Soil Fungistasis: Mechanism in Sterilized, Reinoculated Soil

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ABSTRACT

Germination, in aqueous extracts of sterilized soil made fungistatic by reinoculation with various microorganisms, was high for spores which require no exogenous nutrients, and low for spores requiring exogenous nutrients. Chloroform-methanol extracts of reinoculated soils contained no detectable fungistatic activity. Increase in fungistasis in sterilized subsoil, which was amended with glucose and reinoculated, was correlated with loss of glucose. The results support the nutrient deficiency hypothesis for soil fungistasis. Phytopathology 60:89-91.

Sterilized soil, normally not fungistatic, may become so following inoculation with natural soil or with specific microorganisms (5). It is often assumed that inhibitory substances produced by the introduced microorganisms account for the restoration of fungistasis. However, autoclaved soil was made fungistatic by inoculating it with nonantibiotic- as well as with antibiotic-producing microorganisms (1, 6). Griffin (1) attributed the restored fungistasis by nonantibiotic microorganisms to staling products. Lockwood & Lingappa (6) suggested that the inhibition was caused by enhanced activity of soil microbes at the spore-soil interface which resulted either in the production of inhibitory substances or deprived the spore of required nutrients (4). Subsequently, Ko & Lockwood (2) presented evidence that soil fungistasis is a consequence of a microbe-induced nutrient deficiency in soil. They showed that most fungal spores require exogenous nutrients for germination, and that such nutrients are in short supply in the soil mass apart from organic debris. As a further test of the nutrient deficiency hypothesis, the mechanism of fungistasis in sterilized and reinoculated soil was studied.

Materials and Methods.—The following microorganisms were maintained on potato-dextrose agar: *Aspergillus ustus* (Bainer) Thom & Church; *Glomerella cingulata* (Ston.) Spauld. & Schrenk; *Fusarium solani* f. sp. *phaseoli* (Burk.) Snyd. & Hans.; *Penicillium frequentans* Westling; *Verticillium albo-atrum* Reinke & Berth.; *Bacillus subtilis* Cohn emend. Prazmowski; and *Agrobacterium tumefaciens* (E. F. Sm. & Tow.) Conn., two isolates of *Streptomyces* sp. *Helminthosporium sativum* Pam., King & Bakke and *H. victoriae* Meehan & Murphy, were maintained on V-8 juice agar (per liter: 200 ml V-8 juice [Campbell Soup Co.] 2 g $CaCO_3$, 20 g agar). Fungal conidia, actinomycete spores and mycelia, and bacterial cells were removed from agar slants with sterile distilled water and washed twice by centrifugation before use in experiments.

Conover loam topsoil and subsoil from a depth of 75 cm were sieved and autoclaved in 200-g quantities for 1.5 hr in one-liter Erlenmeyer flasks. Sterilized topsoil was inoculated singly or with different combinations of *F. solani* f. sp. *phaseoli*, *P. frequentans*, *V. albo-atrum*, the two bacteria, and the two streptomycetes; or with a small amount (<1 g) of natural soil (Table 1). Soil moisture was adjusted to about 15% of dry wt. Sterilized subsoil was amended with 0.8 ml of a sterile 10% glucose solution and 4 ml of sterile Czapek's salts solution/flask. The concentration of glucose was 400 µg/g dry subsoil. Sterilized subsoil was inoculated with *Streptomyces* sp., *V. albo-atrum*, or a small amount of natural soil, and was adjusted to 10% moisture. The inoculated soils were incubated at 24 C for 14 days. Flasks were shaken daily to insure thorough colonization of the soil.

The method of Lingappa & Lockwood (3) was used to observe spore germination on soil; 200 spores were counted for each treatment. Aqueous extracts of soil were prepared by shaking soil with an equal volume of distilled water on a wrist action shaker for 5 min, then centrifuging at 8,000 *g* for 5 min. The supernatant solution was sterilized by Millipore filtration (0.22 µ). The anthrone method was used to determine the carbohydrate content of soil extracts (7), using glucose as the standard. Glucose was determined with the Glucostat reagent (Worthington Biochemical Corp.) used according to the manufacturer's instructions. About 80% of the added glucose was recovered in a single extraction. To test for antibiotic activity, soil was extracted with chloroform-methanol (1:1, v/v), and the extract was concentrated approximately 6-fold in a vacuum evaporator at 38 C. Filter paper discs (1.27 cm diam) were saturated with the concentrated extracts and placed on 0.5% peptone agar seeded with conidia of *G. cingulata* or *V. albo-atrum*.

All experiments were done in duplicate, and were repeated two or more times.

Results and Discussion.—All of the combinations of inoculant microorganisms and natural soil restored fungistasis to the sterilized topsoil (Table 1). Germination in aqueous extracts of these soils was high for conidia of *G. cingulata*, which require no exogenous nutrients for germination, and low for the nutrient requiring conidia of *P. frequentans*. Chloroform-methanol extracts of inoculated topsoil gave no zones of inhibition on seeded agar. The fungistatic effect of inoculated soil on *G. cingulata* was attributed to the nutrient sink provided by the inoculant microorganisms; germination of conidia of *G. cingulata* and certain other nutrient-

TABLE 1. Some properties of sterilized Conover loam topsoil and subsoil to which fungistasis was restored by inoculation with microorganisms

Soil	Organisms used to reinoculate sterilized soil	% Germination				Carbohydrates, µg/g soil	Antibiotic activity of soil extract[a]
		Glomerella cingulata		*Penicillium frequentans*			
		Soil	Soil extract	Soil	Soil extract		
Topsoil	2 Streptomycetes[b]	19	88	0		115	0
	3 Fungi[c]	16	87	0		126	0
	2 Bacteria[d]	0	97	0	35	72	0
	Streptomycetes + bacteria	28	97	C	50	104	0
	Fungi + bacteria	4	94	1	0	76	0
	Streptomycetes + bacteria + fungi	3	97	1	24	85	0
	Natural soil	5	99	0	28	38	0
	Sterilized soil control	99	98	97	81	185	0
	Natural soil control	1	99	0	14	8	0
Subsoil	*Streptomyces* sp.	36	76	0	0	0	0
	V. albo-atrum	15	78	0	0	0	0
	Natural soil	7	84	0	0	0	0
	Sterilized soil control	98	89	4	0	0	0
	Natural soil control	3	74	0	0	0	0

[a] Chloroform-methanol extracts were tested by an agar diffusion assay.
[b] Two isolates of *Streptomyces* sp. used in combination.
[c] *Helminthosporium victoriae, Fusarium solani* f. sp. *phaseoli,* and *Penicillium frequentans* in combination.
[d] *Bacillus subtilis* and *Agrobacterium tumefaciens* in combination.

independent spores is known to be inhibited by artificial nutrient sinks (2).

Although germination results were as expected if utilizable nutrients had been depleted, the presence of residual carbohydrates in topsoil prevented an unequivocal conclusion that nutrient deprivation was the cause of the restored fungistasis. To avoid problems with unknown carbohydrates, subsoil was used in subsequent experiments. No carbohydrates were detected in aqueous extracts of autoclaved subsoil (Table 1). The lack of germination of conidia of *P. frequentans* on sterilized subsoil also indicated that it was essentially free of nutrients capable of supporting germination.

Subsoil which was reinoculated with *Streptomyces* sp., *V. albo-atrum,* or natural soil became fungistatic to *G. cingulata* (Table 1), although this fungus germinated well in aqueous extracts of the reinoculated subsoil (Table 1). Conidia of *P. frequentans* failed to germinate on the reinoculated soils or in equeous extracts of them, but germinated over 75% in extracts supplemented with glucose. Mineral salts without glucose did not stimulate germination. Results similar to those with *G. cingulata* were obtained with *H. victoriae,* whose conidia also require no exogenous nutrients for germination, and results similar to those with *P. frequentans* were obtained with conidia of *V. albo-atrum, H. sativum* and *A. ustus,* all of which require exogenous nutrients for germination.

The relationship between glucose utilization and restoration of fungistasis in inoculated subsoil was studied (Fig. 1, 2). Amount of residual glucose and germination of conidia of *H. sativum* on the surface of inoculated subsoil was determined at different times after amendment of subsoil with glucose and inoculation with microorganisms. Decrease in glucose level in the inoculated soil was significantly correlated (r = 0.87, $P < 5\%$) with decreased germination of *H. sativum* (Fig. 2). At about 3 days, glucose was completely utilized and germination was zero. The amount of *sativum* in soil was about three times more than that glucose which supported 50% germination of *H.* required in sterile solutions of glucose (8). Possibly, glucose is more readily available to the spores in solution than in soil. Another explanation might be that germination time is extended when nutrients are at a reduced level. Solutions of glucose at concentrations of 500, 200, 100, and 50 µg/ml gave 50% germination of conidia of *H. sativum* in 5, 6.5, 9.5, and 13 hr, respectively. If these longer germination times are considered, particularly after 42 hr, when glucose was

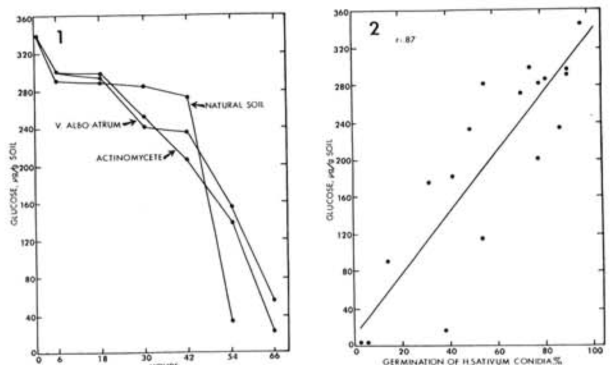

Fig. 1-2. 1) Loss of glucose from sterilized soil inoculated with *Verticillium albo-atrum, Streptomyces* sp., or natural soil. **2)** Relation between amount of glucose remaining in sterilized and reinoculated subsoil and the germination of *Helminthosporium sativum* conidia on the same soil.

being utilized rapidly (Fig. 1), the spores were probably exposed to lower levels of glucose than are shown in Fig. 2, based on a 5 hr germination period.

No inhibitory substances were detected in concentrated chloroform-methanol extracts of the inoculated subsoils. If staling products or self-inhibitors were present in the subsoil, the rate of glucose loss would be expected to decrease with increasing time; instead, the rate increased sharply with increasing time (Fig. 1). The results support the nutrient-deficiency hypothesis for soil fungistasis (2, 5).

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