The Nature of the Widespread Soil Fungistasis

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SUMMARY

Fungistasis of Conover loam, muck, and hardwood forest soils to conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici*, and *Penicillium frequentans* was demonstrated by indirect methods such as agar disks, double agar layer plates, and cellophan folds, as well as by direct placement of spores on or in soils. All attempts to extract toxic substances from soil with water or organic solvents failed. Fungistatic volatile substances could not be demonstrated in soil. Redox, pH, and osmotic conditions were not responsible for soil fungistasis. Various lines of evidence led to the conclusion that the so-called widespread soil fungistasis as observed by indirect methods is the result of production of antibiotics by soil microbes growing on the surface of the assay media, and is not due to a reserve of toxic substances in soils. The possibility is suggested that individual fungus spores serve as nutrient microsubstrates in soil and stimulate the rapid growth of soil microbes on their surface or in their immediate vicinity, and that this results in the production of sufficient fungistatic substances to prevent spore germination. Preliminary evidence in support of this suggestion are results with concentrated 50 % ethanol washings from teliospores of *Ustilago zaeae* which markedly stimulated growth of mixed soil microbes and of pure cultures of *Streptomyces* sp. and *Pseudomonas* sp., known antibiotic producers, in agar media.

INTRODUCTION

Spores of fungi which are able to germinate readily in distilled water or nutrient substrates fail to germinate in natural soils. Since the work on this problem by Dobbs & Hinson (1953), many authors have demonstrated fungistasis in different soils by using spores of numerous fungi (Chinn, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Jackson, 1958*a*; Lockwood, 1959; Stover, 1958). This apparently widespread fungistasis has been attributed to diffusible toxic factors in soil, and appears to coexist with biological activity since soils sterilized by heat or fumigants are not inhibitory (Dobbs & Hinson, 1953; Jackson, 1958*b*; Lockwood, 1959; Stover, 1958). Toxicity to fungi has been restored to autoclaved soil by inoculating with a specific bacterium (Park, 1956*a*) or with various actinomycetes (Lockwood, 1959). Fungistasis has been decreased by addition of plant residues (Chinn & Ledingham, 1957), or organic compounds such as glucose (Dobbs & Hinson, 1953; Jackson, 1958*a*) to soil.

Many attempts have been made to elucidate the precise nature of soil fungistasis.

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On the basis of the similar inhibitory spectrum of various soils, Jackson (1958*b*) speculated that the inhibitors are likely to be a simple mixture of a few components, probably of microbial origin. Some workers have reported unidentified fungistatic substances in soil extracts and diffusates (Dobbs & Hinson, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Stover, 1958). Except for extraction of fungitoxic lignin-like material from soil (Lingappa & Lockwood, 1960) these results have usually been inconclusive, and the factors responsible for fungitoxicity have not been isolated and identified.

Special kinds of soil fungitoxicity such as those of the highly calcareous New-borough sands (Dobbs & Bywater, 1959) and the very acid (pH 2·8) Charnwood forest soils (Jackson, 1958*b*) seem to differ from the general fungistasis expressed by almost all soils. There is need to explain the nature of the widespread soil fungistasis. In the present investigation, the methods used by previous workers were repeated, and work was extended to include additional controls and new experiments. Attempts to determine whether the results from indirect assay methods in common use might lead to erroneous interpretation of soil fungistasis were of particular interest.

METHODS

Conidia from 4- to 7-day-old cultures of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* maintained on potato dextrose agar were used. Conidia of *G. cingulata* do not germinate well in distilled water, but do so on water agar, whereas those of *F. oxysporum* f. *lycopersici* and *P. frequentans* germinate readily in distilled water and on water agar. It was necessary to wet spores of the latter with a $1/10^5$ dilution of polyoxyethylene sorbitan monooleate (Tween 80) to obtain uniform dispersion. A *Streptomyces* sp. and a *Pseudomonas* sp. which produced wide antibiotic inhibition zones on agar media were grown in yeast extract agar (Difco) and nutrient agar (Difco), respectively. All agar media used in these experiments contained 2 % (w/v) Bacto agar (Difco). This material contains, according to the manufacturer, about 0·4 % total protein (calculated from total N), 0·04 % lipids (ether extractable), and 3·86 % mineral content (ash). Bacto peptone (Difco) was used in 0·05 % (w/v) concentration unless specified otherwise.

The soils used were Conover loam, muck, and a hardwood forest soil, all at 50–60 % of saturation, from East Lansing, Michigan, U.S.A. Talc, diatomaceous earth (Celite), kaolin, bentonite, decolorizing carbon (Norit A), cellulose filter paper, or fibre glass filter paper were sometimes used in place of soil.

Many of the methods used by previous workers for assaying soil fungistasis were repeated in this laboratory. These included: (a) cellophan packets or folds (Dobbs & co-workers, 1953, 1957, 1959); (b) double agar layer method (Lockwood, 1959); (c) buried slide coated with seeded agar (Chinn, 1953); (d) water or peptone agar disks (2 mm. thick; Jackson, 1958*a, b*); (e) a modification of the method of Stover (1958) in which a layer of seeded agar was poured over a sterilized cellophan dialysis membrane. All these methods were used in Petri dishes filled to a depth of $\frac{1}{4}$ in. with soil and with the surface smoothed. Dishes were sealed with rubber bands and incubated at 28°. Assay media, except for buried slides, were pre-incubated on soil for 4–8 hr. (7 days for double agar layer method) followed by application of spores, then by continued incubation for another 16 hr. before counting germination. Three

microscope fields in each of 3 plates and a total of about 300 spores for each treatment were counted.

Direct methods of observing spore germination in soil were also used. About 50,000 conidia of the test fungi in 0.2–0.4 ml. of water were placed in a $\frac{1}{4}$ in. diameter area on moist soil in Petri dishes. Samples of these spores were removed after 16 hr. at 28° by lightly pressing 7 mm. diameter water agar disks on the demarked area. Disks and adhering spores were inverted on a glass slide for observation. In another method microscope slides containing a suspension of conidia of *Glomerella cingulata* were air dried to fix the spores to the glass surface, then were buried in soil and treated like those of Chinn (1953). In an extract method $\frac{1}{2}$ –1 million conidia of *G. cingulata* were mixed with 1 g. soil. After incubation for 16 hr. the soil samples were shaken with 10 ml. of 50 % (v/v) glycerol in water containing 0.5 % household detergent (Tide), and centrifuged for 2–20 min. at 1200 rev./min. (160 g); 70–80 % of the spores remained in the supernatant fluid which was free from all but colloidal soil particles. Spores were counted with a haemocytometer. Only ungerminated spores were recovered by this method. By comparison of the recovery from natural and autoclaved soils with controls refrigerated to prevent germination, estimates of germination could be calculated.

Several types of soil extracts were made. (1) Samples (100 g.) of loam or muck were extracted twice by shaking with 200 ml. of various C.P. grade solvents. (2) Drainage water from a tilled agricultural field of Conover loam of about 40 acres was collected from man-holes during dry weather when seepage was slow, and also after heavy rainfall; 16 l. samples were collected and passed through coarse filter paper. (3) Nearly 1000 lb. (about 450 kg.; 8.5 cu.ft.) of Conover loam soil in a greenhouse bench was periodically irrigated with excess water and up to 8 l. of drainage water collected each time in a polyethylene sheet. (4) Loam soil to a depth of 1 in. was placed on a $\frac{1}{2}$ in. deep bed of Norit A in glass trays and irrigated for 24, 48 or 72 hr. The recovered Norit A was extracted with various organic solvents. Water extracts of soils, or drainage water from the greenhouse bench were also treated with Norit A which was subsequently recovered and extracted with organic solvents.

Water extracts larger in volume than 1 l. were evaporated to $\frac{1}{5}$ volume in 20 min. at 40°–50° in a Precision Evaporator (Precision Scientific Co., Chicago, Illinois, U.S.A.). The resultant extracts, water extracts less than 1 l., and those of organic solvents were evaporated to a small volume under vacuum at 28–30° in a flash evaporator and assayed in seeded agar by direct incorporation or in filter paper disks ($\frac{1}{2}$ in. diameter).

Measurements of pH value and redox potential were made with a Beckman Model H2 pH meter.

All experiments were in triplicate and were repeated at least once. Sterile methods were used. In the case of natural soil, experiments were limited to 18 hr. in length when sterility was a consideration, due to the contaminating effects of motile soil microfauna.

RESULTS

Comparison of assay methods

Soil fungistasis was demonstrated by various methods, both indirect and direct. Some of the methods and typical results are given in Table 1. In addition, the modified method of Stover (1958) and the buried slide method of Chinn (1953) also

showed fungistasis in soil. Muck soil and hardwood forest soil gave inhibition of germination similar to that of Conover loam. Loss of fungistasis in autoclaved soils was shown by all methods; in these treatments fungi overgrew the entire plates. Factors such as thickness of agar, and temperature and duration of incubation on soil influenced the intensity of soil fungistasis as measured by the agar methods. By standardizing these factors, all three soils expressed a similar spectrum of inhibition of the test fungi in the order: *Penicillium frequentans* > *Glomerella cingulata* > *Fusarium oxysporum* f. *lycopersici*; *P. frequentans* was most readily inhibited.

Table 1. *Fungistasis of Conover loam soil assayed by various methods*

Treatment	Soil	Mean % germination of conidia*		
		<i>G. cingulata</i>	<i>F. oxysporum</i>	<i>P. frequentans</i>
Directly on soil	Natural	0	0	0
	Autoclaved	100	100	100
Glycerol extraction	Natural	0	—	—
	Autoclaved	94	—	—
Double agar layer	Natural	12	26	0
	Autoclaved	90	95	92
Cellophan packets	Natural	0	0	0
	Autoclaved	100	100	100
Water agar disks	Natural	0	0	0
	Autoclaved	98	100	100
Buried slide	Natural	0	0	0
	Autoclaved	100	92	92
Water agar control		98	100	90

* Mean of nine microscope fields with a total of approximately 300 conidia.

Table 2. *Fungistatic effects of different soils and substrates*

Soil or substrate		Mean % germination of conidia of <i>G. cingulata</i> *	
		Direct†	Agar disk‡
Conover loam	Natural	0	6
	Autoclaved	100	100
Muck	Natural	0	12
	Autoclaved	96	100
Forest soil	Natural	0	0
	Autoclaved	96	95
Sand	Natural	4	16
	Autoclaved	40	100
Fibre glass filter paper	Autoclaved	0	90
Cellulose filter paper	Autoclaved	0	90
Charcoal (Norit)	Autoclaved	0	10
Bentonite	Autoclaved	6	98
Kaolin	Autoclaved	0	90
Celite	Autoclaved	0	90
Talc	Autoclaved	0	95

* Mean of nine microscope fields with a total of approximately 300 conidia. Results were similar with conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

† Spores placed directly on soil surface.

‡ Spores placed on agar disks after disks were preincubated for 4 hr. on soil or substrate.

Fungistatic effects of substrates other than soil

There are reports that substances such as kaolin, alumina, slate dust (Dobbs & Hinson, 1953) and a calcareous sand (Dobbs & Bywater, 1959) decrease germination of fungus spores. Autoclaved cellulose filter papers, washed fibre glass filter papers, activated charcoal, bentonite, kaolin, talc, sand and diatomaceous earth (Celite) were tested for fungistatic effects by placing spores directly on them, and by the agar disk method. Results were similar with all three test fungi. The results for *Glomerella cingulata* (Table 2) show that these materials were fungitoxic when autoclaved and assayed by direct methods, but the fungistatic substances were apparently not diffusible, with the exception of those from sand and charcoal. Since fungistasis by soils was removed by autoclaving and the fungistatic substances were diffusible, it seems unlikely that the widespread soil fungistasis is due to clays or other colloidal or mineral materials.

Soil extracts

From experimental results similar to those in Table 1, previous workers have suspected the existence of diffusible fungistatic substances in natural soil. Following their lead, 100 g. samples of Conover loam soil were extracted with 50, 70 and 95 % (v/v) ethanol in water, *n*-butanol, methanol, acetone, chloroform, chloroform + methanol (1+1 by vol.), light petroleum ether, ethyl ether or water. When the concentrated extracts were assayed in water agar no inhibition of spore germination was observed; instead, stimulation of germination and growth was often observed. Likewise, concentrated drainage water from 8.5 cu.ft. of soil in the greenhouse, and from 40 acres of field soil did not inhibit germination. However, a more elaborate extraction of washed and defatted muck or mineral soil with 95 % (v/v) dioxane or ethanol in water yielded a fungistatic extract in the lignin-like fraction (Lingappa & Lockwood, 1960). This work will be discussed elsewhere (Lingappa & Lockwood, to be published).

The presence of stimulatory substances in soil extracts suggested that the expression of fungistatic substances might be masked. Concentrated extracts were chromatographed on filter paper strips in an attempt to separate inhibitory from stimulatory fractions. Chromatograms were developed in ethanol + acetic acid + water (12+6+1 by vol.), and in *n*-butanol + acetic acid + water (4+1+5 by vol.) and assayed on water agar seeded with *Glomerella cingulata*. Localized areas of stimulation of growth but no inhibition of spore germination were detectable in the assay plates. Strips of filter paper were also inserted into moist soil and diffusible materials from the soil allowed to accumulate at the tips of the hanging wicks (Wilson, 1958). After 16 hr. the strips were sterilized by immersing the buried end in 70 % (v/v) ethanol in water until the solvent reached the tip. When assayed on seeded agar no inhibitory substances were shown; again, areas of growth stimulation were evident.

Tests for volatile fungitoxic substances

The production of volatile antibiotic substances by micro-organisms has been reported (Bilal, 1956), and Dobbs & Bywater (1957) suspected that in some of their soils volatile fungitoxic substances might be present. Therefore it appeared possible

that our failure to extract fungistatic substances from soil might be due to loss of such volatile materials during the evaporation of solvents. Experiments were designed to detect such substances in natural soil. A coverglass coated on one side with a thin layer of water agar was inverted over a Van Tieghem ring and sealed with Vaseline. The rings were pressed into moist soil so that only a few mm. of space was left between soil and agar surface. Agar so exposed for 12–18 hr. at 28° was streaked with spores of the three test fungi and incubated again over soil or in moist chambers. Exposure for longer periods was not considered desirable because of inevitable contamination. In other experiments agar seeded with *Glomerella cingulata* was exposed to the soil atmosphere in Petri dishes for 16 hr. No inhibition of spore germination was observed in any of these tests. Absence of volatile fungitoxic materials in soil is also suggested by observations of Dobbs & Hinson (1953) that conidia on buried slides had germinated in pockets of condensed water. Similar observations were made in the spaces of buried Nylon gauze (Waid & Woodman, 1957). It was also our observation that complete contact of cellophan or agar disks with soil was necessary to avoid islands of spore germination over the air pockets. For these reasons it is believed that natural soil fungistasis is not caused by volatile substances from soil.

pH value and redox potential

The well known effects of pH value and redox potential of the medium on spore germination and growth of micro-organisms suggested the examination of these factors in natural soil fungistasis, although there appears to be no evidence that the pH of soils functions in this regard (Dobbs & Hinson, 1953; Jackson, 1958 *a*). Table 3

Table 3. *Relation of pH value and redox potential of soil to fungistasis*

Substrate	Redox potential (mv)*	pH* value	% germination†
Natural Conover loam	216	7.9	—
Autoclaved Conover loam	198	7.5	+
Natural muck	192	6.5	—
Autoclaved muck	176	7.2	+
Natural Conover loam + 0.5 % peptone	— 120	7.6	—
Natural muck + 0.5 % peptone	— 36	7.5	—
Natural Conover loam + 0.5 % sucrose	— 420	6.2	—
Water agar	276	5.8	+
Water agar in contact with soil 1 day	276	7.5	—

* Measurements made 16 hr. after amendments added, at time of taking spore germination data.

† Assays were by direct addition of conidia of *G. cingulata*, *F. oxysporum* and *P. frequentans* to soil. + = 20–100 % germination; — = 0–19 % germination.

shows that addition of small amounts of nutrients to soil did not annul fungistasis although drastically lowering the redox potential, whereas autoclaving soils annulled fungistasis while decreasing redox potential only slightly. Water agar developed strong fungistasis when incubated in contact with soil for 24 hr., but the pH did not change to an unfavourable value and the redox potential did not change at all. The soils in all treatments were at pH values favourable for spore germination, yet such soils were fungitoxic. For these reasons the pH and redox conditions of normal soil do not appear to be the cause of soil fungistasis.

Annulment of fungistasis

Since various workers have reported a decrease in fungistasis when soil was treated with plant residues or glucose (Dobbs & Hinson, 1953; Chinn & Ledingham, 1957), or when glucose was added to soil extracts (Stover, 1958) or agar disks (Jackson, 1958*a*), it was of interest to know whether spores already inhibited could be stimulated to germinate by nutritional supplementation. It has been shown that inhibited spores were viable (Lockwood, 1959). Spores of test fungi which had lain dormant for 5 days on the inhibitory bottom layer of double agar layer plates were removed with uniform disks of agar and transferred to separate Petri dishes. Known amounts of chemical solutions (0.2 ml. of 0.01 M solutions) were placed on these disks. Of the chemicals used only L-cysteine HCl and L-methionine prevented germination of conidia in controls. Similar results were obtained with all three test fungi. Those for *Glomerella cingulata* (Table 4) show the great effectiveness of peptone, even in small amounts (0.05 %, w/v), in annulling fungistasis. Glucose failed to induce germination of inhibited spores under the conditions of these tests. Various amino and organic acids and water were largely ineffective in annulling fungistasis. Osmotic gradients created by solutions of glucose, sorbitol, mannitol or polyglycols of different molecular weight did not annul fungistasis nor did they interfere with spore germination in controls. Therefore lack of osmotic shock probably would not explain failure of fungal spores to germinate in soils.

Table 4. *Annulment of fungistasis of Glomerella cingulata conidia*

Chemical*	% germination†	Chemical*	% germination†
Glycine	0	Succinic acid	15
L-Histidine	0	Glucose (0.1 M)	0
L-Cystine	8	Peptone (0.5 %, w/v)	90
L-Cysteine HCl‡	0	Peptone (0.05 %, w/v)	84
L-Methionine‡	0	Mannitol	10
D,L-Phenylalanine	0	Mannitol (0.1 M)	12
L-Tyrosine	0	Sorbitol	0
L-Aspartic acid	30	Polyglycols (200, 300, 400, 600 mol. wt.)	0
L-Glutamic acid	12	Water	0
p-Aminobenzoic acid	0	Untreated	0

* All are 0.01 M unless otherwise indicated; 0.2 ml. placed on 1.5 mm. thick × 8 mm. diam. water agar disks.

† Average of nine microscope fields with a total of 300 spores. The spores were inhibited for 5 days by the double agar layer method before reversal. Results were similar using conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

‡ Inhibited spore germination in controls.

Destruction of conidia in soil

The conidia of at least some fungi are destroyed rather rapidly in contact with soil, a fate not revealed by indirect methods of observations. Conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* were placed directly on soil. After 3, 6 and 9 days of incubation, direct microscopical observation revealed large numbers of empty or decomposing conidia of *G. cingulata* and *P. frequentans*. It was difficult to detect the microconidia of *F. oxysporum* f. *lycopersici* because of their small size. By plating these soils after 6 days, large numbers

of colonies of *P. frequentans*, a few of *F. oxysporum* f. *lycopersici*, and none of *G. cingulata* developed. After 9 days the number of colonies of *P. frequentans* was decreased and none of *F. oxysporum* f. *lycopersici* developed. No detectable growth of micro-organisms could be seen on the conidia of these fungi. In autoclaved soil the fungi had overgrown the plates as profusely sporulating mycelia. Lysis of spores by soil was also observed by Subramanian (1946, 1950) and by Park (1955).

Source of fungistasis as assayed by indirect methods

When agar methods were used for assaying soil fungistasis, inhibition occurred more rapidly when 0.05 % (w/v) or more peptone was present than with water agar alone. The bottom layer of double agar layer plates became inhibitory in 3 days when the top agar layer contained 0.5 % (w/v) peptone, but without peptone 7-8 days were required. Similarly, peptone agar disks became fungistatic in 4 hr. when pre-incubated on soil at 28°, but 8 hr. were required for water agar disks to become similarly fungistatic. At 21° water agar disks pre-incubated for 4 hr. gave 60 % germination of conidia of *Fusarium oxysporum* f. *lycopersici*, 20 % of *Glomerella cingulata*, and none of *Penicillium frequentans*. Peptone agar disks similarly treated caused complete inhibition of all three fungi.

Table 5. *Effect of pre-incubation temperature on the expression of soil fungistasis by indirect methods*

Method†	Pre-incubation time	Substrate	Pre-incubation temperature	
			1°	28°
			% germination of <i>G. cingulata</i> conidia*	
Double (water) agar layer	5 days	Natural soil	96	6
	14 days	Natural soil	92	0
	14 days	Autoclaved soil	98	96
Water agar disks on cellophane	8 hr.	Natural soil	98	0
	16 hr.	Natural soil	96	0
	48 hr.	Natural soil	92	0
	48 hr.	Autoclaved soil	92	96
Water agar disks	24 hr.	Fungistatic agar layer	0	0

* Average of nine microscopic fields with a total of 300 spores.

† Agar disks or layers were pre-incubated on Conover loam soil or fungistatic agar layer for various time intervals, then streaked with conidia of *G. cingulata* and incubated for 16 hr. at 28°.

These observations raised the question as to whether fungistasis was caused by substances present in soil, or whether fungistatic substances were generated on the surface of the assay medium and then diffused into it during incubation. Since very little or no growth of micro-organisms takes place at 1°, pre-incubation of assay media at this temperature was tested to investigate this point. The double agar (water) layer and agar (water) disk methods were used, and a layer of sterilized cellophan was interposed between soil and agar. Table 5 shows that the agar media were not toxic to spore germination by *Glomerella cingulata* when pre-incubated for extended periods of time on soil at 1°, then removed and incubated with fungal spores at 28°. The soil did not lose the capacity to express fungistasis by exposure to

cold temperatures, since subsequent pre-incubation of new agar disks on the same soils at 28° caused inhibition of spores subsequently applied to the disks. To test the possibility that fungistatic substances might not diffuse at 1°, double agar layer plates were pre-incubated with soil for 12 days at 28°, thus making the bottom layer fungistatic. The fungistatic layers were then covered with sterilized cellophan. Water agar disks were placed on the cellophan and incubated at 1° for 24 hr. the disks then being removed and placed on water agar. Spores of test fungi applied to these disks and incubated at 28° for 16 hr. did not germinate (Table 5). This indicated that fungistatic substances which had accumulated in agar were indeed able to diffuse into assay disks at 1°. Results were the same with all three fungi, strongly indicating that fungistasis as assayed by the indirect methods (agar or cellophan) was due to production of fungistatic substances by soil microbes growing on the contact surface of assay medium and soil, rather than to the presence of a reservoir of such substances in soil.

Table 6. *The effect of amount of soil on expression of fungistasis by indirect methods*

Substrate or method*	Thickness of soil	% germination of <i>G. cingulata</i> conidia†
Double (water) agar layer	1 cm.	6
	1 mm.	8
(Water) agar disks	6 cm.	2
(Water) agar disks	< 0.1 mm.	12
(Peptone) agar disks	< 0.1 mm.	0
Cellophan	1 cm.	0
Cellophan	< 0.1 mm.	0
Mixed soil organisms assayed with water agar disks‡	0	0
Natural soil-direct	1 cm.	0
Autoclaved soil-direct	1 cm.	88

* Pre-incubation was 6 hr. except for double agar layer which was 7 days.

† Average of nine microscope fields with a total of 300 spores.

‡ Diluted soil suspension was mixed with water agar or peptone agar, incubated 3 days, and assayed with water agar disks.

This possibility was examined further by using different amounts of soil in contact with assay media. Fungistasis of *Glomerella cingulata* conidia (Table 6) was expressed equally by agar disks incubated on a soil layer < 0.1 mm. thick as on a soil layer 60 mm. thick. Bottom layers of double agar layer plates incubated for 7 days at 28° became equally fungistatic whether 16 g. soil, 10 mg. soil, or a thin layer of a 1/1000 dilution of soil in water was placed on the top layer. The amount of soil did not affect the time required for the bottom layer to become fungistatic. Furthermore, 1/1000 dilutions of soil incubated for 3 days on peptone or water agar, then assayed with agar disks or cellophan strips gave the usual fungistatic effects. Similarly, mixed cultures of soil microbes isolated from soil dilution plates and grown for 3 days on peptone agar caused water agar disks placed on them to become fungistatic. Peptone agar seeded with an antagonistic *Streptomyces* sp. or *Pseudo-*

monas sp. also produced fungistasis in agar disks. Inhibition, as compared with *G. cingulata*, was somewhat greater with *Penicillium frequentans*, and slightly less with *Fusarium oxysporum* f. *lycopersici*, in these tests.

The possible production of fungistatic substances on the assay medium surface was further tested by incorporating antibacterial antibiotics into agar disks, pre-incubating them on soil for 2 hr., then removing them to moist fibre glass filter papers which were soaked with the antibiotics, and assaying the generation of fungistasis in these disks. Results were similar with all three test fungi. Those for *Glomerella cingulata* (Table 7) indicated a decrease in soil fungistasis by these methods. The presence of antibiotic-resistant organisms in soil no doubt prevented complete suppression of fungistasis. These results support the view that soil fungistasis as assayed by indirect methods is due to growth of micro-organisms on the assay medium.

Table 7. *Effect of antibacterial antibiotics on soil fungistasis as assayed with peptone or water agar disks**

Antibiotic	% germination of <i>G. cingulata</i> conidia		
	Water agar disks	Peptone agar disks	Water agar disks (no soil)
K Penicillin G	40	90	98
Streptomycin sulphate	90	20	95
Neomycin	67	25	100
Vancomycin	70	90	92
Chloramphenicol	30	75	96
Control (no antibiotic)	12	0	—

* Agar disks containing antibiotics (1 mg./ml.) were pre-incubated directly on soil for 2 hr., then transferred to fibre glass filter paper moistened with the same antibiotic.

The above results were further supported by microscopical observation of the surface of assay media. Water agar or peptone agar disks, or sterile cellophan strips were placed on a smooth moist soil surface and pressed so as to make complete contact. After 8, 12, 24 and 48 hr. they were carefully lifted, inverted on a microscope slide, stained with phenol + rose bengal and washed gently in water. Such preparations revealed extensive colonization of the surface in contact with soil by bacteria within 8 hr. (Pl. 1, figs. 1, 2), and colonization by actinomycetes in 24 hr. (Pl. 1, figs. 3, 4). In this connexion, water agar has been found to be a good substrate for development of soil actinomycetes (Y. Lingappa & Lockwood, 1961).

Antibiotic and nutritional substances from fungal spores and their possible relation to fungistasis in soil

Freshly collected teliospores of the maize smut fungus *Ustilago zaeae* did not germinate when placed directly on natural loam soil, but 40 % germinated on water agar in 16 hr. Germination and production of aerial sporidia occurred on autoclaved soil. Samples (5 g.) of air-dry smut teliospores were washed twice by shaking for 10 min. at a time with 100 ml. 50 % (v/v) ethanol in water. Another 5 g. sample was

spread on the surface of a layer of 20 g. natural soil, moistened, and incubated for 4 days at 24°, after which the spores were scraped off and extracted similarly. When the washings from the air-dry spores were evaporated on filter paper disks and assayed by the agar diffusion method against the organisms arising from a diluted soil suspension, they showed a strong antibiotic zone against these organisms as well as against *Fusarium oxysporum* f. *lycopersici*, *Penicillium frequentans*, *Glomerella cingulata*, *Pseudomonas* sp., and the *Streptomyces* sp. A very wide zone of stimulation occurred outside the zone of inhibition. On the other hand, washings from spores incubated in soil for 4 days showed no zone of inhibition, and only a slight zone of stimulation, suggesting exhaustion or biological breakdown of these materials. On the basis of these scanty observations with one fungus, the possibility is suggested that individual spores might behave as microsubstrates in the soil. Nutrients released by fungus spores could stimulate growth of antagonistic microflora in the vicinity of the spore, preventing its germination. The release of antibiotics from the spore could prevent or delay colonization of the actual spore surface.

DISCUSSION

In spite of numerous attempts to obtain fungistatic substances from soil, a characterized fungistatic principle has not been isolated with the exception of the lignin-like preparation of Lingappa & Lockwood (1960). In the work reported in the present paper, soil extracts were stimulatory to the growth of fungi. In spite of the failure to extract fungistatic substances, the fact that soil extracts are often stimulatory, as reported here and by others (Dobbs & Hinson, 1953; James, 1958; Park, 1956*b*), and the fact that spores of fungi which germinate readily in water are inhibited by soil (Dobbs & Hinson, 1953) show clearly that soil fungistasis cannot be the result of insufficient nutrients in soil.

A possible group of suspected fungistatic substances in soil includes the antibiotics. On the basis of researches to date (Brian, 1957), the production and accumulation of detectable quantities of antibiotics from natural unamended and uninoculated soil is considered to be unlikely. The present workers carried out very extensive and intensive extractions of natural soils and failed to demonstrate any antifungal antibiotics. While the accumulation of antibiotics in natural soil has not been demonstrated, evidence generally favours the production of antibiotics on fresh organic substrates in micro-environments of the soil (Brian, 1957). As the amounts produced are likely to be extremely small, when the bulk of the soil is considered, their demonstration has been by necessity indirect. The addition of a fresh organic substrate, such as agar or cellophan, might supply a source of nutrients and support microbial growth and antibiotic production. Such a possibility is supported by the short generation time of many bacteria and the very large number of micro-organisms in soil. An average soil containing 10 million individual micro-organisms/g. would provide an initial and enormous inoculum of up to 100,000 microbes in contact with a 1 cm. square agar block placed on it. Present information (Brian, 1957) permits us to assume that 40–50 % of the soil micro-organisms produce antibiotics. The present work and that of others (Y. Lingappa & Lockwood, 1961) shows that antibiotics are produced by actinomycetes growing on deficient media such as water agar, and that fungistatic substances are produced as well from mixed cultures as from single cultures of antibiotic-producing micro-organisms.

This explanation of the inhibition of fungal spore germination on assay media in contact with soil was supported by five lines of evidence. (1) Peptone agar became fungistatic more rapidly than water agar in contact with natural loam soil. (2) When agar media were pre-incubated on soil at 1°, a temperature which permits diffusion of fungistatic substances but largely prevents microbial growth, no fungistasis occurred. (3) The same degree of fungistasis was obtained with agar disks pre-incubated with thick layers of soil, minute amounts of soil, mixed cultures of soil microbes, or pure cultures of *Streptomyces* sp. or *Pseudomonas* sp. (4) Decrease of fungistasis was observed when high concentrations of antibacterial antibiotics were added to agar disks used for assaying soil. (5) Microscopic observations showed dense growth of micro-organisms within short periods on the contact surfaces of agar and cellophan with soil.

These indirect techniques, then, do not demonstrate the presence of fungistatic substances in soil, but provide a substrate for growth of micro-organisms which produce antibiotic substances, thus rendering the assay media fungistatic. Nevertheless, failure of fungal spores to germinate in natural soil is indeed a fact as shown by direct methods of observation. Understanding of the action of the indirect assay methods may provide some insight about the mechanism of inhibition involved. The introduction of spores into soil can create, qualitatively, as much a nutrient substrate as a piece of water agar, cellophan or straw. The experiments with smut spores showed that these liberated diffusible substances which stimulated growth of a variety of micro-organisms. If speculation is extended to individual spores in soil one might expect, as a result of nutrients diffusing from the spores, stimulation of surrounding microflora and resultant inhibition of fungal spore germination. An analogous situation is the reported stimulation of vegetative growth of the fungus *Fusarium oxysporum* f. *lycopersici* and the production of the antibiotic fusaric acid in the vicinity of the root surface of tomato plants growing in unsterilized soil or sand (Kalyanasundaram, 1958). Observations of various workers (Subramanian, 1946, 1950; Park, 1955; Chinn, 1953; Lockwood, 1959) and the present work showing that fungus mycelium and conidia are destroyed ultimately by soil, and reports of the colonization of the surface of fungal structures in soil (Mitchell, Hooton & Clark, 1941; Subramanian, 1946, 1950; Waid & Woodman, 1957; Stover, 1958) support the assumption that growth of soil micro-organisms on or in the vicinity of fungal structures is involved in the over-all fungitoxicity. Lysis of fungi appears to be the end result of extended microbial activities in the vicinity of spores and mycelium in soil.

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EXPLANATION OF PLATE 1

Fig. 1. Bacteria colonizing water agar disks after incubation on moist Conover loam soil for 8 hr. at 28°. × 400.

Fig. 2. Bacteria colonizing cellophan after incubation on moist Conover loam soil for 8 hr. at 28°. × 800.

Fig. 3. Actinomycetes colonizing water agar disks after incubation on moist Conover loam soil for 24 hr. at 28°. × 800.

Fig. 4. Actinomycetes colonizing water agar disks after incubation on moist Conover soil for 24 hr. at 28°. × 400.