Abiotic Generation of a Volatile Fungistatic Factor in Soil by Liming

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ABSTRACT

Application of lime as 1:1 $Ca(OH)_2$: $CaCO_3$ to raw soil, and to soil either before or after autoclaving, resulted in the production of fungistatic volatile(s). The volatile factor(s) could be induced in a slightly acid aqueous soil extract made alkaline by lime or tris(hydroxymethyl)aminomethane (tris) solution in the absence of any culturable organisms.

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Additional key words: liming, volatile inhibitor.

The ancient Greeks and Gauls recognized the value of liming soils (20). Modern technology has verified the value of this practice since liming not only alleviates acidity in soil but also increases availability of some essential plant nutrients while reducing concentrations of available iron, aluminum, and manganese which may be toxic under very acid conditions (4). Information on the production of inhibitory substances in soil resulting from this practice, however, is presently nonexistent.

There have been recent reports (11, 12, 17, 18) that the germination of fungal spores and seeds is reduced in natural soil by a volatile inhibitory factor. This phenomenon has been observed more frequently and has more profound effects in alkaline than in acid soils, and may be a significant factor (3, 11, 14, 17, 18) in the widespread soil fungistasis (9) in which viable fungal propagules do not germinate in soil under conditions of temperature and moisture favorable for their germination. Soil fungistasis appears to have a biological origin (9, 16) and a volatile fungistatic factor has been detected in sterilized soil reinfested with actinomycetes, raw soil amended with chitin, and in pure cultures of odoriferous *Streptomyces* (13). Thus, studies on both the origin and effects of this volatile factor have significance in contributing to an understanding of soil microbial ecology and in explaining the mechanisms of biological control of soil borne plant pathogens (2). In this paper, occurrence of a volatile fungistatic factor in limed soil is described and evidence is presented that at least one step in the chemical transformations leading to its generation is physical rather than biological in nature.

MATERIALS AND METHODS.—Ten soils obtained from different parts of the United States were used in this investigation. Their origin and general properties are in Table 1.

Lime was applied to air-dry soil samples in the form of 1:1 $Ca(OH)_2$:$CaCO_3$ or 1:1 MgO:$MgCO_3$ in appropriate proportions, mixed in a twin shell blender, and moistened to 65-70% of water holding capacity. Hydrogen ion concentration was determined in a suspension of 1:2 soil:$CaCl_2$ solution (0.01 M) at the end of 24 h incubation at 25 C. At the termination of experiments, pH was again determined. An occasional deviation of ±0.1 - 0.2 was noticeable in soil pH values.

The proportion of lime in soil necessary to raise pH to the appropriate values was determined by experimentation. For instance, in a loam test soil (No. 70), pH values of 7.0, 7.5, 8.0, and 8.5, were developed from an initial pH of 6.3 by the application of $Ca(OH)_2$:$CaCO_3$ in the amounts of 1, 1.5, 2.5, and 5 mg/g soil, respectively.

Conidia of *Gonatobotrys simplex* Corda, *Penicillium chrysogenum* Thom, *Trichoderma viride* (Fr.) Pers., and *Zygorhynchus vuilleminii* Namyslowski were used to detect the presence of inhibitory factor(s) in soil samples using three assay techniques. First, the soil emanation agar (SEA) method detected the volatile inhibitor; 2% Difco purified water agar disk were suspended over

TABLE 1. Properties of test soils collected from various parts of the USA

Soil number	Soil pH	Origin and texture	Organic matter (%)	Lime	NO_3^--N (μg/g)	NH_4^+-N (μg/g)	P_2O_5 (μg/g)	K_2O (μg/g)	Na (μg/g)	DPTA[a] extractable micronutrients Zn (μg/g)	Fe (μg/g)	Mn (μg/g)	Cu (μg/g)
46	6.6	Colorado loam	2.0	low[b]	33	11.7	11	193	16.1	0.87	25.0	11.9	0.21
48	5.7	Colorado loam	4.3	low	380	9.1	38	395	17.3	7.40	96.0	10.0	0.32
49	5.7	Colorado loam	2.9	low	225	19.0	16	315	10.4	8.70	63.0	10.0	0.28
61	6.7	Colorado sandy loam	1.4	low	1	11.4	28	600	10.4	2.25	11.6	34.0	0.44
65	6.0	Colorado sandy clay loam	1.1	low	0.4	9.4	17	146	34.5	32.5	35.4	35.0	11.84
68	6.3	Nebraska loam	1.5	low	7.1	40.0	16.3	285	40.25	1.23	29.4	69.0	1.83
70	6.3	Colorado loam	1.2	low	22.6	12.2	13.0	263	8.5	1.50	39.3	91.0	2.56
74	6.4	Colorado loamy sand	0.7	low	74	9.6	16	260	5.8	1.26	12.4	24.0	0.52
95	6.3	Illinois loam	4.2	low	2	10.0	59	483	20.7	153.8	92.0	43.0	1.70
98	6.2	Iowa	3.1	Medium[c]	5	5.7	14	216	16.1	72.5	50.2	52.0	0.69

[a]DPTA, diethylenetriamine penta-acetic acid extraction.

[b]Low = < 1%.

[c]Medium = 1-2%.

moistened soil samples in closed Petri dishes at 25 C for 24 h and germination of the test fungi was subsequently followed on these disks. Second, the sterile "Nuclepore" agar diffusion (SNAD) method detected inhibition of conidial germination on agar disks in contact with soil; agar disks were placed on a sterile Nuclepore filter in contact with soil for 24 h at 25 C in a closed Petri dish and were subsequently tested for spore germination. Third, the direct (D) method measured inhibition of germination of conidia in direct contact with soil; conidium-embedded "Millipore" membrane filter strips (1 cm^2) were impregnated with conidial suspension, buried in soil in a closed Petri dish for 24 h at 25 C and examined for the germination of conidia. Four hundred conidia were observed over several microscopic fields (0.32 cm^2) in two replicates for each treatment. Controls consisted of incubation of agar disks (for the first two methods) or "Millipore" membrane filters (for the direct method) without soil, but otherwise under identical conditions. Details of all these techniques have been previously described (11, 13, 17, 18).

Sterilization was accomplished by autoclaving moistened soil samples in 50 g portions for 50 min at 121 C twice consecutively over 2 days. Tests to determine soil sterility were made by introducing particles of autoclaved soil into Difco AC medium (composition: proteose peptone, 2% Bacto beef extract, 0.3%, Bacto dextrose, 0.5% ascorbic acid, 0.2%; Bacto agar, 0.1%; final pH 7.2 at 25C) and incubating at 25 C for 3-4 days. Soil was also tested for sterility following completion of experiments.

Levels of CO_2 in both nontreated and limed soil samples remained below 0.06% by volume during experiments.

RESULTS.—Relatively small inhibition (0-42% of controls) due to volatiles was detected by SEA technique immediately after moistening ten soils of pH 5.7 - 6.7 (Fig. 1). With the application of lime to these soils [1:1 $Ca(OH)_2$:$CaCO_3$ at 5 mg/g of soil], germination of conidia of *P. chrysogenum, T. viride,* and *Z. vuilleminii* decreased 40-100% relative to control except for soil No. 65. While the germination of *G. simplex* over limed soils was not as much affected as the conidia of other soil fungi, length of the germ tube was drastically reduced.

Soil No. 70 was selected for more extensive study. Lime was applied to raw soil and to soil before or aseptically as aqueous suspension after autoclaving. SEA, D, and SNAD bioassays were made for fungistatic activity using four soil test fungi. In general, a decline in conidial germination of test fungi was evident in all bioassays when the soil pH was raised from 6.3 to 8.5 (Fig. 2). Inhibition curves for all test fungi in each soil treatment were approximately parallel with the possible exception of the response observed in SNAD method with limed soil used before or after autoclaving. A 5(pH values) X 3(bioassays) X 3(treatments) factorial analysis of variance of orthogonal comparisons confirmed a significant ($P = 0.05$) decrease in conidial germination in all bioassays for all treatments between soil pH 6.3 and 8.5.

Application of 1:1 MgO:$MgCO_3$ to soil was equally effective in the generation of volatile inhibitor (Table 2).

These results suggested that the volatile factor

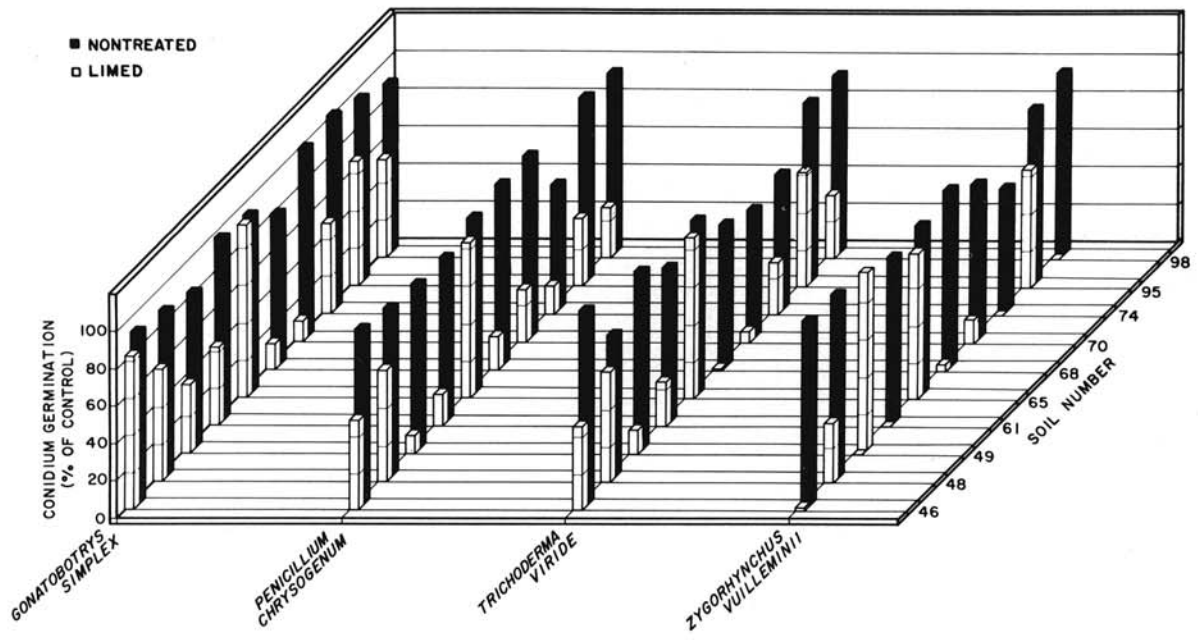

Fig. 1. Volatile fungistatic activity in ten test soils limed with 1:1 $Ca(OH)_2$:$CaCO_3$ measured by the soil emanation agar (SEA) method.

might be generated from the liquid phase when soil was made alkaline. Accordingly, a sterile extract was obtained from an aqueous suspension (1:1) of a slightly acid soil (No. 68) using Pyrex filtration apparatus with a fitted glass disk of pore size 0.9-1.4 μm. Failure to detect any contamination in AC medium verified the sterility of the extract. One ml of 0.2 M tris [tris(hydroxymethyl)aminomethane] solution or 0.5 ml of aqueous lime suspension containing 0.5 mg of 1:1 $Ca(OH)_2$:$CaCO_3$ was added aseptically into petri plates containing 20 ml portions of sterile aqueous soil extracts (final pH 9.0 and 8.8, respectively). Water agar disks were held over the extracts in the closed petri dishes for 24 h at 25 C. Conidia of four test fungi were seeded on agar disks and spore germination was observed. High volatile fungistatic activity was present in the extracts treated with tris or lime (Fig. 3). Attempted culture for contaminants in AC medium again confirmed the sterility of the treated extracts.

DISCUSSION.—Extensive interpretation of the data on SNAD and D bioassays (Fig. 2) is not possible. These methods expose propagules to nutrients which become available when soil is moistened or autoclaved. Nutrients nullify fungistatic activity (10, 16). Again, spores are exposed to the direct effects of pH in soil and the quantitative effects of this factor are extremely complex,

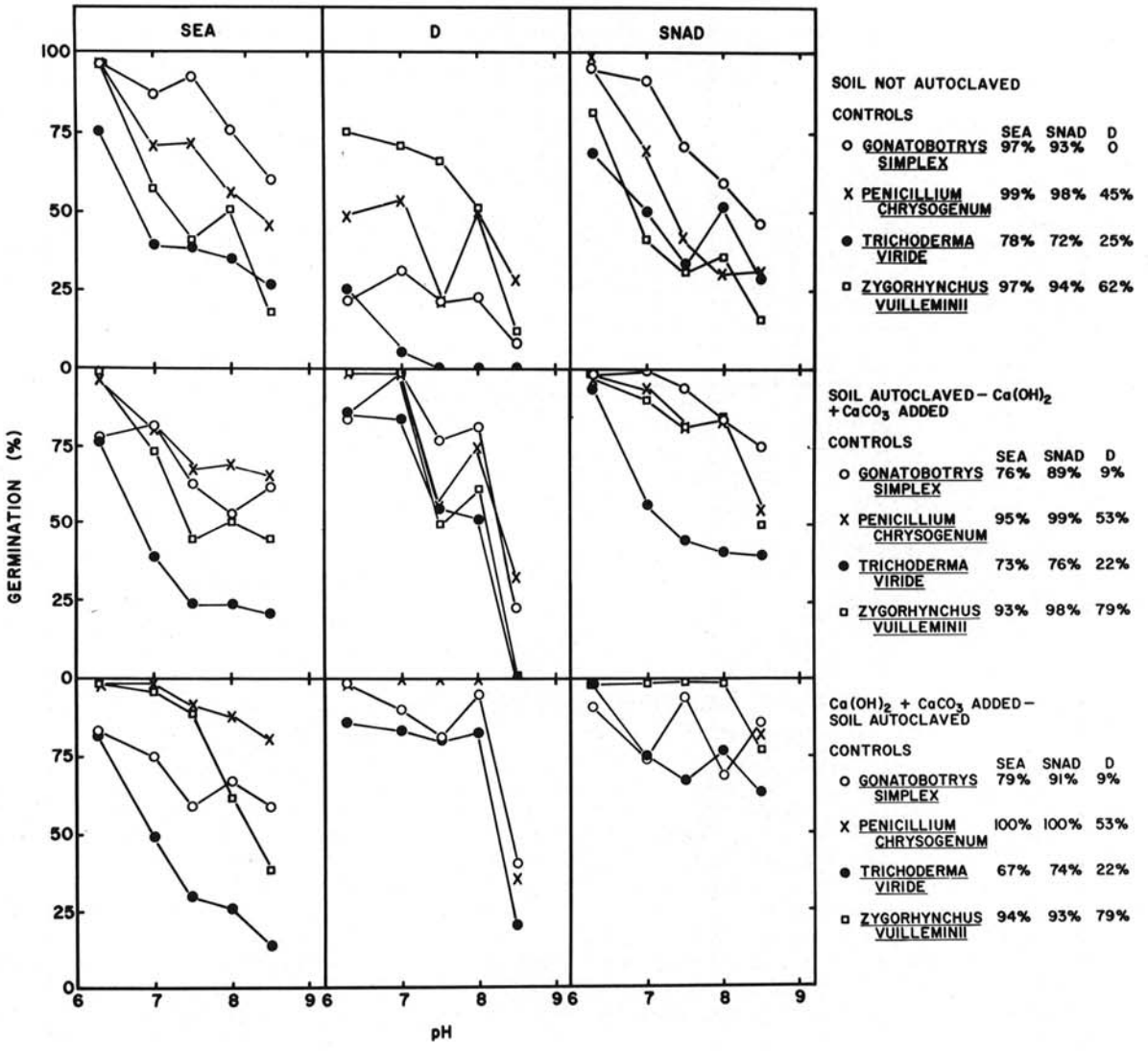

Fig. 2. Conidial germination of four test fungi in three bioassays at an original soil pH 6.3, and at pH 7.0, 7.5, 8.0, and 8.5, developed by liming raw soil and soil either before or after autoclaving. Abbreviations: SEA, soil emanation agar method; D, direct method; and SNAD, sterile "Nuclepore" agar diffusion method. Controls indicate germination obtained for each bioassay without soil.

TABLE 2. Fungistatic activity in a loam soil (Sample No. 70), limed with 1:1 $Ca(OH)_2$:$CaCO_3$ or MgO:$MgCO_3$

Fungus	Conidia germination (%)[a]							
	Control		Nontreated soil (pH 6.3)		Soil + 1:1 $Ca(OH)_2$:$CaCO_3$ (pH 8.5)		Soil + 1:1 MgO:$MgCO_3$[b] (pH 8.4)	
	SEA[c]	SNAD[d]	SEA	SNAD	SEA	SNAD	SEA	SNAD
1) *Gonatobotrys simplex*	95	94	95	96	68	58	70	57
2) *Penicillium chrysogenum*	99	99	97	96	74	52	72	53
3) *Trichoderma viride*	81	82	77	78	36	29	28	23
4) *Zygorhynchus vuilleminii*	98	97	92	95	43	28	39	33

[a]Percentage germination based on 400 conidia observed over several microscopic fields (0.32 cm^2).
[b]1:1 MgO:$MgCO_3$ applied 4 mg/g soil.
[c]SEA = Soil emanation agar method.
[d]SNAD = Sterile "Nuclepore" agar diffusion method.

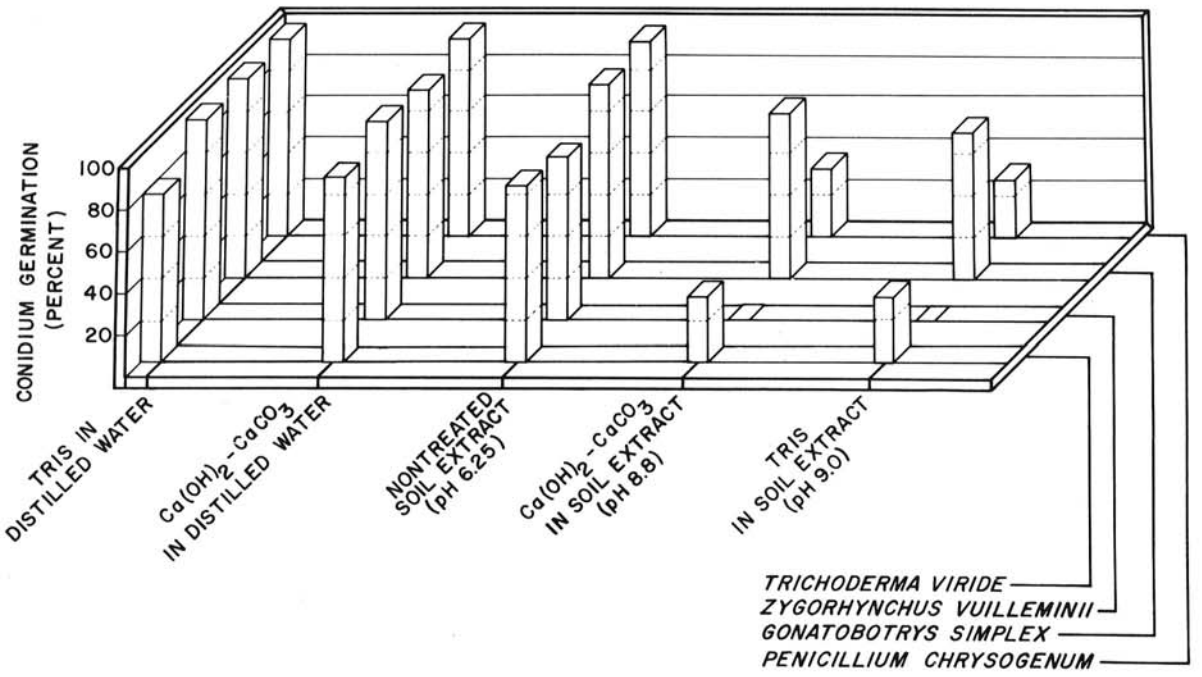

Fig. 3. Volatile fungistatic activity in sterile aqueous soil extract by the addition of 0.2 M tris solution or 1:1 $Ca(OH_2$:$CaCO_3$ suspension.

especially in interaction with nutrients (5). Finally, diffusible inhibitors (volatile and perhaps nonvolatile) complicate the situation still further. Therefore, the firm conclusions from the data presented in Fig. 2 are: (i) germination of conidia of test fungi was reduced in all bioassays with an increase in soil pH; (ii) fungistatic activity was evident when propagules were in contact with soil or exposed to volatiles above alkaline soil whether the soil system was sterile or not.

Krüger (15) concluded that reduced spore germination of *Sphacelotheca reilian* (Kühn) Clint. in soil after liming could be explained by the direct effect of pH. Evidence presented here indicates that liming also results in generation of a volatile factor which reduces spore germination.

Generation of the volatile inhibitory factor occurred in aqueous soil extract in the absence of biological entities (Fig. 3). Since tris is an organic buffer base, these results, together with those obtained in soil systems (Table 2), also suggest that a change from a slightly acid to an alkaline soil reaction was responsible for the generation of the volatile factor and not the associated compounds or metallic cations added to the treatments.

Earlier workers (6, 7, 8, 19) failed to observe consistent fungistatic activity in sterile aqueous soil extracts. These observations were confirmed in our

nontreated controls (Fig. 3). Apparently, substrates essential in the formation of the inhibitory materials were soluble in water and filterable; however, an alkaline reaction in the aqueous soil extracts was necessary to promote the production of the volatile inhibitory factor in concns sufficient to affect the germination of the test fungi.

Previous studies have shown that *Streptomyces* capable of producing earthy smelling compounds may at least be one source of inhibitory volatile(s) (13). Further, populations of *Streptomyces* spp. are abundant in soil between pH 6.5 - 8.0 and are favored by liming (1). This, together with other evidence in the literature, indicates that the origin of soil fungistasis is biological. These studies, however, provide the first evidence that a step in the series of reactions leading to the release of a volatile fungistatic factor in soil can be initiated in the absence of biological activity.

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