various time periods.

but otherwise under identical conditions.

Samples of seven soils of various hydrogen ion concentrations and textures (some properties listed in Table 1) were stored air-dried and moistened to 50 to 60% field capacity immediately before use. Moistened soils were incubated in Erlenmeyer flasks or petri plates in polyethylene plastic bags in an environmental chamber held at 22 C and 60% RH. Soil moisture content during the course of the experiments was not significantly altered.

RESULTS.—Germination results from experiments with seven soils using *H. sativum* as a test organism in the various bioassays are shown in Fig. 2. The means for all values for germination using each bioassay were 16% (D), 16% (SNAD), 18% (SEA), and 91% (SNAD postgermination) of controls. Thus substantial reduction in germination occurred using the first three methods. A 7 (pH levels) X 3 (test intervals) X 4 (bioassays) factorial analysis of variance of orthogonal comparisons was done to determine whether results from the various bioassays were different. The probability level ($P = 0.85$) indicated there were no differences between the data obtained using the D and SNAD methods. A P of 0.0028 was obtained between D-SNAD and SEA, indicating the former were different from the latter. When SNAD, postgermination data was compared with the other three methods, P was zero so there were very distinct differences.

The differences between D-SNAD bioassays and the SEA method were not extensive but were concentered in soils of extreme pH; i.e., 3.8 and 8.8 (Fig. 2). This may reflect the direct effect of pH on conidium germination especially in the D method. In Fig. 3, germination responses of *H. sativum* on buffered 2% Difco purified agar discs compared with that in distilled water at various hydrogen ion concentrations (pH adjusted with 0.1 M sodium phosphate buffer) is presented. Depression of germination was apparent at either high or low pH when conidia were suspended in buffered water solutions; this was not true for nonwashed agar discs.

Similar experiments were attempted using four of the soils and *Fusarium solani* (Mart.) Appel & Wr. f. sp. *phaseoli* (Burk.) Snyd. & Hans. as the test organism. Conidia, washed three times by centrifugation and resuspension in sterile distilled water, were used. Macroconidia of this organism at the densities used in these experiments (5) may not require exogenous nutrients (4); these conidia readily germinated after 12 hr of incubation on agar discs washed for 90 min. Germination was inhibited in the D, SNAD, and SEA bioassays in comparison to controls (Table 2). An exception was noted in the highly alkaline soil (pH 8.8), in which little inhibition due to volatiles was observed. Again very low germination was observed in soils with extreme hydrogen ion concentrations in the D method. No inhibition of germination was observed on nonwashed purified agar discs in the SEA bioassay.

DISCUSSION.—Similar germination responses were obtained using D, SNAD, and SEA bioassays.

TABLE 1. Properties of soils assayed for fungistatic activity

pH[a]	Texture	Origin	Organic matter (%)	NO_3-N (μg/ml)	NH_4-N (μg/ml)	Conductivity (salts) (mmhos/cm)
3.8	Clay loam	Viriginia (yellow podzolic)	0.3	2.6	8.84	0.4
4.8	Clay loam	Tennessee	0.7	4.3		
6.2	Silt loam	Kansas	2.9	7.1	6.52	0.5
6.9	Silt loam	Nebraska	1.3	50.0	7.25	1.4
7.6	Clay	Colorado (Mesa)	2.8	4.7	8.0	0.9
7.7	Silty clay	Colorado (Limon)	2.5	33.0	10.32	4.0
8.8	Clay	Colorado (Swale)	2.4	61.7	5.6	48.0

[a]Soil pH in 1:2 soil: 0.01M $CaCl_2$ suspension.

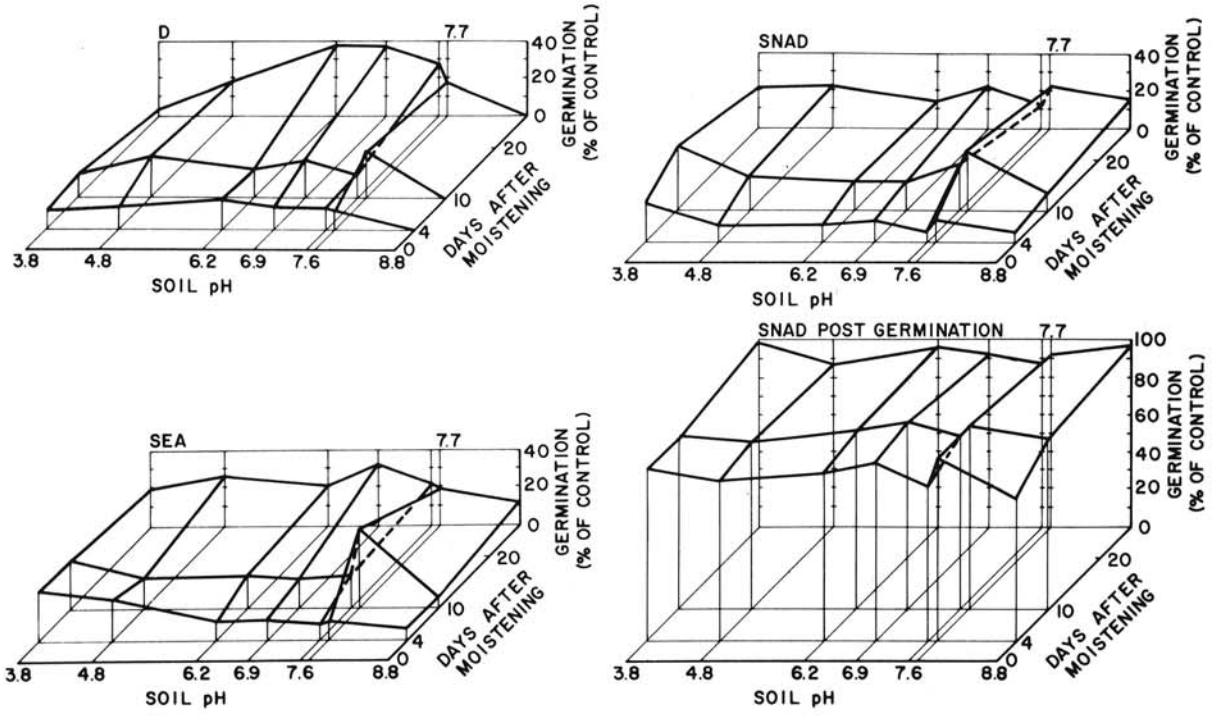

Fig. 2. Conidial germination of *Helminthosporium sativum* using the direct (D) assay, the sterile Nuclepore agar diffusion (SNAD) assay, and the soil emanation agar (SEA) assay in seven soils 4, 10, and 26 days after moistening. Postgermination response was also observed in the SNAD assay (SNAD-POSTGERMINATION). Germination in controls was 83-95%.

Relatively lower germination in soils of either high or low pH, especially in the D assay, may have reflected the direct effect of pH. Germination of *H. sativum* was reduced in distilled water buffered to relatively high or low hydrogen ion concentrations but not on nonwashed agar discs. The former situation is more likely when conidia on nutritionally inert Nuclepore filters were exposed directly to the soil in the D assay. Byther (1) also demonstrated profound influences of pH on germination of conidia of *F. solani* f. sp. *phaseoli* in solutions of low nutrient status.

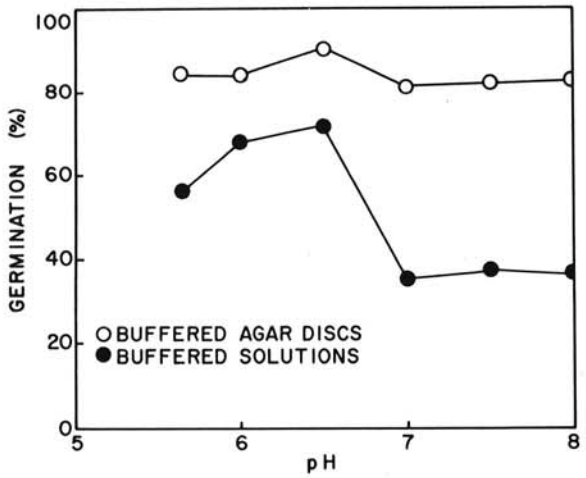

Fig. 3. Conidial germination of *Helminthosporium sativum* at various hydrogen ion concentrations using sodium phosphate buffers in water and in water agar discs.

The inhibition obtained using the SEA bioassay with soils having different properties, from different localities, and moistened for relatively long periods indicates that volatiles capable of suppressing germination may have widespread ecological significance. Two observations alleged to be inconsistent with this conclusion have been advanced (8). Firstly, inhibition due to volatiles persists after autoclaving, especially in soils of high pH. We have also observed this phenomenon (6). Presumably this conflicts with observations (using direct methods) indicating that spores inhibited in raw soil may germinate after soil has been sterilized (2). Interpretation, however, is not possible at present owing to lack of essential data. Certainly nutrients are released during autoclaving which can mask activity of possible inhibitors if direct methods are used. Secondly, delay in germination of ascospores of *Neurospora tetrasperma* Shear & Dodge, normally considered insensitive to soil fungistasis, by volatile inhibitors from a swale soil was reported (8). The impropriety of drawing conclusions from experiments involving soil using fungi not usually associated with this medium has been treated recently (15). But little inhibition of conidia of *F. solani* f. sp. *phaseoli* due to

TABLE 2. Macroconidium germination (expressed as percent of controls[a]) of *Fusarium solani* f. sp. *phaseoli* at various intervals after moistening soils of different hydrogen ion concentrations using the direct (D), sterile Nuclepore agar diffusion (SNAD), and soil emanation agar (SEA) assays for soil fungistasis

Soil pH	Days after moistening[b]											
	11				18				25			
	D	SNAD	SEA	SEA-NW[c]	D	SNAD	SEA	SEA-NW	D	SNAD	SEA	SEA-NW
	%	%	%	%	%	%	%	%	%	%	%	%
3.8	1	77	31	98	0	26	16	97	0	45	2	100
6.2	10	27	3	96	15	5	4	99	13	39	9	101
7.6	22	59	18	102	27	58	8	100	13	26	12	101
8.8	4	92	102	101	0	90	94	99	0	97	96	98

[a]Germination in controls ranged from 86-90% in D, 88-99% in SNAD, and 81-97% in SEA assays.
[b]Soils stored air-dried and moistened at 50-60% of field capacity at the beginning of the experiment.
[c]SEA-NW designates the SEA assay using nonwashed 2% Difco purified agar discs.

volatiles was also observed (Table 2) in the same (swale) soil. Apparently volatiles in some soils may affect germination differently in various fungi. Interpreted in this way, conidia of *F. solani* f. sp. *solani* would not germinate in this swale soil using direct methods and apparently soil fungistasis is operating; but in this case reduced germination is not due to volatiles but to the overriding and direct effect of pH.

Washed agar discs in contact with soil for 24 hr were not very fungistatic to *H. sativum* after their removal although incubation of conidia on similar discs on the soil (SNAD bioassay) resulted in germination inhibition. Interpreted according to the nutritional hypothesis, these agar discs were nutrient "sinks" only when they were in contact with soil (9); however, this would seem unlikely since discs not in contact with soil but exposed to volatiles (SEA bioassay) were fungistatic. The same reasoning would not support the alternate idea that fungistasis in the discs was generated by microbial activity at the agar-soil interface (10).

Whether soil fungistasis can be attributed solely to competition resulting in inadequate nutrients to support germination or to inhibitory materials in the soil mass cannot be resolved presently. The results reported in this paper, however, indicate that volatiles in soil can account for the observed depression of germination of conidia in the test fungi used. Further, need for washing of agar discs to improve sensitivity in the SEA assay underscores the importance of low nutrient status, an essential feature of the nutritional hypothesis (9), for detection of volatile inhibitory factors in soil. Perhaps the key question in resolving the current controversy (15) over the nature of soil fungistasis is whether spores with or without exogenous nutrient requirements could germinate in soil if inhibitory factors were removed. The data presented here, however, suggest that soil fungistasis involving propagules having no exogenous nutrient requirements for germination is due to an inhibitory factor which can be annulled with appropriate nutrients.

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