

Experimental Evaluation of Hypotheses Explaining the Nature of Soil Fungistasis

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ABSTRACT

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The quantity of nutrients available for germination of fungal propagules in soil has been a subject of controversy. A bioassay was used to detect the available nutrients and/or inhibitors in 17 soils (pH 5.1-8.6) at various intervals after moistening. To allow diffusion of soil solutions, washed agar disks were placed aseptically on sterile Nuclepore membrane filters resting on soil. After 22-24 hr of incubation, agar disks were removed and tested for ability to support germination of four test fungi. Both increases and decreases in germination of the test fungi were detectable in the bioassay. Increased

germination was best attributed to enhanced nutrient status of agar disks resulting from exposure to soil. Decreased germination was attributed primarily to inhibitory substances. Sterile filtrates of soil: water (1:1, w/v) extracts diluted to 1:50 supported germination of conidia of *Verticillium albo-atrum* which have an exogenous organic carbon requirement for germination. The results are best explained by hypotheses explaining the nature of soil fungistasis based on interactions between inhibitors and nutrients in soil.

Additional key words: spore germination, volatile fungistatic factor, *Gonatotryps simplex*, *Penicillium chrysogenum*, *Fusarium solani*.

Viable spores of most soil fungi do not germinate in natural soil except in the vicinity of suitable nutrients. This basic definition of the phenomenon of soil fungistasis is one of the few statements not under dispute in this area of research.

The nutritional hypothesis explains lack of germination of fungal propagules in soil solely as a consequence of the unavailability of required nutrients (16, 18). For spores that do not have an exogenous nutrient requirement, but still germinate poorly in soil, a microbial nutrient "sink" in soil prevents germination. The inhibitory hypothesis (1, 2, 6, 15, 22) suggests that propagules do not germinate in soil because of fungistatic substances. Appropriate nutrients overcome this inhibition. At any given time the relative impact of effective stimulators or inhibitors determines whether spore germination occurs.

In an experimental test of the nutrient hypothesis, it is necessary to know the nutrient status of soil: are there enough nutrients to permit germination of propagules in soil if inhibitors were not present? Is competition so intense that nutrients are actually removed from spores? Appropriate experimental evidence is lacking on these subjects (1, 6, 8, 16, 18, 19, 22). Indeed, current analytical methods are invalid since they do not assess the actual nutritional microenvironment surrounding a spore in

soil. Previous methods for direct bioassay are suspect because postulated and experimentally-detectable (9, 19, 23) germination inhibitors complicate interpretation because they mask the stimulators. An indirect method recently published (20) requires use of data on labeled spore exudates calculated from a control based on an analog (17) of a "sink" not yet sufficiently duplicated and demonstrated experimentally to exist in soil.

There is general agreement that bacteriologically filtered or sterilized aqueous soil extracts contain enough nutrients for spore germination of most soil fungi (6, 10, 12, 21). This may be evidence of sufficient nutrients for promoting germination in soil, assuming inhibitors (supposedly lost or masked during sterilization) are not present. Still, this is not conclusive since such recovered nutrients might be extracted from microsites more enriched with nutrients than the soil mass as a whole (16, 18).

In this paper, we describe indirect bioassays for nutrients promoting spore germination of soil fungi. We attempt to determine objectively whether the nutritional or inhibitory hypotheses of soil fungistasis can best interpret the results. A short report has been published (14).

MATERIALS AND METHODS

Fungi.—*Fusarium solani* (Mart.) Appel & Wr. emend. Synd. & Hans. isolated from peanut fruit (7),

TABLE 1. Properties of soils used in bioassays for nutrient status and fungistatic activity

Soil number	Soil pH	Soil Texture	Organic matter (%)	Lime ^a	DTPA-extractable micronutrients ^b								
					NO ₃ -N (μg/g)	NH ₄ -N (μg/g)	P ₂ O ₅ (μg/g)	K ₂ O (μg/g)	Na (μg/g)	Zn (μg/g)	Fe (μg/g)	Mn (μg/g)	Cu (μg/g)
1	5.0	Sandy clay loam	0.7	Low	<1	26	29	153	20	0.76	30.0	14.1	0.3
2	5.1	Loamy sand	2.6	Medium	51	35	30	85	37	2.40	106.0	2.5	2.9
3	5.2	Sandy clay loam	1.6	Low	<1	99	6	40	12	1.37	12.0	23.0	1.2
4	5.4	Silt clay loam	0.9	Low	<1	43	20	88	9	1.70	15.6	41.5	0.8
5	5.7	Silt clay loam	3.6	Low	<1	315	7	368	14	3.00	30.40	100.0	1.6
6	5.8	Sandy clay loam	1.1	Low	<1	9.4	17	146	345	32.50	35.40	35.0	11.8
7	5.9	Loam	4.3	Low	380	9.1	38	395	17	7.40	96.0	10.0	0.3
8	5.9	Loam	2.0	Low	33	11.7	11	193	16	0.87	35.00	11.9	0.2
9	6.0	Clay loam	2.9	High	42	25.0	48	430	10	1.28	20.9	58.0	1.4
10	6.1	Loam	ND	Low	ND ^c	ND	ND	ND	29	ND	ND	ND	ND
11	6.1	Clay loam	4.2	Low	165	15	8	393	13	2.20	27.80	2.2	41.0
12	6.6	Clay loam	4.5	Low	1	445	22	205	12	4.70	44.0	67.5	1.5
13	7.0	Sandy clay loam	1.5	Low	5	26	6	650	24	2.20	5.9	16.0	1.0
14	7.0	Sandy clay loam	2.6	Low	21	36	61	780	11	1.32	8.8	10.7	1.8
15	7.5	Sandy clay loam	7.8	High	16	129	9	480	7	5.50	16.0	24.0	0.8
16	7.8	Clay loam	0.7	High	100	282	360	70	1581	1.40	8.5	7.0	1.0
17	8.6	Clay loam	3.4	High	62	29.0	24	500	ND	10.00	10.0	6.4	1.8

^aLime, low = > 1%; medium = 1-2%; and high = <2%.

^bDTPA, diethylenetriamine penta-acetic acid extraction.

^cND = not determined.

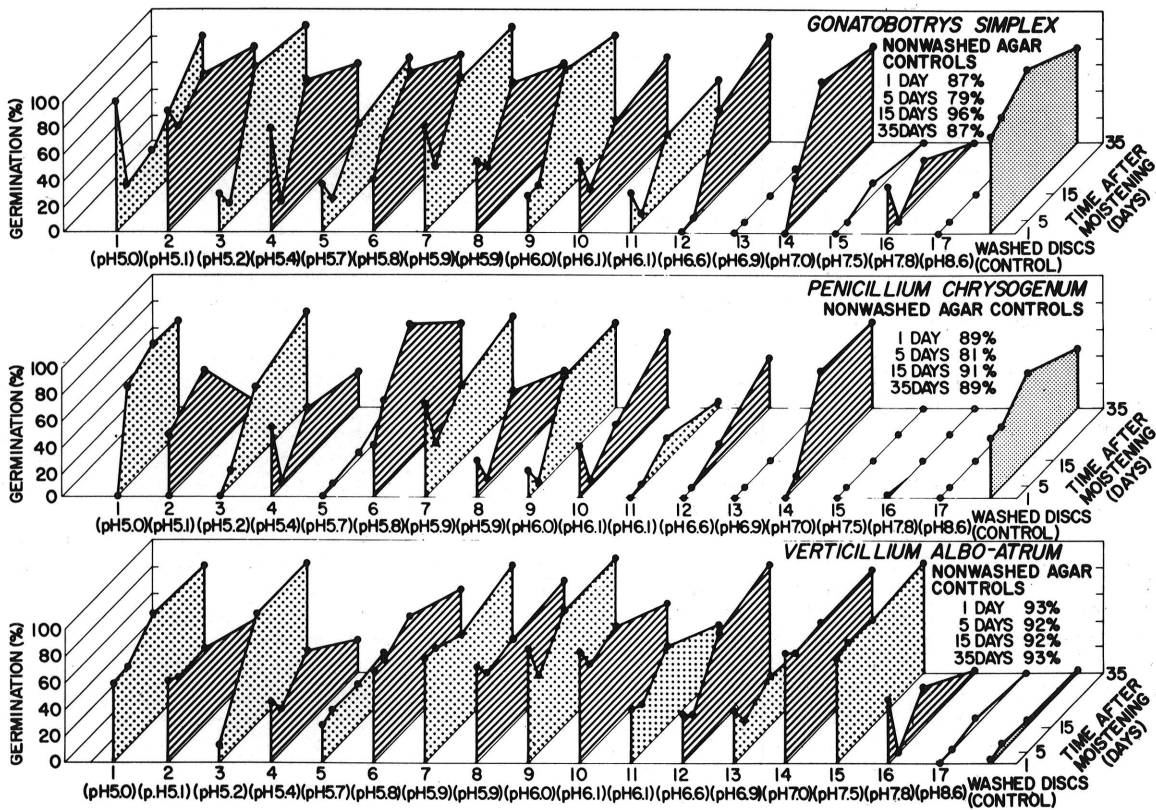


Fig. 1. Conidial germination of three soil fungi on washed water agar disks exposed at 25 C for 24 hr to 17 soils (soils 1 through 17) of different pH and incubated for 1, 5, 15, and 35 days, using sterile 'Nuclepore' agar diffusion (SNAD) technique.

Gonatotryps simplex Corda, *Penicillium chrysogenum* Thom., and *Verticillium albo-atrum* Reinke & Berth. were used as test fungi. *Fusarium solani* was grown on malt extract agar under light at 27-29 C for 2-3 wk. The other three fungi were cultured on potato-dextrose agar for 5-7 days at 25 C.

Soils.—Seventeen soils, collected from various parts of the United States, were used. General properties of 11 of these soils are outlined in Table 1. Some of these (soils 2, 11, 13, 15, 16, and 17) also were used in another investigation (9). Soils were air-dried before use and, at the beginning of experiments, dispensed in 50-g portions in deep 9-cm diameter petri dishes. Each sample was moistened to approximately 50% of field capacity by pipetting appropriate amounts of distilled water gently over the flattened soil surface and allowing equilibrium to be established. Water content of soils 2, 11, 13, 15, and 16 at 0.33 atm. has been reported (see Table 1, reference 9). These moistened samples in covered petri dishes were incubated at room temperature until assays were done. During incubation, soil moisture level in the samples was maintained weekly by appropriate additions of distilled water.

Hydrogen-ion concentration was determined in a suspension of 1:2 soil: CaCl₂ solution (0.01 M) for each soil sample at the beginning and again at the termination of an experiment. An occasional deviation of ±0.1-0.3 in pH values was observed.

Preparation of conidial suspensions.—Conidia of *F. solani* were washed four times by centrifugation at 150 rpm in 45-ml portions of sterile B solution (7). Conidia of *G. simplex*, *P. chrysogenum*, and *V. albo-atrum* were washed in sterile glass-distilled water or B solution using Tween-20 [polyoxyethylene sorbitan monolaurate, Atlas Chemical Industries, Inc., Wilmington, Delaware] as a surfactant, and centrifuged at 3,000 rpm for 15 min to sediment the spores.

Preparation of water agar disks.—In tests with *F. solani* water agar disks, 10 mm in diameter and 5 mm in thickness, were made from 2% Difco purified agar. To remove nutrients, the disks were washed in a 250-ml screw-cap Erlenmeyer flask (50 disks in 125 ml of sterile glass-distilled water), by shaking on a Burrell wrist-action shaker for 15 min 16 times. Water was replaced aseptically after each shaking. For *F. solani*, the spacing of macroconidia on agar disks chosen was such that macroconidial germination in washed agar disk controls was low and dependent on exogenous organic carbon sources (6, 7). Spore density in B solution was adjusted to

4×10^6 conidia/ml. Two hundredths milliliter of this suspension was placed on each disk.

Since conidial germination of *V. albo-atrum* was still high on the agar disks washed as described above, the approach was modified as follows: agar disks, 6 mm in diameter and 4-5 mm thick (150-200 disks in 200 ml sterile glass-distilled water), were shaken in a 500-ml screw-cap Erlenmeyer flask on a rotary shaker for at least 30 min. This was done six to seven times, and the water replaced aseptically after each cycle. After the last cycle, water was changed again, and the disks were left submerged at 1-2 C overnight. One day later, the disks again were washed six to seven times. This sequence was repeated over 3-5 days. Germination of spores of *V. albo-atrum* on disks receiving this treatment was below 20%. Germination of conidia of *G. simplex* and *P. chrysogenum* on such disks was lower than on nonwashed controls, but was not restricted as severely as for *V. albo-atrum*.

Assay.—Three assay techniques were applied to determine the presence of nutrients in soil.

Sterile Nuclepore agar diffusion (SNAD) method.—Washed agar disks were placed on sterile Nuclepore membrane filters (4.5 cm diameter; Nuclepore Corp., Pleasanton, California) in contact with soil samples moistened, as described previously, and incubated for 1, 5, 15, and 35 days in deep petri dishes. All soil samples were saturated with water before placement of the agar disks on filters, to facilitate diffusion of the soil solutions into the disks. After 24 hr at 25 C, the agar disks were removed aseptically, transferred to sterile glass slides in covered petri dishes containing moistened filter paper or distilled water, and conidia of the test organisms applied to the surfaces of the disks. Macroconidia of *F. solani* were incubated at 27-29 C for 7 hr at 27-29 C. Conidial germination of *G. simplex*, *P. chrysogenum*, and *V. albo-atrum* was measured after 20 hr of incubation at 24-25 C.

Aqueous soil extract dilution (ASED) technique.—Soil extracts were prepared from soils 2 (pH 5.1) and 11 (pH 6.1) using soil: glass-distilled water (1:1, w/v), and were sterilized by passage through a Morton filtration apparatus. The extract thus obtained was diluted aseptically 1:5, 1:10, and 1:50, with sterile 0.01 M sodium phosphate buffer (pH 6.0) solution. Washed spores of *V. albo-atrum* were added to small (5-ml) portions of the individual dilution series and the concentration of spores was adjusted to $2.0-2.5 \times 10^5$ conidia/ml. Five hundredths milliliter portions of the spore suspension from each dilution series was pipetted into double-depression slides in moist petri dishes, and

TABLE 2. Germination of *Fusarium solani* macroconidia on washed agar disks exposed to nine soil samples for 24 hr at room temperature (27-29 C), using the SNAD (sterile Nuclepore agar diffusion) technique

Experiment number	Time after moistening (days)	Controls		Conidial germination ^a on disks exposed to soil number									
		Nonwashed agar disks (%)	Washed agar disks (%)	1 (%)	2 (%)	7 (%)	8 (%)	11 (%)	14 (%)	15 (%)	16 (%)	17 (%)	
I	1	50	25		7				5	12	7	2	17
	7	52	21		3				3	15	2	3	17
II	2	79	13		25	47	36						
	8	77	13		20	67	32						

^aGermination after 7 hr on the surface of the disks.

incubated 25 C. Germination counts of conidia were recorded after 12 hr. A total of 400 spores were examined in each treatment. Controls consisted of spore germination tests in sterile glass-distilled water or buffer solution.

Direct (D) assay.—Conidial germination of *G. simplex* and *V. albo-atrum* was examined by the direct assay technique with six soils (soils 2, 11, 13, 15, 16, and 17) incubated for 1, 5, 15, and 35 days as described previously (11).

Germination tests in buffer solution.—Tests also were initiated to determine the influence of pH on the germination of *G. simplex*, *P. chrysogenum*, and *V. albo-atrum*. Washed agar disks were soaked in Good buffer (concentration 5 mM) solutions adjusted at pH 6.0, 7.0, 8.0, and 8.6, as previously described (14). Tests were also done with sodium phosphate (pH 8.0) and Tris (pH 8.6) buffer (concentration 0.03 M) solutions.

RESULTS

Germination on nonwashed agar disk controls was high, usually above 80% (Fig. 1). On washed agar disks, germination of *G. simplex* was comparable to nonwashed controls. For *P. chrysogenum*, germination on washed disks was approximately 50% of nonwashed controls. There was very low germination (8% or less) of conidia of *V. albo-atrum* on washed disks.

For all soils, germination was less than controls for *G. simplex* 5 days after moistening as measured by the SNAD bioassay (Fig. 1). Reduced germination was apparent at all times during the incubation period for soils 11, 13, 15, 16, and 17. In general, there was reduced germination of *P. chrysogenum* at all incubation periods

for most soils and most reduction of germination occurred over soils with lower hydrogen-ion concentrations. There were exceptions, however, in soils 1, 3, 6, and 14 after 6-15 days, where increased germination over washed-disk controls was observed. Substantial increases in germination of *V. albo-atrum* conidia occurred after moistening, in all except soil 17.

In a separate study of nine soils, *F. solani* macroconidia were tested by the SNAD bioassay. Germination was reduced to 13-25% on washed disks; but after the macroconidia were exposed to soils, substantial increases in germination were observed for soils 1, 7, and 8 (Table 2). Disks exposed to six other soils (soils 2, 11, 14, 15, 16, and 17) typically reduced germination of macroconidia in comparison with those on washed agar disk controls. In repeated experiments, trends for soils 7 and 14 were not as consistent as for the seven others.

Germination of *V. albo-atrum* in dilutions of filtered sterile soil extracts from soils 2 and 11 was measured (Fig. 2). Little or no germination of conidia was observed in buffer solution corresponding to responses on washed agar disks (Fig. 1). Increases of germination compared to the control were observed in the diluted aqueous soil extracts for soil 11 in almost all cases even to a 1:50 dilution. Increases were observed also for soil 2, but were not as substantial, especially at the highest dilution.

Direct assays for fungistatic activity were done for six of the soils 1-35 days after moistening. Soil fungistasis was present in all soils during the course of the experiment (Fig. 3). Spore germination of *G. simplex* was reduced 33-100% compared to controls on Millipore filters not exposed to soil. Germination in soils also was low in most instances using *V. albo-atrum*. Fifteen days after moistening, germination slightly exceeded the level observed on Millipore filter controls in soil 14 (pH 7.0).

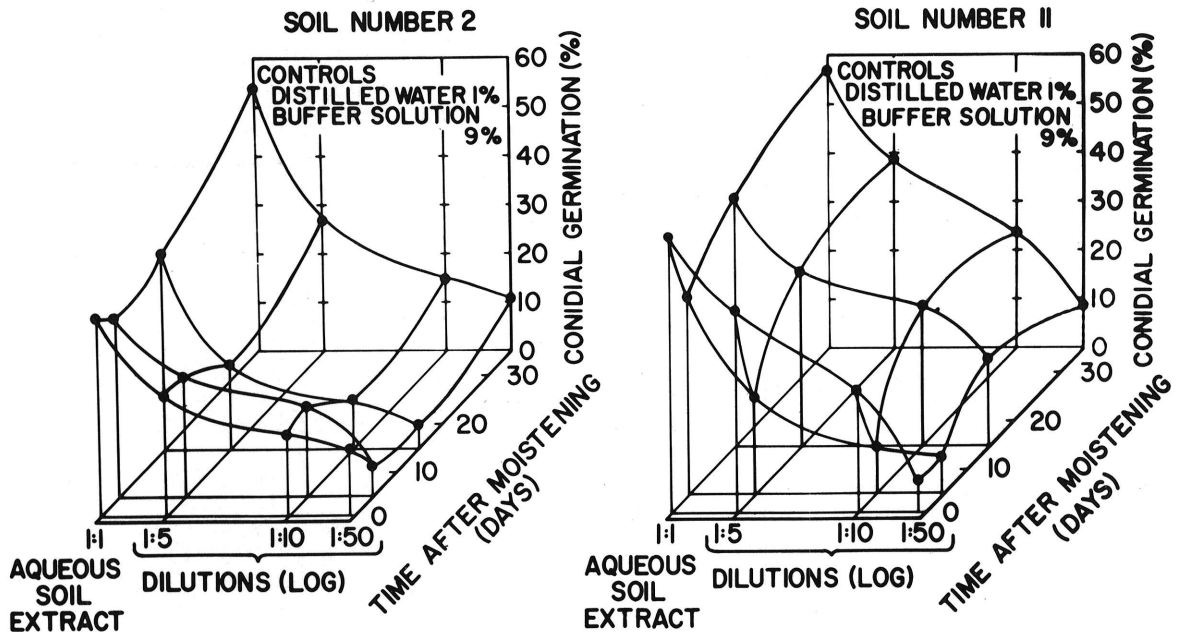


Fig. 2. Germination of *Verticillium albo-atrum* conidia in sterile aqueous soil extracts obtained from soils 2 and 11 (pH 5.1 and pH 6.1) incubated for 1, 5, 15, and 35 days, and diluted 1:1, 1:5, 1:10, and 1:50 with sodium phosphate buffer (0.01 M) solution.

In buffer solutions, germination of *V. albo-atrum* was unaffected between pH 6.0-8.6 (Fig. 4). In contrast, conidial germination of *P. chrysogenum* and *G. simplex* decreased sharply (60-90%) between 7.0 and 8.6 in Good buffers. Lowest germination was observed in Tris buffer at pH 8.6. Germination of *P. chrysogenum* conidia in sodium phosphate (pH 8.0) buffer was similar to that using Good buffer at pH 8.0. *P. chrysogenum* conidia germinated 80% in the former solution; however, germ tubes were severely restricted in length.

DISCUSSION

As demonstrated in Fig. 3, representative soils used in this investigation were fungistatic to conidia of the test organisms. The reason(s) for this is (are) still in controversy even though almost a quarter of a century has passed since the first clear demonstration of soil fungistasis (5).

The selection of the test fungi used for bioassay in this study was opportune. Germination of conidia of *G. simplex* on washed agar disk controls was comparable to that observed on nonwashed disks. In contrast, conidia of *V. albo-atrum* germinated readily on nonwashed disks but very poorly on washed disks. The response of *P. chrysogenum* and *F. solani* was intermediate (Fig. 1 and Table 2). Thus, bioassays using these organisms had the

potential of demonstrating either stimulation or inhibition of conidial germination.

Disks in contact with the soil solution, through an intervening sterile filter, supported much higher germination of conidia of *V. albo-atrum* in comparison with washed agar disk controls for all soils except one (soil 17); this was very alkaline and had a high salt content (Table 1). Similar results were obtained for some soils with conidia of *P. chrysogenum* (Fig. 1) and *F. solani* (Table 2). The plausible interpretation is that nutrients moved from the soil into the agar disks and supported germination when conidia were subsequently placed on their surfaces.

That this nutrient level in the extracted soil solution was significant is apparent from the results given in Fig. 2. Some increase in germination over that observed in distilled water controls of *V. albo-atrum* was seen even in soil solution dilutions of 1:50 from soil 11. What nutrient concentration does this actually represent in the soil solution? Soil 11 should contain approximately 0.2 ml water/g oven-dry soil (see Table 1, reference 9) in which nutrients would be dissolved. The soil-water suspension (1:1, w/v) used to prepare an extract, then, contained about 1.2 ml of water and 0.8 g of (oven-dry) soil, actually a 1:6 dilution of the soil solution. By the same reasoning, the 1:50 dilution of soil gave a dilution of at least 1:300 in terms of the soil solution. Even if inhibitors were not a factor, there must be nutrients in the soil solution in concentration many times the requirement of 5.3×10^{-3} ng carbon/conidium for complete conidial germination by *V. albo-atrum* (10); however, this does not obviate the possibility that nutrients are not distributed generally in the soil solution but may be concentrated at microsites (16, 18).

How can the results in this paper be interpreted in terms of either the nutritional or inhibitory hypothesis of soil fungistasis? The following points are relevant:

(i) Depression of germination (compared with washed agar controls) of conidia of conidia of *G. simplex* and *P. chrysogenum* on agar disks exposed to the soil solution (SNAD method) was observed in all soils sometime after moistening (Fig. 1). The observed suppression in alkaline soils for these two fungi can be attributed in large part to the direct effect (19) of pH (Fig. 4). To explain reduced germination in neutral or acid soils, however, the inhibitory hypothesis suggests that a fungistatic entity present in soils diffused into the disks. The nutritional hypothesis explains this phenomenon by assuming "loss of nutrients [essential for germination] from the agar" to the soil (16). The nutritional status of the agar disks used in this investigation was very low as evidenced by the inability of the disks to support germination of conidia of species having an exogenous requirement for germination (19); e. g., *V. albo-atrum*. To explain depression of germination of *G. simplex* and *P. chrysogenum* on agar disks, after exposure to soil, by the nutritional hypothesis, one must assume that the nutrient "sink" extracted even more nutrients from the disks. The same reasoning could be used to explain reduction of conidium germination of *F. solani* in some soils (Table 1).

(ii) When air-dried soil is moistened, nutrient substrates capable of supporting germination become available for microorganisms (8, 10). As illustrated in Fig.

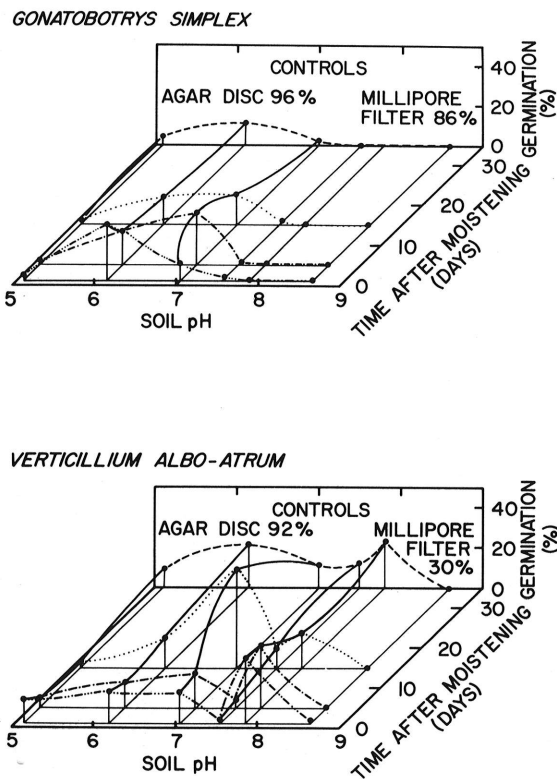


Fig. 3. Conidial germination of two soil fungi in six soils (soils 2, 11, 13, 14, 15, and 16) incubated for 1, 5, 15, and 35 days, by direct (D) assay.

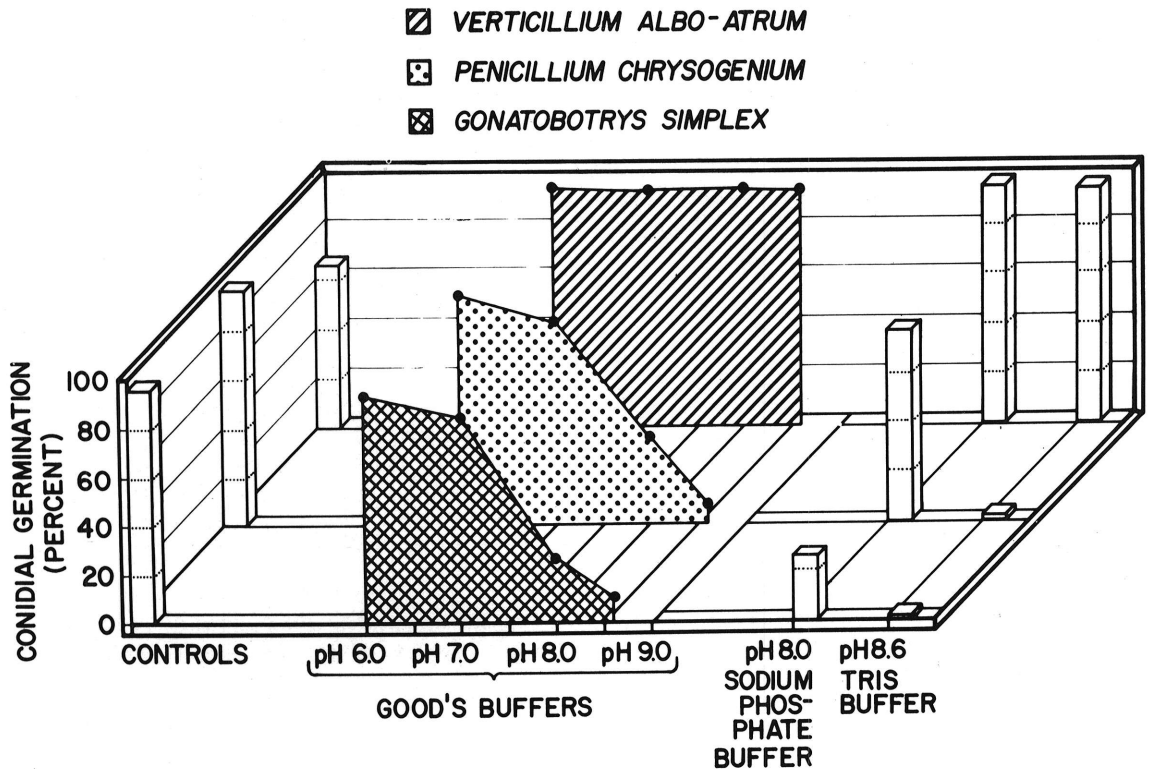


Fig. 4. Conidial germination of three soil fungi on washed agar disks pre-soaked in sterile Good ($5 \mu\text{M}$), sodium phosphate (0.02 M), and Tris (0.03 M) buffer solutions. Germination on washed agar disks constituted controls.

1, and discussed under (i), this expected stimulus to germination for *G. simplex* and *P. chrysogenum* did not occur; in most instances inhibition of germination was observed 1-4 days after moistening soils. In instances when soils incited depression of germination, *F. solani* responded similarly, and in some soils (e.g., soils 1, 3, and 12) *V. albo-atrum* germinated at a lower level during this period than 15-30 days after moistening. The nutritional hypothesis explains this in terms of more intense competition during the period immediately following moistening resulting in nutrients being very limited in disks, even on the first day. The inhibitory hypothesis, however, predicts more inhibitors being produced during periods of increased metabolic activity (the first 2 days after moistening) swinging the effective balance between germination inhibitors and stimulants absorbed by the disks in the direction of the former.

(iii) The nutritional hypothesis demands intense metabolic activity of microorganisms in the general soil solution as well as in the microsites, following moistening of soil, eventually reducing nutrients to an extreme deficit level over time. If this be true, germination on agar disks in the SNAD method should decrease with time after moistening. Just the opposite occurred (Fig. 1). The same response over time was observed when conidia of *V. albo-atrum* were germinated in soil extracts (Fig. 2). On the other hand, decreased metabolic activity over time should reduce generation of fungistatic compounds. The

same inhibitors would depress fungal and bacterial (3, 4) activity leading to a very small but unavailable residual pool of persistent nutrients: detectable by the techniques used to obtain data in Fig. 1 and 2. Increased germination in these bioassays 15 days after moistening would not be explained if inhibitors accumulated over time; however, the volatile nature of at least some of these has been established (11, 12, 13, 14, 19).

(iv) Finally, there was an increase (compared with washed disk controls) in conidial germination of *P. chrysogenum* on disks exposed to soils 1, 3, 6, and 14 at some period following moistening of soils (Fig. 1). In the same kind of experiment, *V. albo-atrum*, germinating at low levels in washed agar disk controls, germinated at high levels following exposure of disks to every soil but one. Macroconidia of *F. solani*, used on agar disks at densities high enough to suppress germination (i. e., self-inhibition) also were stimulated the same way in some soils. This germination is attributed to the increased nutrient status of the agar disks and the nutrients must have originated from the soil. The foregoing discussion of the results [especially (iii)] tends to decrease the importance of enriched microniches as the sole source of these nutrients; however, conidia of *V. albo-atrum* exposed to volatiles over some of the same soils used in these investigations, germinated on agar disks previously not capable of supporting germination (9). This forces the conclusion that enough nutrient in the volatile form alone

is present in soil to allow germination of conidia of this organism. It is not likely that volatiles are concentrated solely in discrete microniches. The nutritional hypothesis explains suppression of germination of *V. albo-atrum* in soil (Fig. 3) by the overriding effect of the nutrient "sink" even though there are adequate volatile nutritional stimulators present. Alternatively, the inhibitory hypothesis visualizes a dynamic situation involving both spore germination inhibitors and stimulators (6, 22). The net effect of these opposing influences suppresses conidium germination of *V. albo-atrum* conidia in soil unless nutrients adequate to overcome suppression are present.

We conclude that hypotheses concerning the nature of soil fungistasis involving inhibitory substances as well as nutritional factors in soil best explain the results reported in this paper.

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