ABSTRACT

SOIL FUNGISTASIS: SENSITIVITY OF SPORES;

MECHANISM IN REINOCULATED STERILIZED SOILS

By Gary W. Steiner

Experiments were designed (a) to determine the relative sensitivities of a number of fungi to soil fungistasis, and to attempt to establish possible reasons for such differences and (b) to determine the mechanism of fungistasis in sterilized, reinoculated soil as a test of the nutrient deficiency hypothesis.

The sensitivities of 19 fungi to soil fungistasis were compared by determining the proportions of sterile and natural soil in mixtures which would support 50% germination of the spores. Spores requiring a high ratio of sterile:natural soil were considered to be more sensitive to soil fungistasis. Sensitivity to soil fungistasis was a function of spore volume and time required for germination. For example, Aspergillus terreus, which has small spores and a long germination time, germinated 50% when the ratio of sterile:natural soil was 23. Cladosporium cucumerinum, which is intermediate in spore volume and germination time, germinated 50% when the ratio of sterile:natural soil was 2.5. Helminthosporium victoriae, which has large spores and germinates quickly, germinated 50% when the ratio of sterile:natural soil was 0.5. Conidia between the ages of 1 and 9 weeks were not significantly different in their sensitivity to soil fungistasis. Chlamydospores, microconidia and macroconidia of 3 Fusarium species were exceptions to the correlations of spore volume and germination time with sensitivity to soil fungistasis. These spore

types were all relatively insensitive to soil fungistasis while their volumes and germination times were intermediate. Among all the fungi tested, including the Fusaria, spore volume and germination time on sterilized soil were inversely correlated.

Neither spore volume nor sensitivity to soil fungistasis were correlated with the concentration of glucose in a solution required for 50% germination of the spores. Spore volume and sensitivity to soil fungistasis also were not correlated with the concentration of aqueous extracts of sterilized soil required for 50% germination.

Neither germination time of nutrient dependent conidia nor their sensitivity to soil fungistasis were affected by pretreatment in water at 1 or 24 C or in nutrient solution at 1 C. Incubation in nutrient solution at 24 C reduced germination time and sensitivity on soil. Nutrient independent conidia followed a similar pattern except that incubation at 24 C in water reduced germination time and sensitivity, but not as much as did incubation in nutrient solution. There was a direct correlation between reduction in germination time and reduction in sensitivity to soil fungistasis for all fungi pre-incubated at 24 C in nutrient solution.

Mycelia of 8 different fungi were less sensitive to soil fungistasis than were the corresponding spores. For all 8 fungi, there was a significant correlation between the ratio of sterile:natural soil for spore germination and that for mycelial growth.

The time required for germination of conidia on sterilized soil or in a nutrient solution was neither shortened nor lengthened by intermittent incubation periods in natural soil or water. Thus, germination time was cumulative and irreversible. Germination of a population of partially germinated conidia was immediately stopped when Millipore filters containing the conidia were placed on natural soil, or on washed-sterilized sand through which a nutrient-free buffer solution was flowing.

When sterile subsoil was amended with glucose and inoculated with any of several microorganisms, a significant correlation was established between loss of glucose and restoration of fungistasis.

From these results it can be concluded that the behavior of fungi in soil is largely determined by the presence of utilizable carbon nutrients and length of time that they are available.

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INTRODUCTION

It has long been known that most fungal spores will not germinate in natural soil in the absence of utilizable nutrients (10, 15, 19, 26). Even in the presence of small amounts of nutrients many spores still fail to germinate (31). Numerous papers have been written on the nature of this soil fungistasis. From these have emerged essentially 2 possibilities to explain its nature, (1) inhibitors in soil and (2) lack of essential nutrients in soil. Recently Ko and Lockwood presented new evidence which indicated that the lack of essential nutrients in soil is the cause of soil fungistasis (31).

My research was directed toward 2 aspects of soil fungistasis for which definitive information is lacking. One phase of the research was designed to determine the relative sensitivities of a number of fungi to soil fungistasis, and to attempt to establish possible reasons for such differences. The other was to determine the mechanism of fungistasis in sterilized, reinoculated soil as an approach to test the nutrient deficiency hypothesis.

LITERATURE REVIEW

The inability of fungal spores to germinate in soil under conditions considered favorable for germination has been termed soil fungistasis or mycostasis (14, 19). In the last 15 years numerous papers have been written attesting to the existence of soil fungistasis in most areas of the world. Lockwood has recently reviewed the literature related to this subject (37). My review will consider literature dealing with the nature of fungistasis, important papers on the subject since Lockwood's review, and material related to specific areas covered in the present research.

Nature of soil fungistasis: Numerous mechanisms have been proposed to explain the nature of soil fungistasis. Most of these have been speculative with little convincing evidence to support them (37). One area of common agreement is that microorganisms are in some way responsible for the inhibition of fungi in soil. Essentially, there are 2 possible explanations of soil fungistasis: (1) a diffusible inhibitory substance of microbial origin and (2) a deficiency of nutrients essential to fungal growth, as a result of microbial activity in soil.

The inhibitor hypothesis is supported by most workers, but their views differ as to the inhibitor source and nature. Dobbs and co-workers believe that the inhibitor is a soluble, unstable substance which exists in the soil solution (17, 18, 19, 20). Jackson has shown, however, that fallow soils which are very extensively leached with water are still fungistatic (27). He believed the inhibitors were complex organic substances which were resistant to chemical and biological breakdown. Lockwood postulated that antibiotics could be the inhibitory substances causing fungistasis (36). Lingappa and Lockwood later ruled this out in view of the fact that antibiotics could not be isolated from soil, and the areas where antibiotics are most likely to be produced, if at all, are also the places where spores will germinate (33). Such loci include rhizospheres and areas adjacent to crop residues. These workers also obtained results showing that lignin decomposition products and many compounds related to lignin building blocks were fungitoxic (34). They did not suggest, however, that this was the cause for the widespread fungistasis, since fungal inhibition occurs in soils low in organic matter, and even in sand, and the type of inhibition was not characteristic of that in soil (16, 33). Dobbs and Bywater once suggested that volatile substances of fungal origin might be present in soil (14), but Lingappa and Lockwood were unable to confirm this suggestion (33).

Park (49, 50, 51, 52, 54) has suggested that staling products may explain soil fungistasis. He followed the growth, sporulation and autolysis of Fusarium oxysporum in culture. This model system reproduces many of the features of behavior of fungi occurring in the soil. Although he explains the morphological changes of the fungi as due to the action of an inhibitory compound, it is known that similar morphological changes also occur when nutrients become limiting (10, 24). That fungal morphogens do exist in culture, however, is well illustrated by Park (50, 54, 55), but they have not been detected with certainty, nor isolated from natural soil. Griffin found that autoclaved soil could be made fungistatic by inoculating it with nonantagonistic as well as with antagonistic microorganisms (23). Since the nonantagonistic organisms were incapable of producing antibiotics in culture, he attributed the induced fungistasis to a build-up of staling products.

Many attempts have been made to demonstrate the presence of fungal inhibitors in soil extracts (37). Failure of spores to germinate in nonsterile soil extracts has been interpreted as indicating the presence of inhibitory compounds in soil (18, 20, 24, 29). However, this type of observation does not prove the existence of inhibitors in soil, since the microorganisms present in the extracts originally may be the cause for failure of spore germination, either through the production of inhibitory compounds or through utilization of nutrients essential for fungal spore germination. Frequently when these extracts are sterilized they lose, wholly or in part, their inhibitory qualities (20, 24, 29, 45, 57). Some non-sterile extracts are also stimulatory to spore germination (33).

Sterile soil extracts that retained inhibitory activity have been reported in a few cases (37). Dobbs et al. succeeded in obtaining sterile inhibitory extracts from garden soils under special conditions of reduced aeration (20). Conidia of *Penicillium frequentans* germinated 22% in the sterile extract, 14% in the non-sterile extract and 45% in the water controls. Using extracts from a mineral soil, conidia of *Mucor ramannianus* germinated 66% in the extract and 83% in conductivity water (15). To obtain these results it was necessary to make the extractions and perform the germination tests under nitrogen gas. Extracts from other soils were not inhibitory. None of the proposed inhibitors found have been chemically characterized nor have they shown the features characteristic of soil fungistasis, i.e., strong inhibition, widespread occurrence and broad inhibitory spectrum. Moreover, these compounds have not been shown to occur in soil at levels high enough to cause inhibition (37).

Vaartaja and Agnihotri have recently collected leachates from a nursery soil which was originally a plantation of *Pinus silvestris* L. (58, 59). The leachates were collected in lysimeters buried in soil and were tested for their effect on growth of *Pythium ultimum* and *Thanatephorus praticolus*. They reported that the extracts were inhibitory to fungi after pasteurization, autoclaving, Millipore filtration, 3-fold concentration and storage at 0 C for 6 months. However in the case of *P. ultimum*, the only reliable data showing inhibition were with autoclaved extracts. The inhibition with other treatments cannot be ascribed with certainty to the presence of inhibitors since there is no proof that the extracts were sterile. With *T. praticolus*, growth was inhibited when the leachates were filtered, pasteurized at 60 C or stored cold for 1 day, but when the same extracts were autoclaved the growth of *T. praticolus* was equal to the control. When nonsterile leachates were concentrated, there was stimulation of growth for both fungi at low concentrations and approximately 50% inhibition at a 3-fold concentration. Nutrients did not completely overcome this inhibition. They concluded that an inhibitor was concentrated to the point where it caused inhibition regardless of nutrients. However, they did not rule out the possibility that the inhibition was due to a toxic concentration of salts or an increased concentration of microorganisms in the nonsterile leachates. For any of their data to be reliable, a control lysimeter receiving only water should have been included to eliminate the possibility of any inhibitory substances coming from the leachate-collecting mechanism.

The failure of spores to germinate on agar discs which are in contact with natural soil is often interpreted as due to the presence of fungistatic substances which have diffused from the soil to the agar. Griffin obtained inhibitory agar even when it was incubated on soil at low temperatures (24). He felt that the low temperature ruled out the loss of essential nutrients since microbial activity would be low. However, Ko and Lockwood have shown clearly that agar can rapidly lose nutrients to soil at either 1 or 24 C (31). Weltzien used gel electrophoresis on agar made fungistatic by incubation on soil, and found that a new inhibition zone developed at the anode (60). He explained this apparent migration in terms of a mobile inhibitor. Results of this kind can also be interpreted as due to the migration of essential nutrients creating new zones of deficiency or to inhibitory pH levels due to the migration of ions. Attempts to extract an inhibitor from agar previously incubated on soil have so far failed (33).

Dobbs and Gash have reported a thermostable inhibitor in certain dune sands in Great Britain (16). This type of inhibition was termed residual mycostasis to distinguish it from the common "microbial" mycostasis. They suggested that $CaCO_3$ and inorganic iron were responsible for the residual mycostasis. Since both these materials are nearly insoluble in water it seems unlikely that agar incubated on sand was made fungistatic because of the diffusion of such substances from sand into the agar. The possibility of nutrient loss from the agar was not ruled out.

Most workers have not considered the cause of fungistasis to be a lack of essential nutrients in soil because it was assumed that most fungal spores would germinate in distilled water. There are now sufficient data available, however, to prove otherwise (10, 31, 2). Most fungal spores need an exogenous source of carbon in order to germinate. Nitrogen and even minerals are often required, in addition. Any deficiency of an essential element in soil could be responsible for fungistasis. Critics of the nutrient hypothesis point also to the fact that water extracts of a fungistatic soil will often support germination. However, spores will germinate in soil if placed near an organic amendment such as alfalfa residue (31). Such isolated sources of nutrients within a generally deficient soil mass can contribute sufficient nutrients in the total soil extract to support spore germination (31).

Cook and Schroth have argued that a balance between inhibitory substances and nutrient supply is responsible for fungistasis (11). However, if nutrients are lacking, there seems to be no need to postulate inhibitory substances being present at any time for inhibition of fungal spore germination and growth.

Cooke has found soil close to retreating ice-caps in Iceland to be fungistatic to *Penicillium frequentans* (12). Since microbial activity and organic matter were low in this soil, he suggested, without providing evidence, that the failure of spores to germinate was due to deficiency of nutrients.

Powellson showed clearly that soil can be deficient in carbon sources which are required for germination of *Verticillium dahliae* conidia (53). He used a nutrient-free gel supplemented with KNO_3, which is also required for germination of the test fungus. When different soils were added to half the gel plate surface, conidia would not germinate, even close to the soil, until an appropriate carbon source such as glucose was added. No chemical tests were done to determine the amount of soluble carbohydrates in the natural soil, but other data indicate that soil is very deficient in soluble carbon (31).

Recently Ko and Lockwood published an extensive paper dealing with the relation of soil fungistasis to fungal spore nutrition (31). In 18 of 22 fungi tested they found a direct correlation between the ability of spores to germinate without an exogenous source of nutrients and the ability of spores to germinate on soil. Those fungal spores that germinated poorly or not at all in water, responded in a similar manner on natural soil. Four out of 22 fungi were exceptional in that they were inhibited in soil but germinated in distilled water without exogenous nutrients. However, spores of these 4 fungi failed to germinate when leached gently with dripping water or buffer. This artificial sink for exudates from the spore was suggested to be similar to the sink existing in natural soil as a result of microbial activity. Extracts of natural soil were shown to have insufficient nutrients to support spore germination of fungi requiring exogenous nutrients, but nevertheless they allowed germination of nutrient independent spores. Only when amendments such as alfalfa were added to natural soil were carbohydrate and amino acid levels high enough in water extracts to support germination of nutrient dependent spores.

Ko and Lockwood have also provided evidence for the loss of nutrients from agar placed on soil. There was a direct correlation between the loss of nutrients from the agar to soil and decreased germination of nutrient dependent spores. Conidia of *Helminthosporium victoriae* and ascospores of *Neurospora tetrasperma*, both of which do not require exogenous nutrients, germinated freely on the agar. Agar could be made fungistatic also by leaching it with distilled water. They have concluded that soil fungistasis is a consequence of nutrient deficiency in soil or the loss of nutrients from spores when in the soil.

Sensitivity of different fungal spores to soil fungistasis: A wide array of fungi has been tested for susceptibility to soil fungistasis. Spores of most fungi are completely or substantially suppressed by natural soil, although a few exceptions such as ascospores of *N. tetrasperma*, urediospores of *Puccinia coronata*, and conidia of *Erysiphe graminis* f. sp. *tritici* germinate freely in soil (31,35).

Jackson tested 17 fungi for sensitivity to soil fungistasis using agar disks placed on soil and the buried slide technique (27, 28). He found differences in sensitivity among the different fungi and grouped them according to whether they were inhibited, unaffected or stimulated. Most fungi were inhibited and only 1 was stimulated. When spores of the inhibited fungi were allowed to incubate on agar disks prior to placing the disks on soil, the sensitivity to fungistasis was reduced (28). Recently Dix used agar blocks of different thicknesses to measure sensitivity of spores to soil fungistasis (13). He grouped the spores into 4 broad classes: sensitive, low degree of sensitivity, high degree of sensitivity and complete inhibition. Spores from 7 of 11 fungi tested were highly sensitive or inhibited completely. Only one fungus was insensitive. These data were used to classify fungi with low sensitivity to fungistasis as pioneer colonizers of roots and those with high sensitivity as secondary or noncolonizers.

Differential sensitivity among groups of fungi was also illustrated in a series of papers by Park (44, 47, 48). He found that 6 soil-inhabiting fungi could germinate and grow in partially sterilized soil, whereas those that were exochthonous to the soil remained quiescent in such soil. Neither group was active in natural soil. Little work has been reported on the sensitivity of different spore forms of the same species. There are indications, however, that *Fusarium solani* f. *phaseoli* chlamydospores are more sensitive than the conidia of the same fungus (41). Conidia will germinate to some extent on natural soil but the chlamydospores will not. Spores from different isolates of *Cochliobolus sativus* were also found to differ in their sensitivity to soil fungistasis (7, 8, 9). This same condition is true for isolates of *Fusarium solani* (25). There are insufficient data available to determine if mycelia of spores are more sensitive to soil fungistasis. Conidia of *Helminthosporium sativum* (42) and *Trichothecium roseum* (26) were more readily inhibited than hyphae when placed directly on soil. In nonsterile extracts, however, hyphae of *Fusarium oxysporum* f. *cubense* was more strongly inhibited than was spore germination (57).

A limiting factor in the comparison of sensitivity of different fungi has been a technique which would give a quantitative measurement of differences. Most methods are indirect since spores are difficult to recover and observe on soil. The indirect methods usually involve nutritive substances such as agar discs or cellophane placed between the test fungus and soil. Whether the nature and degree of fungistasis by these methods was similar to that in soil was difficult to ascertain. Chacko and Lockwood developed a suitable method by mixing different amounts of natural and sterilized soil (6). Spores were added directly to the soil mixtures and recovered by using a plastic membrane (35). Using mixtures in different proportions, dosage-response curves could be made and the ratio of sterile:natural soil for 50% germination determined. Of 3 fungi tested in this system, *H. victoriae* was least sensitive, *Glomerella cingulata* next and *Penicillium frequentans*, the most sensitive fungus.

Materials and Methods

Maintenance of fungi: The following fungi were maintained on potato-dextrose agar: Fusarium solani (Mart.) Appel & Wr. f. phaseoli (Burk) Snyd. & Hans., F. solani f. pisi (F. R. Jones) Snyd & Hans.,F. oxysporum Schlecht, f. melonis (Leach & Currence) Snyd. & Hans., Glomerella cingulata (Ston.) Spauld. & Schrenk, Cladosporium cucumerinum Ell. & Ev., Botrytis cinerea Pers. ex Fr., Trichoderma viride Fr., Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr., Thielaviopsis basicola (Berk. & Br.) Ferr., Verticillium albo-atrum Reinke & Berthold, Aspergillus ustus (Bainer) Thom & Church, A. fumigatus Fresenius, A.terreus Thom, Penicillium frequentans Westling, P. variabile Sopp and Gliocladium sp. Corda. V-8 juice agar (per liter: 200 ml V-8 juice (Campbell Soup Co.), 2 g $CaCO_3$, 20 g agar) was used to maintain Helminthosporium sativum Pam., King, & Bakke, H. victoriae Meehan & Murphy, Curvularia lunata (Wakker) Boedijn, Alternaria tenuis Nees, and Stemphylium sarcinaeforme (Cav.) Wiltshire. The actinomycetes and Neurospora tetrasperma Shear & Dodge were grown on a nutrient agar (per liter: 10 g maltose, 4 g yeast extract, 4 g dextrose, 20 g agar). Puccinia recondita Rob. ex Desm. f. sp. tritici Eriks. and Erysiphe graminis DC. f. sp. tritici Em. Marchal were grown on seedling wheat (Triticum compactum Host, cv. 'Little Club') in a greenhouse.

Preparation of spores and mycelia: The age of all fungal cultures when used was 8 - 20 days unless stated otherwise.

For spore germination experiments, conidia of E. graminis f. sp. tritici and urediospores of P. recondita f. sp. tritici were shaken directly from the wheat seedlings onto the germinating medium. Conidia of the other fungi were removed from the agar slants by adding sterile glass distilled water and gently rubbing the colonies with a transfer loop. The conidial suspension was centrifuged at 3000 X G for 3 minutes and the supernatant liquid was discarded. The conidia were resuspended in sterile water, thoroughly shaken, and centrifuged again to remove any nutrients that may have carried over from the agar slants. After removing the supernatant liquid the conidia were resuspended in glass distilled water and transferred immediately to the germination medium.

Chlamydospores of F. solani f. phaseoli, F. solani f. pisi and F. oxysporum f. melonis were obtained by a method similar to that of Alexander et al. (1). An extract from Conover loam soil was sterilized with a Millipore filter (0.22 μ). Fifty ml of the filtrate were added to 250 ml erlenmeyer flasks. The solution was inoculated with unwashed conidia and mycelium from the different Fusarium isolates and incubated on a shaker at 24 C for 8 or more days. The chlamydospores formed were detached from the mycelia by blending in a Servall Omnimixer for 1 min. at approximately 5000 rpm. The chlamydospores were washed by centrifugation.

Mycelia were prepared by allowing conidia of different fungi to germinate in a nutrient solution of 0.5% peptone and 0.5% glucose. When the germ tubes were 4-6 times the length of the spores, the fungi were washed in the same manner as the spores.

Determination of spore volumes and germination times: The volumes of the different fungal spores were determined immediately after an aqueous suspension was made. The volumes of conidia of

Aspergilli, Penicillia, Puccinia recondita f. sp. tritici and chlamydospores of F. oxysporum f. melonis were determined by the formula for the volume of a sphere ($4/3 \times \pi \times \text{radius}^3$). Volumes of other spores were obtained using the formula for a prolate sphaeroid ($0.5236 \times \text{length} \times \text{width}^2$) (4, 39).

Germination times of fungal spores were determined on sterilized soil. A spore was considered germinated when a visible germ tube could be seen. For comparison with other fungi, the time required for 50% germination was used.

Source and preparation of soil: A Conover loam soil, possessing the following characteristics, was used in all experiments requiring topsoil: pH 6.7, organic matter 3.8%, water holding capacity (WHC) 42.7%, clay 7.5%, silt 42.8% and sand 49.7%. This soil, collected from the Michigan State University Botany and Plant Pathology Farm, was sieved and stored at 35% WHC in plastic containers at approximately 25 ± 2 C. When topsoil was to be inoculated with microorganisms, 50 g samples in 250 ml erlenmeyer flasks were first autoclaved for 1-1.5 hr. Washed fungal spores, bacterial cells and actinomycetes which had previously been blended in a Servall Omnimixer were then pipetted into the soil. The soil moisture was adjusted to 15% and then incubated at 24 C about 2 weeks. The soil was shaken daily to insure thorough mixing of the soil and microorganisms.

Subsoil was also inoculated with microorganisms. The subsoil was obtained from the same area as the topsoil and from a depth of approximately 30 in. It was stored in the laboratory in plastic bags at 12 C. Before use the soil was air dried with a fan, sieved and adjusted to about 10% moisture. Two hundred g of soil were added to 1 liter erlenmeyer flasks and autoclaved for 1-1.5 hr. Eight-tenths ml of a sterile 10% glucose solution and 4 ml of sterile Czapek's solutions (per 100 ml: 1.0 g K_2HPO_4, 2.0 g NH_4NO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$) (30) were added to each 200 g of sterilized subsoil. Thus, the concentration of glucose in subsoil was 400 μg/g of dried soil. After inoculation the soil was again adjusted to 10% moisture, and stored at 4 C for 4-6 hr. This was sufficient time to allow moisture to spread evenly in the soil; the low temperature prevented microbial activity. Thereafter, the inoculated soil was incubated at 24 C. The soil was shaken twice daily to insure thorough mixing of the contents.

Soil assays: The method of Lingappa and Lockwood (35) was used for observation of spore germination on soil (Fig. 1). Eighty g of topsoil was added to a petri dish (90 X 15 mm) and a smooth surface was made with a stainless steel spatula. The surface of the soil was cut into 6 equal sectors with the spatula. Two or three drops of a spore suspension of various fungi were applied to each soil sector. Subsoil was handled in the same manner except that smaller petri dishes (60 X 15 mm) were used and soil was divided into 2 or 3 sectors. After the appropriate incubation period, the spores were killed and stained with phenolic rose bengal. The spores were taken up with a polystyrene membrane and observed under the microscope for germination. One hundred spores were counted for each

Figure 1. Method used to test germination of fungal spores on soil. These materials were used in the following manner: (a) spatula for making smooth soil surfaces and cutting the soil into different sectors, (b) phenolic rose bengal to kill and stain the fungi, (c) polystyrene solution to pick up the spores from the soil and (d) mineral oil to prepare slide mounts for microscopic observation.

treatment. All experiments were run in duplicate and were repeated two or more times.

The quantitative assay for soil fungistasis developed by Chacko and Lockwood (6) was used to measure sensitivities of different fungi to soil fungistasis. Sterilized and natural soils were mixed in different proportions. The ratios of sterile: natural soil used were 20:1, 6:1, 3:1, 1:1, 1:3 and 1:6. Mixed soil was then added to petri dishes, made smooth and then divided into 6 equal sectors (Fig. 1). Controls consisted of plates with only sterilized or natural soil. Spores were added to the soil surfaces, and germination determined. These data were used to obtain dosage-response curves. The ratio of sterile:natural soil giving 50% germination of the fungal spores was obtained from these curves. This value was called the sensitivity index.

Chemical assays: Glucose and carbohydrates were extracted from soil to which an equal volume of water was added. This mixture was shaken on a wrist action shaker for 5 min, then centrifuged at 8000 X G for 5 min. The supernatant solution was sterilized when necessary by Millipore filtration (0.22 μ). About 70 to 85% of the original glucose added was recovered in a single water extract.

The method of Morris was used to determine the soluble carbohydrate content of soil (40). The anthrone reagent was made by dissolving 0.2 g of anthrone in 100 ml of dilute H_2SO_4 (prepared by adding 500 ml concentrated H_2SO_4 to 200 ml of H_2O). Nine ml of the reagent was mixed with 1 ml of the sample and heated in boiling water for 10 min. After cooling in ice water, the optical density was read at 600 mu in a colorimeter. A standard curve was made by using 1 ml samples of 20, 40 and 80 μg of glucose.

The amount of glucose in subsoil was determined with the Glucostat reagent (Worthington Biochemical Corporation). The soil extracts were diluted to contain between 0.05-0.3 mg glucose/ml, then mixed with 9 ml of the Glucostat reagent. After 10 min. incubation the reaction was stopped and the solution stabilized by the addition of one drop of 4 M HCl. A standard curve was made using 1 ml samples containing 20, 50, 100 and 300 μg glucose. Optical density readings were taken at 400 mμ in a colorimeter.

<u>Determination of carbohydrate levels needed for spore germination</u>: Extracts of natural soil and autoclaved soil were obtained by shaking equal volumes of water and soil for 30 min. The soil suspensions were centrifuged at 8000 X G for 5 min. The supernatant solutions were sterilized by filtering through a Millipore filter (0.22 μ). The extracts of autoclaved soil were diluted with extracts of natural soil. Solutions containing 100, 50, 30, 10, 1 and 0.1% autoclaved soil extract were used. One and one-half ml of each dilution was added to small petri dishes with an appropriate number of washed spores. Germination of spores in different concentrations of glucose in Czapek's solution was also determined.

A silica gel medium was used also for spore germination tests (21). This medium was prepared by mixing 60 ml of a solution containing 12 g of powdered silica gel (100-200 mesh) and 8.5 g of KOH with 27 ml of a solution of 20% aqueous o-phosphoric acid (85%).

Before this mixture solidified different dilutions of soil extract were added and thoroughly mixed. Six petri dishes (90 X 15 mm) were poured with this solution. To assure thorough mixing of all the ingredients before solidification, this process was carried out at 1 C. The water of syneresis, which forms upon gelation, was removed, and washed spores were added to the medium surface. After a germination period the spores were killed and stained with phenolic rose bengal and germination determined microscopically.

RESULTS

Sensitivity of fungal spores to soil fungistasis: Twenty-nine different fungal spores from 24 species were tested for their sensitivity to soil fungistasis by incubating them on different mixtures of sterile and natural soil (Table 1). The percent germination of the fungal spores on the different mixtures was used to establish a dosage-response curve for each fungus. For example, with ratios of sterile:natural soil equal to 20, 6, 4, 1, 0.3 and 0.2 the conidia of C. lunata germinated 96, 96, 89, 33, 4 and 1%, respectively. On the same mixtures conidia of T. viride germinated 86, 40, 14, 10, 2 and 0%, respectively. When plotted on log-probability paper, the data gave straight lines (Fig. 2). The slopes of the dosage-response curves for most fungi were similar with the exception of P. variabile, Gliocladium sp. and V. albo-atrum, which had steeper slopes (Fig. 2). From the dosage-response curves, the ratio of sterile:natural soil giving 50% germination of each spore type was determined. A low sensitivity index means that a relatively small amount of sterile soil in the mixture would give 50% germination of the spores.

There was a wide range in sensitivity indexes among the different fungi (Table 1, Fig. 2). Three fungi germinated freely on natural soil and are not included in Fig. 2. These were conidia of E. graminis f. sp. tritici, ascospores of N. tetrasperma and urediospores of P. recondita. Therefore, these fungi are considered to be completely insensitive to soil fungistasis (sensitivity index = 0). This confirms the results of Ko and Lockwood with N. tetrasperma, E. graminis f. sp. tritici and P. coronata (31). Other fungi which

Table 1. Volumes, germination times and sensitivities to soil fungistasis of spores of various fungi

Fungus	Spore type	Spore Volume (μ^3)	Germination time (hr.)[a]	Sensitivity index[b]
Helminthosporium sativum	Conidia	10537	2.5	2.5
Stemphylium sarcinaeforme	Conidia	6583	3.5	1.3
Helminthosporium victoriae	Conidia	6333	1.5	0.5
Puccinia recondita	Urediospores	5219	2.0	0
Neurospora tetrasperma	Ascospores	3839	2.0	0
Erysiphe graminis sp. tritici	Conidia	1985	2.0	0
Alternaria tenuis	Conidia	1772	4.0	3.8
Curvularia lunata	Conidia	1025	1.3	1.5
Fusarium solani f. phaseoli	Macroconidia	568	4.5	0.2
Fusarium solani f. pisi	Macroconidia	427	4.0	0.1
Fusarium solani f. phaseoli	Chlamydospores	309	7.0	0.8
Fusarium oxysporum f. melonis	Chlamydospores	220	4.5	1.4
Fusarium oxysporum f. melonis	Macroconidia	218	6.0	0.4
Glomerella cingulata	Conidia	212	6.0	3.4
Thielaviopsis basicola	Conidia	210	5.0	6.2

Botrytis cinerea	conidia	182	4.0	11.5
Fusarium solani f. pisi	Chlamydospores	124	4.5	0.9
Cladosporium cucumerinum	Conidia	92	5.0	2.5
Fusarium solani f. pisi	Microconidia	64	5.5	0.4
Trichoderma viride	Conidia	45	17.0	9.1
Myrothecium verrucaria	Conidia	36	6.5	3.8
Fusarium oxysporum f. melonis	Microconidia	36	7.0	1.7
Verticillium albo-atrum	Conidia	22	12.0	7.5
Aspergillus ustus	Conidia	20	8.0	5.8
Penicillium frequentans	Conidia	19	7.0	20.0
Gliocladium sp.	Conidia	16	4.5	0.6
Penicillium variabile	Conidia	14	14.0	>20.0
Aspergillus fumigatus	Conidia	13	13.0	15.5
Aspergillus terreus	Conidia	8	15.0	23.0

[a] Time for germination of 50% of the spores on sterilized soil.

[b] Ratio of sterile:natural soil for 50% germination.

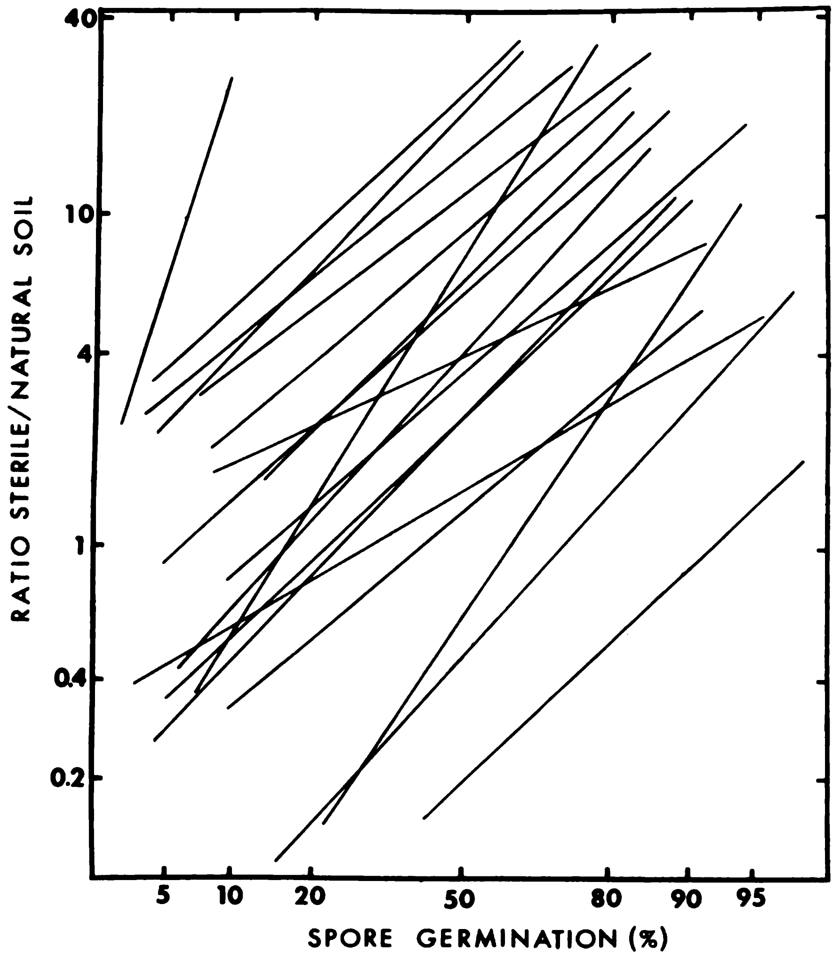

Figure 2. Dosage-response curves for conidia of 19 different fungi. Germination was determined by using various mixtures of sterile and natural soil. Each line represents a different fungus.

were relatively insensitive to soil fungistasis were macroconidia of F. solani f. phaseoli, conidia of H. victoriae and conidia of Gliocladium sp. with sensitivity indexes of 0.2, 0.5 and 0.6, respectively (Table 1).

Fungi with conidia intermediate in sensitivity were G. cingulata, M. verrucaria and C. cucumerinum which had sensitivity indexes equal to 3.4, 3.8 and 2.5, respectively. Fungi with conidia requiring relatively larger amounts of sterilized soil were A. terreus with a sensitivity index of 23, A. fumigatus with 15.5 and B. cinerea with 11.5. One fungus, P. variabile, which germinated 90-100% on sterilized soil, would not germinate more than 20% with a sterile:natural soil ratio of 20. The amount of sterile soil needed for 50% germination was not determined but the steep slope of the dosage-response curve indicates that its sensitivity index would be much higher than that of any other fungus tested (Fig. 2).

Effect of spore age on sensitivity to fungistasis: Three fungi were incubated on agar slants for 1, 3, 5 and 9 weeks to determine if age of the conidia affected their sensitivity to soil fungistasis. The 3 fungi did not change in sensitivity over the time period tested (Fig. 3). G. cingulata had a sensitivity index of 3.0 at the age of 1 week and 2.9 at 9 weeks. P. frequentans had an sensitivity index of 5.5 at 1 week old and 5.6 at 9 weeks. H. victoriae conidia had a sensitivity index of 0.8 at 1 week and 1.5 at 9 weeks, but the difference was not statistically significant. When cultures of the 3 fungi were 9-13 weeks old some of the conidia apparently were

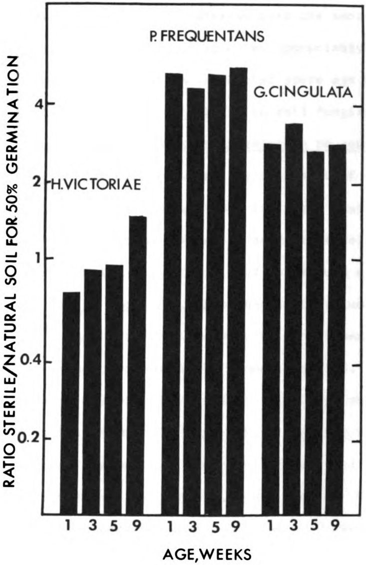

Figure 3. Effect of conidial age on sensitivity to soil fungistasis.

not viable since the percentage germination on sterilized soil had decreased. However, limited data indicated that the sensitivities of the viable conidia were still not affected appreciably.

Results using these 3 fungi indicate that spore age between 1 and 9 weeks is not a factor in sensitivity to soil fungistasis.

Spore volume and germination time in relation to sensitivity to soil fungistasis: The data illustrating the range of sensitivity of the different fungi to soil fungistasis indicated that differences in spore volume and germination time could be factors related to sensitivity to soil fungistasis. Conidia of fungi such as *H. sativum*, *S. sarcinaeforme*, and *H. victoriae*, which had large volumes of 10,537, 6,583 and 6,333 μ^3, respectively, were also the least sensitive among the fungi, having respective sensitivity indexes of 2.5, 1.3 and 0.5 (Table 1). Fungal conidia intermediate in volume, such as those of *G. cingulata* with a volume of 212 μ^3 and *T. basicola* with a volume of 210 μ^3, also were intermediate in sensitivity to soil fungistasis, with sensitivity indexes of 3.4 and 6.2, respectively. Those fungi with small volumes also were the most sensitive to fungistasis. For example, conidia of *A. terreus*, *A. fumigatus* and *P. variabile* had volumes of 8, 13 and 14 μ^3, respectively, and had sensitivity indexes of 23, 15.5 and 20.

The correlation coefficient for spore volume and sensitivity to soil fungistasis for spores of 17 fungi was statistically significant ($r=.81$, $p<5\%$) (Fig. 4). Those fungal spores germinating freely on soil were not included in this correlation or in Fig. 4.

However, these 3 insensitive fungi, E. graminis f. sp. tritici, P. recondita and N. tetrasperma, had relatively large spores with volumes of 5,219, 3,839 and 1,985 μ^3, respectively. Of those fungi sensitive to fungistasis, macroconidia of F. solani f. phaseoli and conidia of Gliocladium sp. also were not included in figuring the correlation coefficient, since they were obviously exceptions to the correlation of spore volume with sensitivity. Spores of these fungi were relatively insensitive to fungistasis, but were intermediate to small in size.

The relationship between sensitivity of spores to soil fungistasis and germination time was considered also. Conidia of C. lunata, H. victoriae and H. sativum were relatively fast germinating, requiring 1.3, 1.5 and 2.5 hr., respectively, for 50% of the spores to germinate on sterilized soil (Table 1). They were also relatively insensitive to soil fungistasis, having respective sensitivity indexes of 1.5, 0.5 and 2.5. Intermediate in germination time were conidia of A. tenuis, T. basicola and M. verrucaria, which required 4.0, 5.0 and 6.5 hr. for germination and had sensitivity indexes of 3.8, 6.2 and 3.8, respectively.

Fungi with conidia that required long periods to germinate were the most sensitive to fungistasis. These included P. variabile, which germinated 50% in 14 hr. and had a sensitivity index greater than 20; A. terreus, with a germination time of 15 hr. and a sensitivity index of 23; and A. fumigatus, with a germination time of 13 hr. and a sensitivity index of 15.5.

There was a significant correlation ($r=.80$, $p<5\%$) between

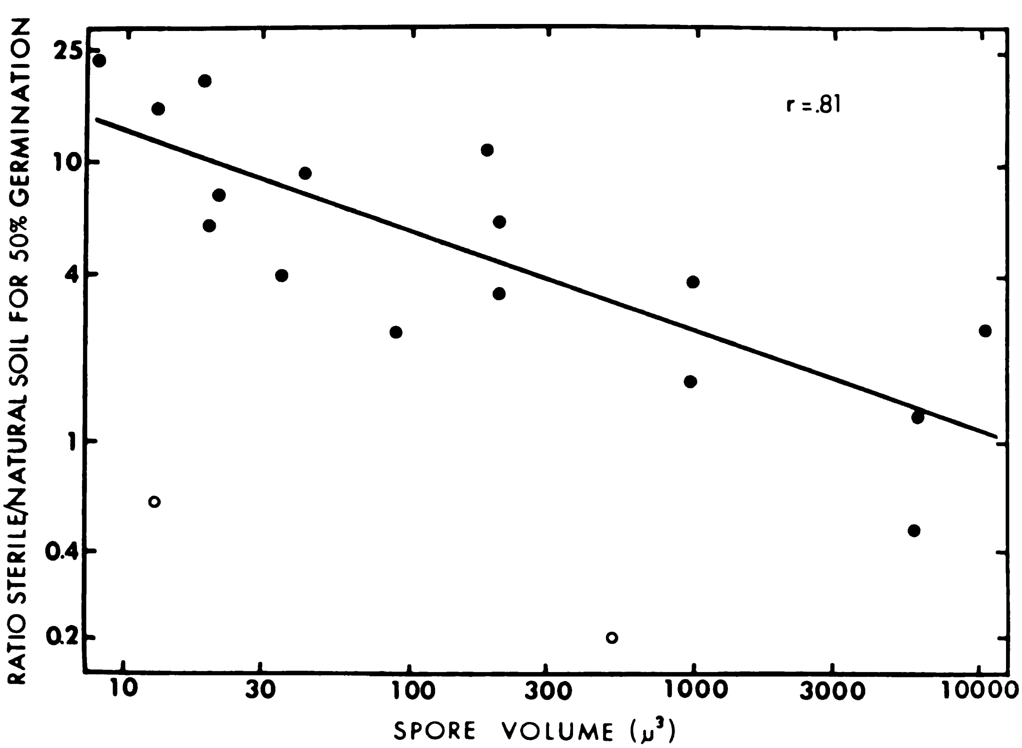

Figure 4. Relationship between spore volume and sensitivity to soil fungistasis. Solid circles represent those fungi used in figuring the correlation coefficient. Open circles represent two exceptions to this relationship, *Gliocladium* sp. and *F. solani* f. *phaseoli*.

the time required for 50% germination and the sensitivity of 17 fungi to soil fungistasis (Fig. 5). Macroconidia of F. solani f. phaseoli and conidia of Gliocladium sp. were exceptions to this relationship also, and were not included in calculating the correlation coefficient. These conidia require 4-5 hr. for 50% germination, but were among the least sensitive of the fungi tested. It was of interest also, that the 3 fungi which were completely insensitive to fungistasis all germinated 50% on sterilized soil within 2 hr. Since their sensitivity indexes were 0, these fungi were not included in calculating the correlation coefficient or in Fig. 5.

The effect of nutrient concentration on spore germination time was also considered. Conidia of P. frequentans, F. solani f. phaseoli and H. sativum germinated faster in high concentrations of aqueous glucose than at low levels. For example, conidia of P. frequentans germinated 50% in 10.5, 13.5 and 15 hr. when the glucose concentrations were 500, 100 and 50 μg/ ml solution, respectively. Results with other fungi followed a similar pattern. Since germination time was correlated with sensitivity to soil fungistasis, these data suggest that factors affecting germination time might in turn affect sensitivity also.

Sensitivity of different spore forms of Fusarium: Since the macroconidia of F. solani f. phaseoli were an exception to the correlations of spore size and germination time with sensitivity to soil fungistasis, the sensitivities of two other species of Fusarium, F. solani f. pisi and F. oxysporum f. melonis, were determined.

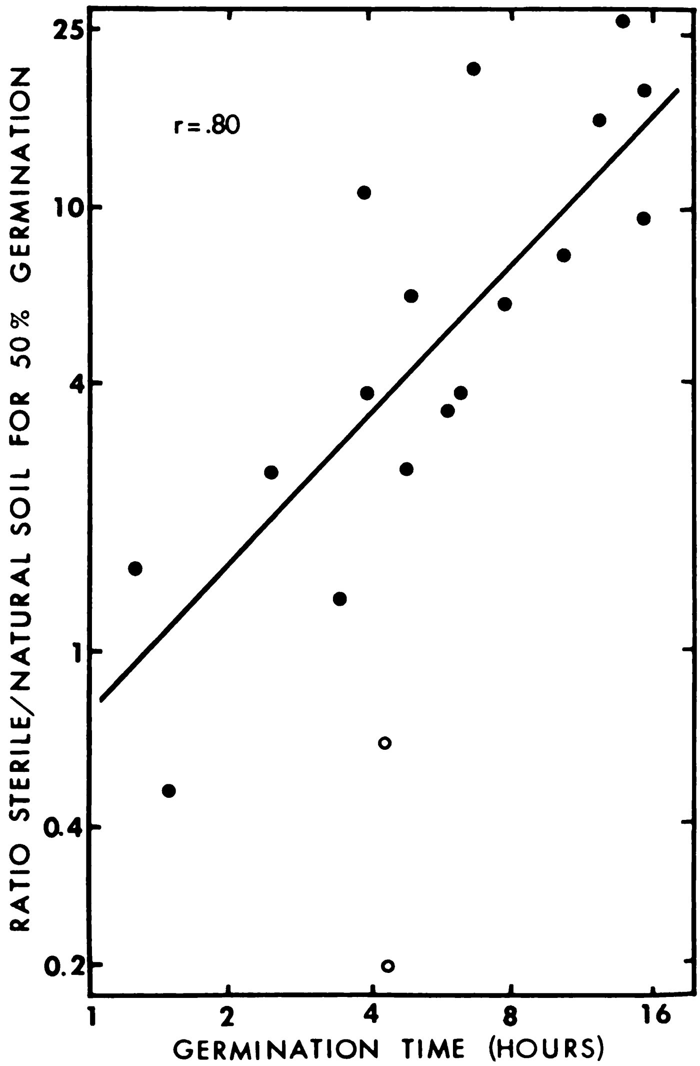

Figure 5. Relationship between germination time and sensitivity to soil fungistasis. Solid circles represent those fungi used in figuring the correlation coefficient. Open circles represent two exceptions to this relationship, *Gliocladium* sp. and *F. solani* f. *phaseoli*.

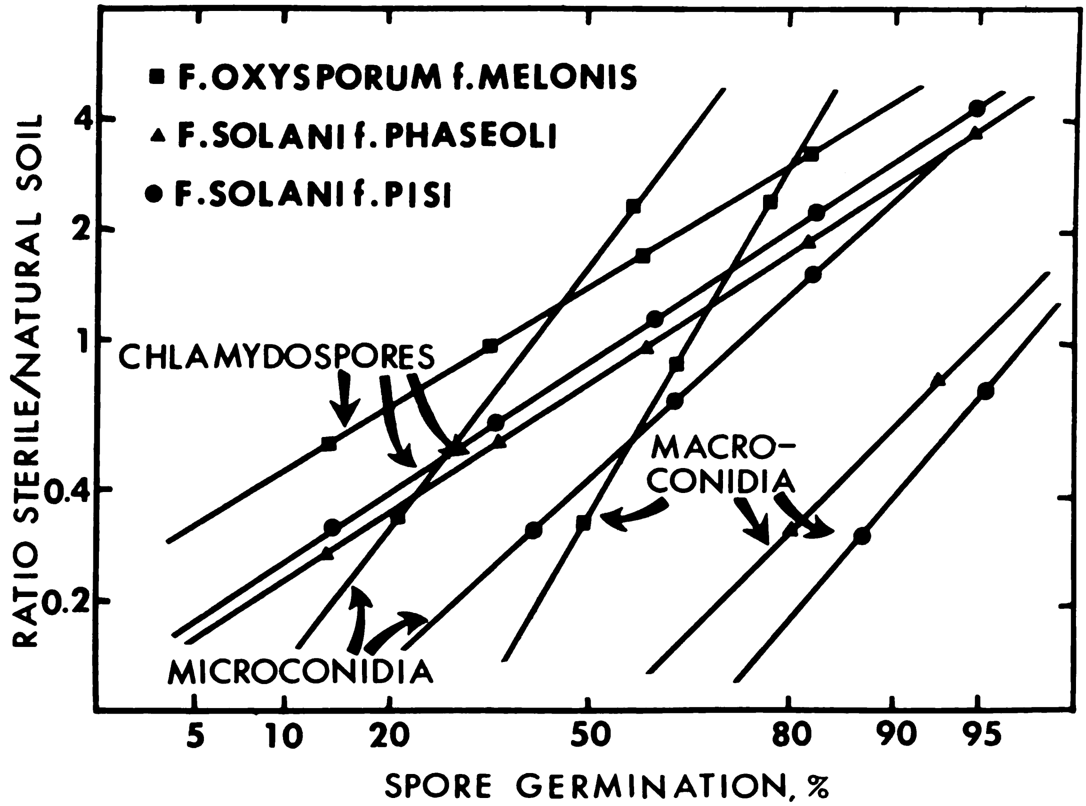

Figure 6. Dosage-response curves for chlamydospores, microconidia and macroconidia of 3 *Fusarium* species.

The sensitivity index for macroconidia of F. solani f. pisi was 0.1 and for F. oxysporum f. melonis, 0.2. These conidia were the least sensitive of any spores tested with the exception of the 3 totally insensitive fungi. Microconidia of F. solani f. pisi and F. oxysporum f. melonis had sensitivity indexes of 0.4 and 1.7, respectively (Fig. 6). The isolate of F. solani f. phaseoli used in this study did not produce microconidia. For chlamydospores, the sensitivity index for F. solani f. phaseoli was 0.8, that for F. solani f. pisi was 0.9, and that for F. oxysporum f. melonis was 1.4. Therefore, these spores were more sensitive to fungistasis than were the macroconidia, but in comparison with other fungal spores, chlamydospores were relatively insensitive to soil fungistasis.

The germination time of all 3 Fusarium spore types was 4-7 hr. on sterilized soil. This is an intermediate time in comparison with the other fungi. However, the volumes of these same spores were intermediate to small (Table 1). Therefore, these spores as a group are exceptions to the correlations of spore volume and germination time with sensitivity to soil fungistasis.

Relationship between germination time and spore volume: Since spore volume and germination time were clearly related to sensitivity of fungal spores to fungistasis, with the exceptions of the Fusaria and Gliocladium sp., the relationship of these two factors was examined without respect to fungistasis (Fig. 7). All 29 different spore types were used including conidia of Gliocladium sp. and different spore forms of the 3 Fusarium species. Also included were the 3 completely insensitive species, ascospores of N. tetrasperma,

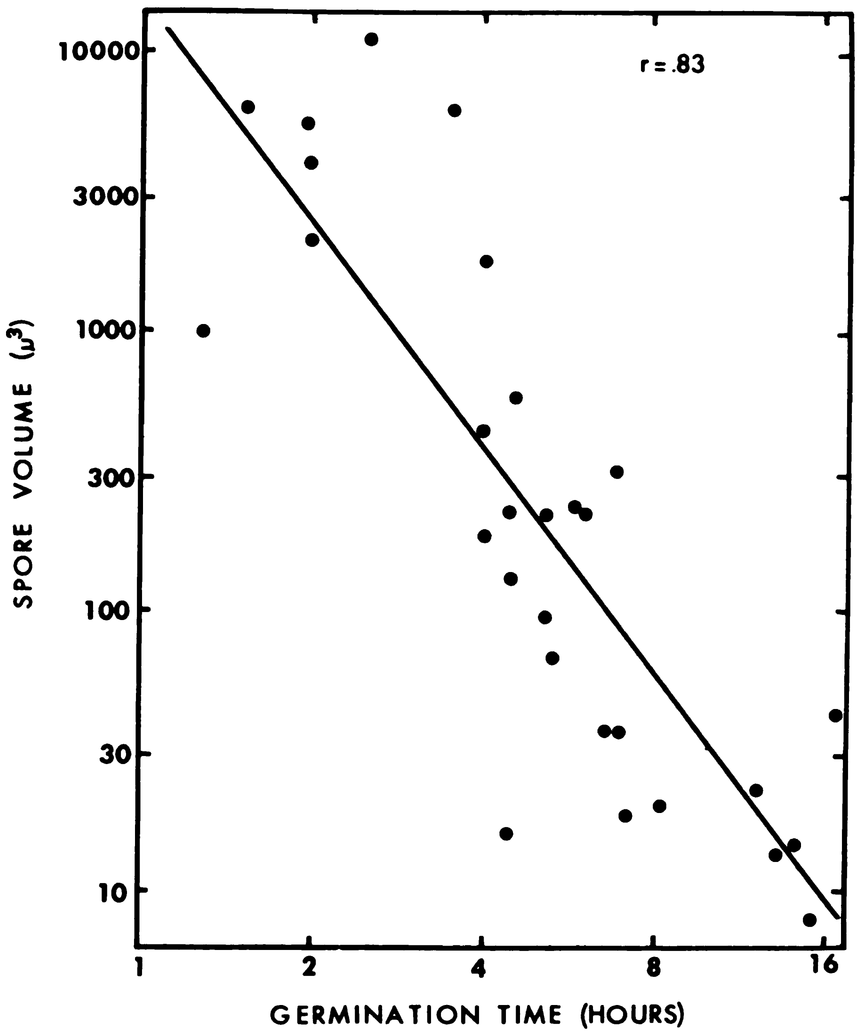

Figure 7. Relationship between spore volume and germination time on sterilized soil. Twenty-nine spores from 24 different fungi are included.

urediospores of _P_. _recondita_ and conidia of _E_. _graminis_ f. sp. _tritici_. Germination time and spore volume were significantly correlated ($r=.83$, $p<5\%$). Thus, as a rule, fungi with large spore types appear to germinate relatively fast with the opposite being true for small spore types.

Concentration of nutrients needed for spore germination: Previous results using different mixtures of sterile and natural soil revealed that a considerably smaller amount of sterilized soil (or a smaller amount of nutrients) was needed for germination of large spores than was required for the germination of small spores. This suggested that large spores might have low sensitivities to soil fungistasis because they required less nutrients to germinate. The respective levels of water soluble carbohydrates in the soil mixtures containing 20, 6, 3, 1, 0.3 and 0.2 parts of sterilized soil for each part of natural soil were 380, 345, 280, 190, 100 and 55 µg/g soil. The concentration of utilizable carbohydrates in these mixtures could be lower than that required by some fungal spores to germinate.

Another factor affecting concentration of nutrients in soil mixtures was the rapid loss of the nutrients from the mixtures due to microbial activity (Fig. 8). For example, when the ratio of sterile:natural soil was 3, the concentration of carbohydrates was 280 µg/g soil at 0 time, 212 µg after 5 hr. and 170 µg after 12 hr. incubation. For those fungal spores requiring a long time to germinate, the nutrient level could easily drop below that required for germination before the germination process was completed, even though the original concentration may have been adequate.

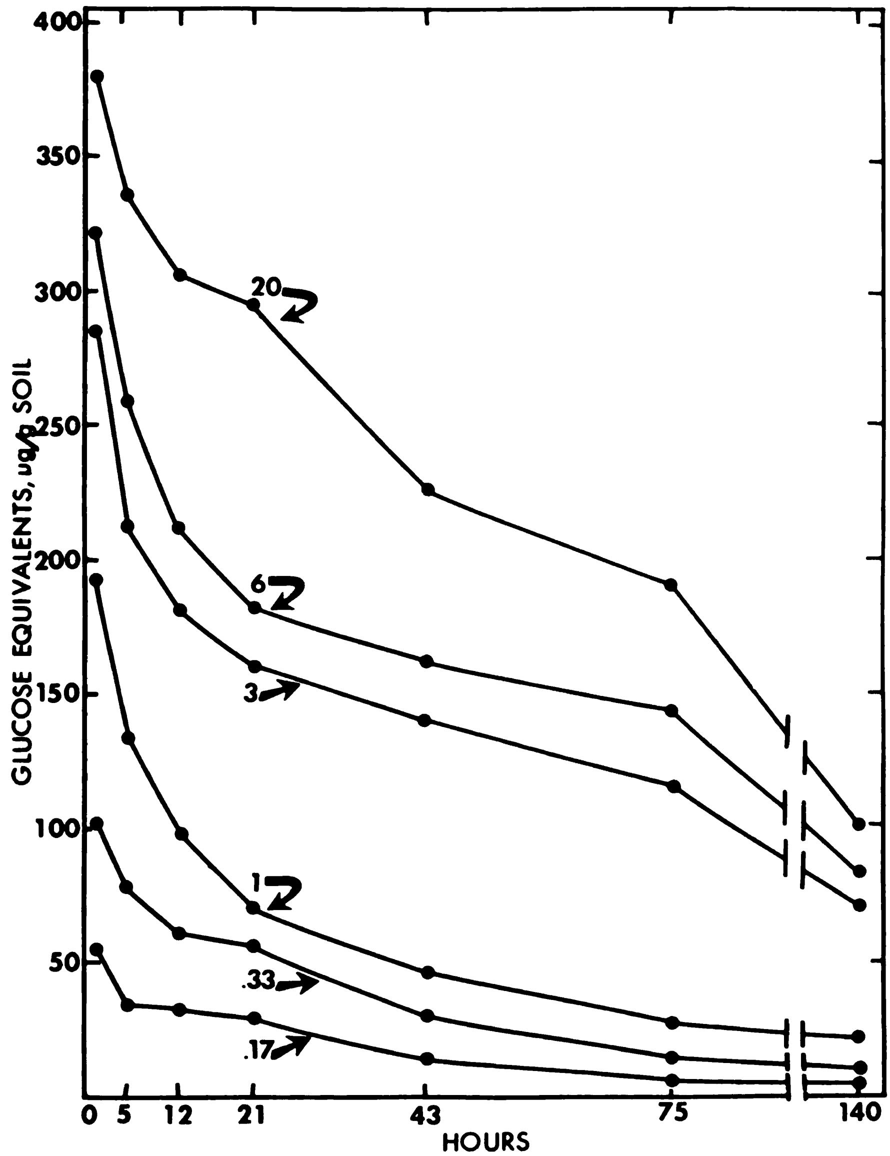

Figure 8. Loss of carbohydrates, as determined by the anthrone reagent, from different mixtures of sterile and natural soil. Numbers beside each arrow indicate the ratio of sterile:natural soil.

To avoid these complications, concentration of nutrients required for spore germination was determined in various concentrations of nutrient solutions. Thus, the spores could germinate in a constant level of nutrients.

Different concentrations of glucose with a constant level of mineral salts were used to determine the level required for 50% germination of 8 fungi with nutrient dependent conidia. The concentration of glucose required for 50% germination was not significantly correlated with the sensitivity index of the conidia ($r = .61$, $p < 5\%$). Further, amounts of glucose needed for germination were not correlated with spore volume ($r = .07$. $p < 5\%$). For example, conidia of _H_. _sativum_, which were the largest spores (10,537 μ^3) and had the lowest sensitivity index (2.5), required 51 μg glucose/ml solution for 50% germination. _P_. _variabile_, which had small conidia (14 μ^3) and the highest sensitive index (>20.0) needed 62 μg glucose/ml solution for 50% germination. _V_. _albo-atrum_, whose small spores (22 μ^3) were intermediate in sensitivity (7.5), required only 26 μg glucose/ml solution for 50% germination. These experiments were repeated 5 times with similar results.

Similar experiments were also done using different concentrations of an aqueous extract of sterilized soil diluted with an aqueous extract of natural soil. This allowed a closer duplication of the nutrients in the soil mixtures. Nutrient dependent conidia of 7 fungi were used to determine the concentration of sterilized soil extract giving 50% germination. These data also showed no correlation between sensitivity of spores to soil fungistasis and concentration of soil extract required for spore germination ($r=.33$, $p<5\%$). A similar lack of correlation was observed for soil extract concentration and spore volume ($r=.14$, $p<5\%$). For example, conidia of A. terreus which were small (8 μ^3) and had a very high sensitivity index of 23, required a solution containing 19% extract of sterilized soil. H. sativum with the largest conidia (10,537 μ^3) and lowest sensitivity index of 2.5 of those tested required 20% extract of sterilized soil for 50% germination. These experiments were repeated 3 times with similar results.

In an effort to duplicate the contact of the spore surface with the soil surface the same soil extract mixtures used above were dispersed in silica gel. The same 7 fungi were used. The results were identical with those using soil extract solutions in showing no correlation of sensitivity to fungistasis or spore volume with nutrient concentration required for 50% germination.

It was concluded from these experiments that concentration of nutrients required for germination of fungal spores do not appear to be related to their sensitivity to soil fungistasis nor to their size.

Effect of reducing germination time on sensitivity to soil fungistasis: The previous results suggested that the time a spore needs for germination could be a significant factor in determining its sensitivity to soil fungistasis, since nutrients are rapidly decreasing in the soil mixtures during the germination process (Fig. 8). For example, slow germinating conidia of A. terreus (15 hr.) and T. viride (17 hr.) might be at a disadvantage insofar as ability to germinate in an environment where nutrients were depleted rapidly.

To test the possibility that germination time was related to sensitivity of spores to fungistasis, conidia of various fungi were incubated in a nutrient solution of 0.5% peptone and 0.5% glucose for periods of about 1-4 hr. less than that required for germination to begin. The spores were then washed and their sensitivities to soil fungistasis were assayed on mixtures of sterile and natural soil. Conidia of the same fungi without any treatment prior to placing them on the soil mixtures were used as controls.

Pretreatment of spores in nutrients reduced germination time and sensitivity in the following manner: conidia of P. frequentans were reduced in germination time on sterilized soil from 8 hr. to 2.5 hr. and were reduced in sensitivity from an index of 20 to 4.5. The germination time of V. albo-atrum was reduced from 12 hr. to 4 hr. and sensitivity from a value of 7.0 to 1.1. C. lunata was reduced in germination time from 1.3 to 0.8 and sensitivity from a value of 1.4 to 0.5. Results with 11 other fungi followed a similar pattern.

For the 14 fungi used, the percent reduction in germination time and percent reduction in sensitivity were significantly correlated ($r=.72$, $p<5\%$) (Fig. 9).

Further experiments were designed to determine if reduction in germination time and sensitivity to fungistasis required the presence of a nutrient environment and occurred only in metabolically active spores. This was accomplished by incubating spores in a nutrient solution of 0.5% peptone and 0.5% glucose or water at 1 or 24 C

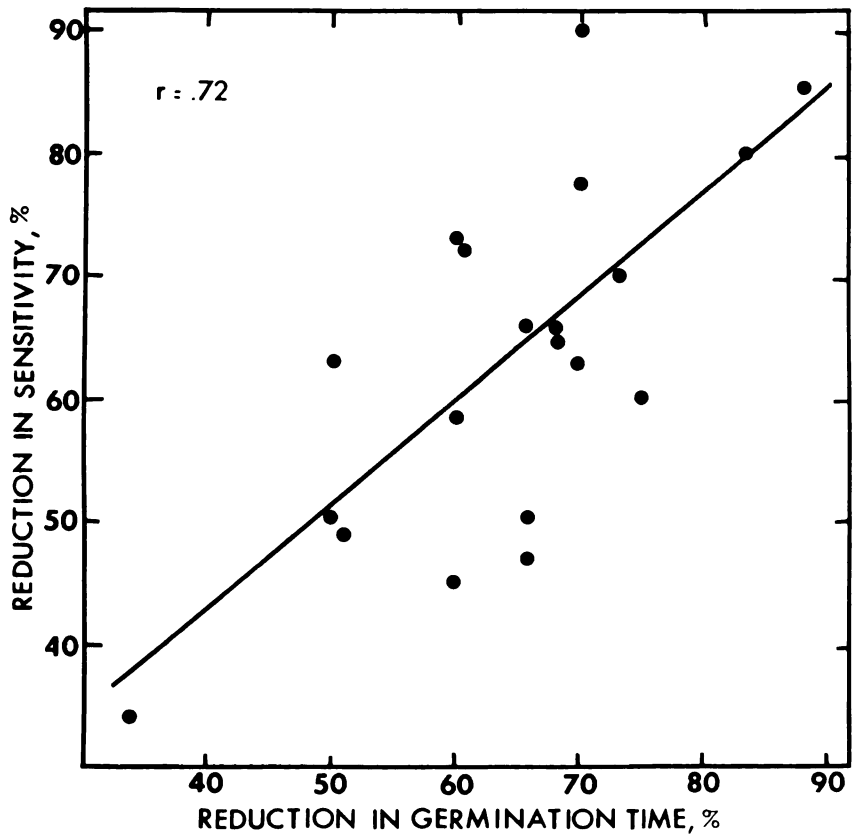

Figure 9. Relationship between reduction in germination time and reduction in sensitivity to soil fungistasis when spores were incubated in a nutrient solution prior to placing them on different mixtures of sterile and natural soil.

before assaying them on soil. Both nutrient dependent and nutrient independent conidia were used.

Neither sensitivity indexes nor germination times of nutrient dependent conidia of _B. cinerea_ and _P. frequentans_ were affected by pretreatment in water at 1 or 24 C or in nutrient solution at 1 C (Fig. 10). Incubation in the nutrient solution at 24 C reduced germination time and sensitivity of the fungi in a manner similar to that in the previous tests. When conidia of _G. cingulata_, which are nutrient independent, were incubated in nutrient solution or water at 1 C prior to placing them on the different soil mixtures, germination time and sensitivity to soil fungistasis were not affected. However, incubation of these conidia in water at 24 C prior to adding them to the soil reduced germination time from 8.5 to 6.0 hr. and the sensitivity index from 3.2 to 0.9. When the conidia were incubated in nutrient solution at 24 C, the germination time was reduced from 8.5 to 2.5 and the sensitivity index from 3.2 to 0.3.

These data indicate that available nutrients can reduce the germination time, and as a consequence, sensitivity to fungistasis is also decreased if the spores are at a temperature favorable for metabolism to take place. With nutrient independent spores, even water can substantially reduce germination time and sensitivity.

Sensitivity of mycelia to soil fungistasis: The preceding results showed that reducing germination time of fungal spores by incubating them in a nutrient environment for periods shorter than that required for germination reduces sensitivity to soil fungistasis. Therefore, mycelia should be less sensitive than the corresponding

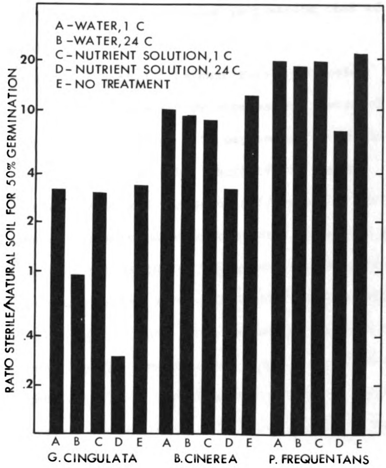

Figure 10. Effect on sensitivity to soil fungistasis of incubating conidia in water or a nutrient solution at 1 or 24 C. Control (no treatment) was conidia placed on various soil mixtures without any prior treatment.

spores, since germination time is not a factor in mycelial growth. Spores incubated in nutrient solutions to reduce germination time should have a sensitivity index value approaching that of mycelium of the same fungus.

Experiments were designed to test this hypothesis. Sensitivity of fungal mycelia was determined by germinating spores of different fungi in a nutrient solution of 0.5% peptone and 0.5% glucose until the germ tubes were 4-6 times the length of the spores. Spores and mycelia were then washed and placed on different soil mixtures. As a control some of the germinated spores were killed and stained with rose bengal so that the amount of new growth on the soil mixtures could be determined. After 12 hr. incubation, the mycelia of all fungi were killed and stained. The new mycelial growth was determined by subtracting the average total length in the control from the average total length of the incubated mycelia. Percent germination of the spores was obtained in the usual manner. These data were used to obtain the ratio of sterile:natural soil for 50% germination of the spores and for 30 μ new growth of the mycelium. On log-probability paper the dosage-response curves for sensitivity of spores and mycelia were straight lines.

Mycelia of all fungi tested were less sensitive to soil fungistasis than corresponding conidia (Table 2), although those incubated in nutrients to reduce germination time had sensitivity indexes much closer to those for mycelia. For example, P. frequentans had sensitivity indexes of 20 for nontreated conidia, 4.5 for conidia incubated in nutrients and 1.1 for mycelia. The sensitivity indexes for V.

Table 2. Comparison between the sensitivity to soil fungistasis of fungal mycelia and the corresponding spores

	Sensitivity Indexes	
Fungus	Mycelia	Spores
F. solani f. *phaseoli*	0.2	0.2
H. victoriae	0.2	0.5
V. albo-atrum	0.2	7.5
A. ustus	0.9	5.8
C. cucumerinum	0.9	2.5
P. frequentans	1.1	20.0
A. fumigatus	1.9	15.5
A. terreus	2.6	23.0

albo-atrum and A. ustus, following the same order of treatments were 7.5, 1.1 and 0.2 and 5.8, 1.5 and 0.9, respectively. The range in sensitivity indexes for mycelia (0.2 to 2.6) was much narrower than for the corresponding conidia (0.2 to 23). For these fungi the ratio of sterilized:natural soil for 50% germination of spores and 30 μ new growth of mycelia were significantly correlated ($r = .77$, $p < 5\%$) (Fig. 11). In other words, those fungi with relatively sensitive spores also had relatively sensitive mycelia.

These data supported the prediction that mycelia are less sensitive to soil fungistasis than corresponding spores and further indicates the importance of germination time in determining sensitivity to soil fungistasis. Mycelia probably represent the least sensitive stage of a fungus to fungistasis.

Effect of intermittent exposure to nutrients on germination time requirement: Spores of fungi require a certain amount of time to germinate when exposed to nutrients suitable for germination. The time requirement for germination on sterilized soil can be shortened if spores are incubated in a nutrient solution prior to placing them on soil. In natural soil small amounts of nutrients are continually being released and quickly used by the microflora. If the nutrients are used by the microflora before some spores have germinated, will the time required for germination of these spores revert to what it was originally or will the spores germinate more quickly when additional nutrients are again supplied? To help answer this question the following experiments were done. Spores of 3 different fungi were placed in separate nutrient solutions for 4 hr., removed, washed

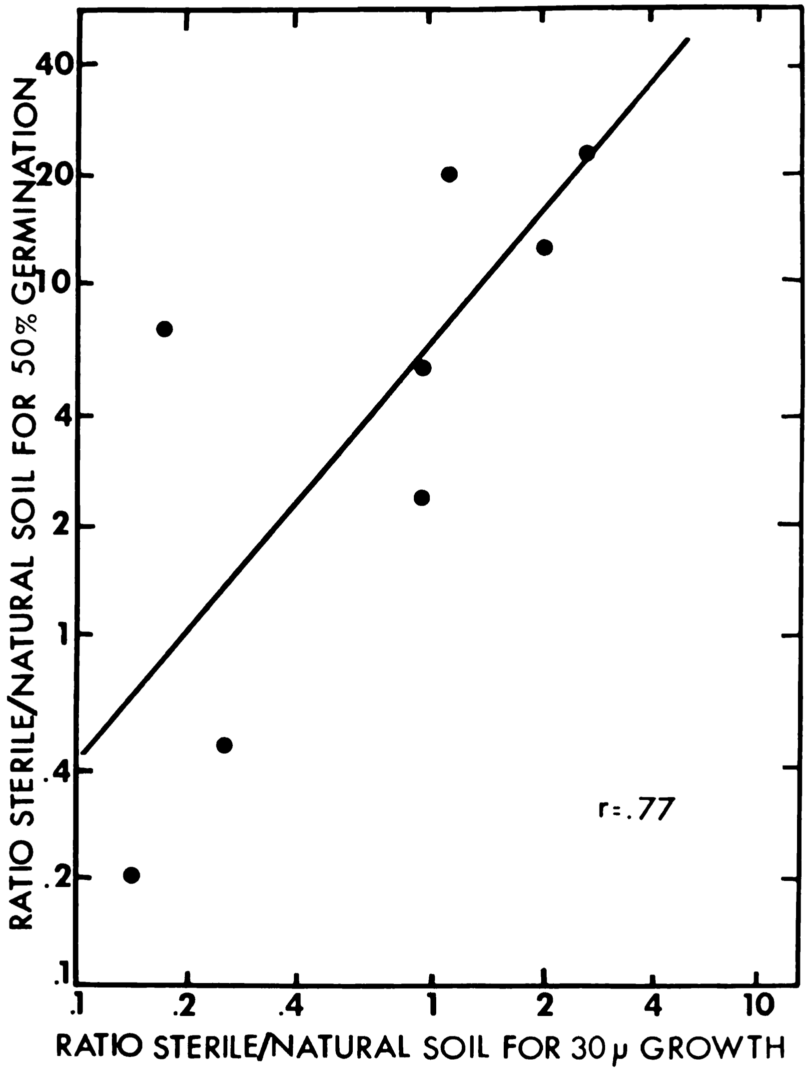

Figure 11. Relationship between sensitivities of conidia and mycelia of 8 different fungi to soil fungistasis.

and placed in sterile-glass distilled water for 4 hr. The same 2 treatments were repeated once again, and were followed by a final treatment in nutrient solution. After each treatment a small portion of the spore suspension was retained in the same solution and the time required for 50% germination determined.

The germination time for conidia of _P_. _frequentans_ continually incubated in nutrients solution was 9.5 hr. (Fig. 12). In the first water treatment no germination occurred after 24 hr. At the end of the second nutrient treatment, no spores had germinated, but spores left in the same solution germinated 50% after an additional 1.5 hr. incubation. In the water treatment that followed, conidia did not germinate more than 10% in 24 hr. When the spores were placed in the last nutrient solution they germinated 50% in 2 hr. Thus, the total time needed for 50% germination in intermittent nutrient sources was 10 hr. as compared with 9.5 hr. in continual nutrients. Conidia of _A_. _ustus_ and _V_. _albo-atrum_ responded in a manner similar to _P_. _frequentans_ when tested in the various nutrient-water treatments.

It was concluded that germination time of fungal spores in a nutrient solution is cumulative and irreversible. Substitution of a nonnutritive medium cannot shorten germination time or substitute for an essential nutrient at any time during the germination process.

Experiments were performed to determine if the germination process of spores on soil was also cumulative in a manner similar to that in liquid media. Conidia of _P_. _frequentans_, _A_. _ustus_ and _V_. _albo-atrum_,

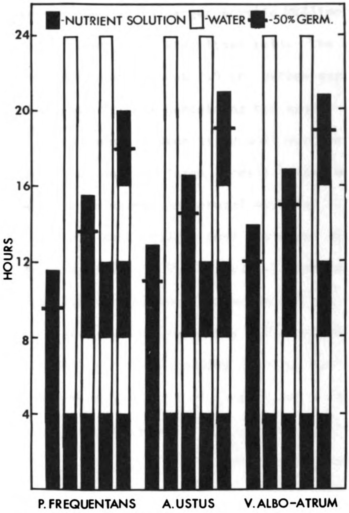

Figure 12. Effect of alternating incubation in a nutrient solution and water on conidial germination time. After each treatment some conidia were left in the solution and time required for 50% germination determined.

were pipetted on small sterile Millipore filters (0.22 μ, 25 mm diam.) which were then placed on larger sterile Millipore filters (0.22 μ, 47 mm diam.) resting on sterilized soil. The conidia were left on the sterile soil until about 1.5 hr. before germination would have started. The small filters containing the spores were then removed and placed on (a) another sterilized soil surface which served as a control, and (b) on another large sterile filter on natural soil. The spores were incubated on natural soil for 2, 4, 6 and 11 days before removing the small filter once again and placing it on the surface of sterilized soil. The additional time needed on sterilized soil for 50% germination of the conidia was determined.

The total time required on sterilized soil for 50% germination of _G_. _cingulata_ conidia was : (a) control 10.5 hr. and (b) after interrupted periods on natural soil of 2, 4, 6 and 11 days, 8.5, 9.0, 10.0 and 10.5 hr., respectively (Fig. 13). Time required for germination did not vary significantly between the various treatments. Time necessary for germination of conidia of _P_. _frequentans_ and _A_. _ustus_ followed essentially the same pattern as that for conidia of _G_. _cingulata_.

These data show that the germination process was cumulative. The fact that germination time did not revert back to the time originally needed for germination when placed on natural soil suggests that the germination process is irreversible. Germination was not induced by a triggering mechanism since a given time in nutrient solution was required whether the exposure was continual or interrupted.

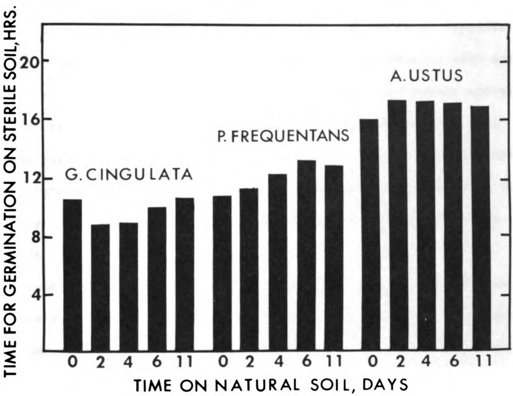

Figure 13. Germination time of conidia incubated first on sterilized soil, then on natural soil for 2, 4, 6 or 11 days, and again on sterilized soil until germination occurred. Conidia were incubated on double Millipore filters (0.22 μ) to maintain sterility.

<u>Establishment of fungistasis on different model systems</u>: Previous results indicated that germination time for nutrient dependent spores can be shortened only with nutrients. These results also suggested that the germination process can be stopped by placing spores in restrictedly nutritive media such as water or natural soil when the time remaining for germination was at least 1.5 hr. Experiments were designed to determine whether spores with little germination time remaining could be stopped by the elimination of the nutritive environment.

Advantage was taken of the fact that most fungal spores do not germinate synchronously. Conidia of 4 different fungi were incubated in glucose-peptone solutions until 10-35% of the conidia were germinated. The conidia were then washed and placed on Millipore filters (0.22 μ). The filters containing the different conidia were placed on (a) natural soil, where the spores were immediately killed and stained to determine the original percent germination of the spores, (b) sterilized soil to determine potential percent germination, (c) natural soil, (d) washed, sterilized sand through which a continuous stream (2 ml/hr.) of buffer solution (0.005 M PO_4, pH = 6.8) was moving and (e) sand moistened with buffer.

Using these model systems, <u>P</u>. <u>frequentans</u> germinated as follows: (a) zero time control, 16%, (b) sterilized soil, 98%, (c) natural soil, 13%, (d) sand plus running water, 17% and (e) moist sand, 28% (Fig. 14). The conidia of <u>G</u>. <u>cingulata</u>, following the same order of treatments, germinated 35, 100, 36, 40 and 47%. Results with partially germinated conidia of <u>B</u>. <u>cinerea</u> and <u>V</u>. <u>albo-atrum</u> were similar to

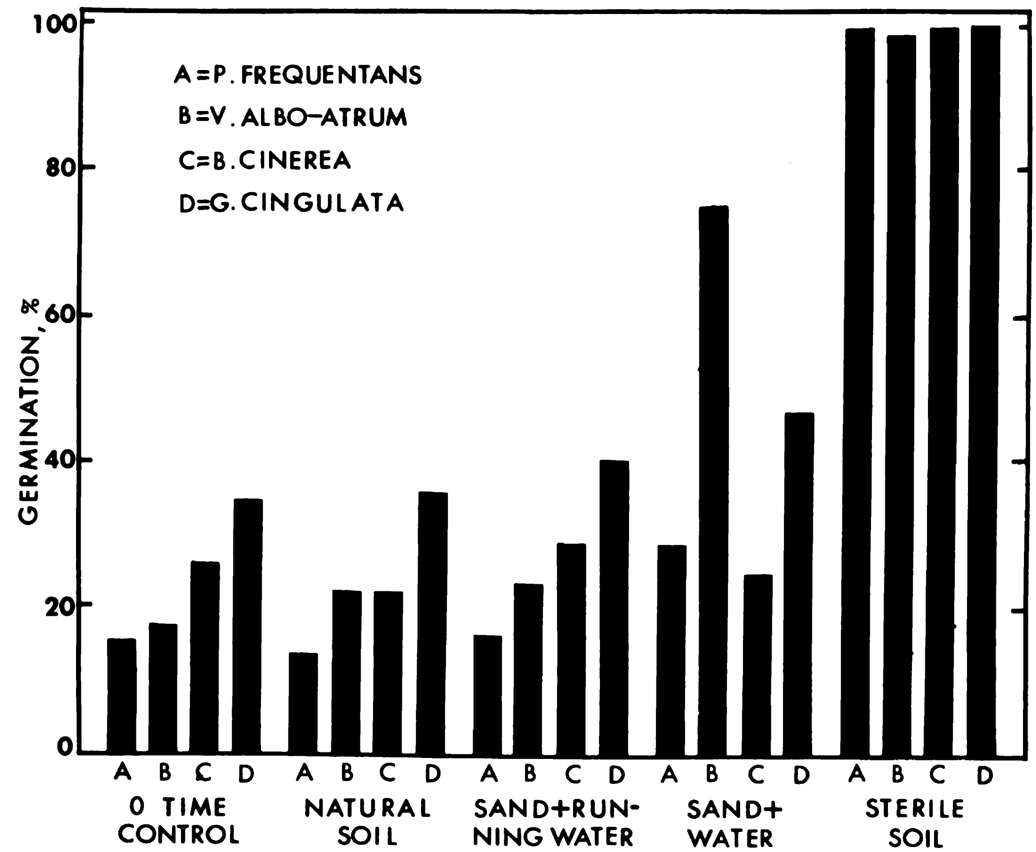

Figure 14. Inhibition of the germination process by different model systems. Conidia were partially germinated, added to Millipore filters (0.22 µ) and then placed on sand or soil. The control was the percent germination of the conidia at the time the spores were added to sand or soil.

those with conidia of G. cingulata and P. frequentans.

These data illustrate that any system which removes essential nutrients from the fungal spore environment during incubation may inhibit spore germination regardless of the stage of germination. This is true for nutrient dependent and nutrient independent spores. Ko and Lockwood have previously shown that germination of nutrient dependent and independent spores could be prevented by leaching them with water (31).

<u>Mechanism of fungistasis in reinoculated sterilized soils</u>: It is well established that fungistasis can be restored to sterilized topsoil by inoculating with microorganisms (36, 38, 43, 46). Data from Lockwood's laboratory indicate that the cause of fungistasis is a lack of nutrients in soil essential for germination of fungal spores (31). According to this concept inoculated sterile soil should be fungistatic only after the essential nutrients are exhausted or are at a low level in the soil. Experiments were designed to establish the role of nutrient depletion by microorganisms in the restoration of fungistasis to sterilized soil. Three fungi (*P. frequentans*, *F. solani* f. *phaseoli*, *H. victoriae*), 2 unidentified actinomycetes and 2 bacteria (*Bacillus subtilis* Cohn emend. Prazmowski and *Agrobacterium tumefaciens* (E.F. Sm. & Town.) Conn) were used singly or in various combinations to inoculate the soil. After two weeks incubation tests were made to determine (a) the fungistatic level of the soil, (b) the level of soluble carbohydrates in the soil and (c) antibiotic activity in soil. Tests for antibiotic activity were done using chloroform-methanol extracts concentrated approximately 6-fold in a vacuum evaporator at 38 C. Filter paper disks were saturated with the concentrated extracts and placed on agar seeded with conidia of *G. cingulata*.

Conidia of *G. cingulata* germinated between 0 and 28% on 8 different inoculated soils (Table 3). *P. frequentans* conidia did not germinate on any of the inoculated soils. In the soil extracts, conidia of G. cingulata, which are nutrient independent, germinated 87-98%. However, the nutrient dependent conidia of *P. frequentans*

Table 3. Some properties of sterilized soil to which fungistasis was restored by inoculation with microorganisms

Organisms used to reinfest sterilized soil	Germination, %				Carbo- hydrates, ug/g soil[b]	Antibiotic activity of soil extract[c]
	G. cingulata		*P. frequentans*			
	Soil	Soil extract	Soil	Soil extract		
A	19	88	0		115	0
F	16	87	0		126	0
B	0	97	0	35	72	0
A + B	28	96	0	50	104	0
F + B	4	94	1	0	76	0
F + A + B	3	97	1	24	85	0
Natural soil	5	99	0	28	38	0
Sterilized soil cont.	99	98	97	81	185	0
Natural soil cont.	1	99	0	14	8	0

[a] A = 2 actinomycetes

B + 2 bacteria, *B. subtilis* and *A. tumefaciens*

F = 3 fungi, *H. victoriae*, *F. solani* f. *phaseoli*, and *P. frequentans*

[b] Carbohydrates were determined by the anthrone reagent.

[c] Filter paper disks were saturated with concentrated chloroform-methanol extracts and placed on agar seeded with *G. cingulata* conidia.

germinated from 0-5%. The germination of conidia in the extracts of sterilized soil was 81% for _P. frequentans_ and 98% for _G. cingulata_.

The amount of soluble carbohydrates remaining in the different soils after 14 days was 72-126 μg/g soil as compared with 185 μg/g soil in the sterilized soil and 8 μg/g in natural soil. Soil which had been inoculated with 1 g of natural soil had 38 μg/g soil. When the incubation period was extended 2-4 days longer, carbohydrate levels in this soil were close to or equal to those of natural soil. Apparently the complex of microorganisms in natural soil was more effective in using the carbohydrates present in sterilized topsoil than were the pure cultures of organisms used in these tests.

Concentrated chloroform-methanol extracts from these soils yielded no zones of inhibition on seeded agar. However, a clear conclusion as to the cause of soil fungistasis cannot be made from these results. Although the presence of antibiotics in the soil could not be demonstrated, residual carbohydrate concentrations were relatively high. It is possible that the organisms used for restoring fungistasis could only utilize part of the carbohydrates present in the sterilized soil and the portion remaining was not capable of supporting the germination of the test fungi.

To avoid possible problems with the unknown carbohydrates in sterilized topsoil and to more accurately assess the role of nutrients in inoculated sterilized soil, a simple system consisting of subsoil was used. The microbial population in natural subsoil was about 90,000 bacteria, 1000 fungi and 100 actinomycetes/g soil. Before and after sterilization of the subsoil no carbohydrates were detectable with the anthrone reagent. When fungal spores were added to the surface of sterilized subsoil, the nutrient independent conidia of H. victoriae and G. cingulata germinated 62% or more, whereas nutrient dependent conidia of V. albo-atrum, H. sativum, A. ustus and P. frequentans germinated 4-29% (Table 4). These data indicated that this soil was essentially free of nutrients capable of supporting germination of nutrient dependent spores. Therefore, this soil was amended with a known amount of glucose and mineral salts and inoculated with (a) Streptomyces sp., (b) V. albo-atrum and (c) natural soil. At different time intervals the amount of glucose in the soil was determined and compared with germination of conidia of H. sativum on the subsoil surface. This fungus was used because it is nutrient dependent and germinates relatively fast (3-5 hr.). The amount of glucose selected for comparison with percent germination of the conidia, was the amount present in the soil at the approximate time of germination. This value was obtained from the curves showing loss of glucose from the inoculated soils with time (Fig. 15).

Subsoil inoculated with Streptomyces sp. or V. albo-atrum lost glucose at about the same rate (Fig. 15). Thirty hours after inoculation the level of glucose dropped from 345 to approximately 240 μg/g soil. In the next 30 hours glucose was reduced to 40 μg. When natural soil was used as the inoculum, glucose was reduced from 345 to 280 μg/g soil in 42 hours. However, within the next 12 hr. the glucose level was lowered to 35 μg.

As the glucose level decreased in each of the inoculated soils, there was a corresponding decrease in germination of H. sativum on

Table 4. Germination of fungal spores on sterilized subsoil with and without added nutrients

Fungi	Germination, %	
	No added nutrients	Added nutrients[a]
H. victoriae	66	80
G. cingulata	62	83
V. albo-atrum	29	75
H. sativum	20	85
A. ustus	15	90
P. frequentans	4	75

[a] Glucose and mineral salts

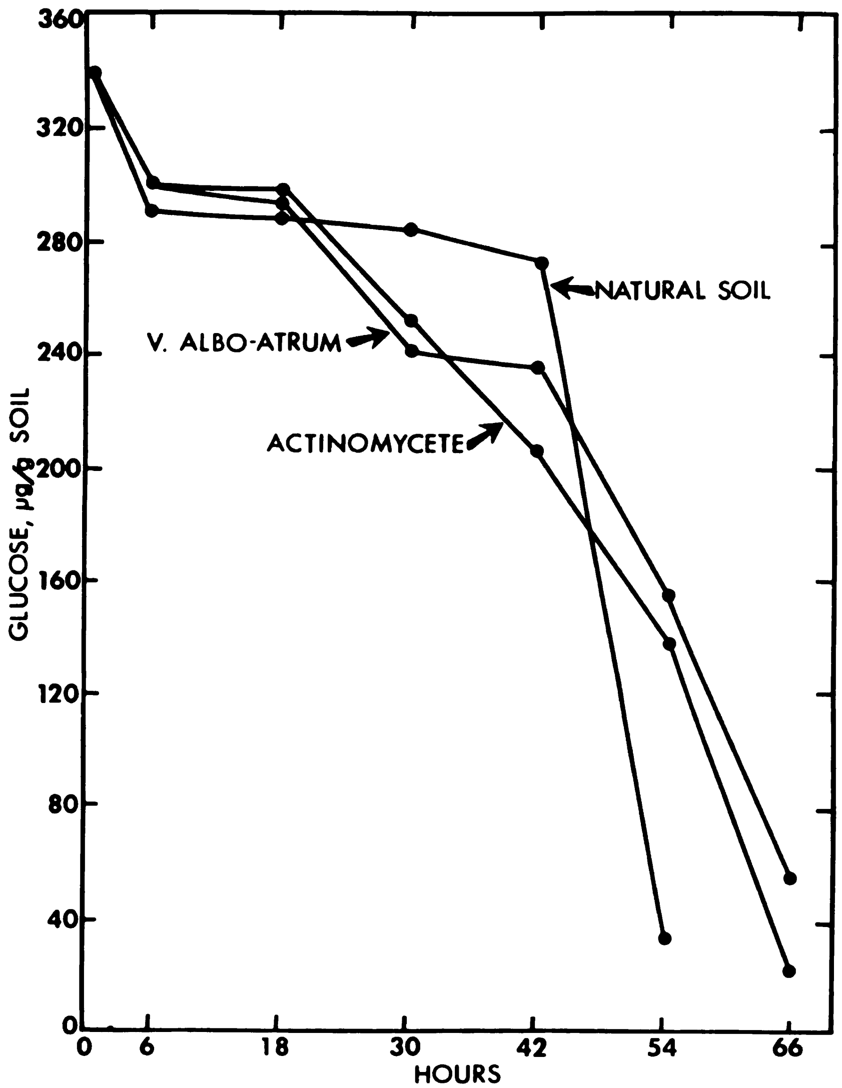

Figure 15. Loss of glucose from subsoil inoculated with <u>V</u>. <u>albo-atrum</u>, <u>Streptomyces</u> sp. or natural soil.

the soil surface. For example, with subsoil inoculated with V. albo-atrum, the conidia germinated 94, 76, 50, 42 and 14% when 345, 300, 230, 180 and 90 μg glucose/g soil, respectively, were present. The correlation between the amount of glucose and conidial germination of H. sativum was significant ($r=.87$, $p<5\%$) (Fig. 16).

In all the inoculated soils, after all detectable glucose was utilized, water extracts were taken and sterilized by Millipore filtration (0.22 μ). Germination of conidia of H. sativum and P. frequentans was tested in these extracts. The extracts would not support germination of these conidia until additional glucose was added. When mineral salts were added without glucose, germination was unaffected.

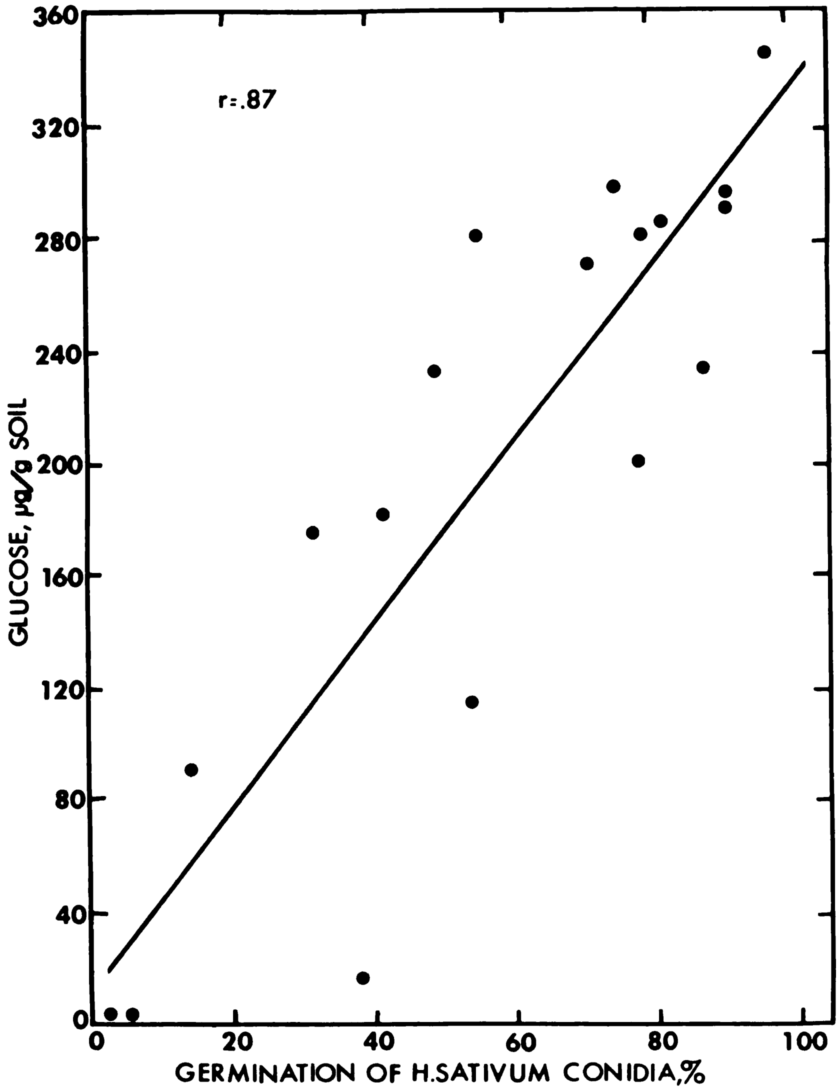

Figure 16. Relationship between amount of glucose remaining in inoculated subsoil and the percent germination of *H. sativum* conidia on the same soil.

DISCUSSION

Characteristically, fungal spores in soil fail to germinate until an exogenous source of nutrients is available for the fungus. If fungi could germinate in soil without nutrients, mycelia would have little chance of growing and eventually sporulating due to starvation (57). Therefore, susceptibility of fungal spores to soil fungistasis is thought to be an advantage to fungi for survival in soil (37).

Results from this research indicate that the degree to which nutrients can effect spore germination in soil is dependent upon the length of time required for its germination. Soluble nutrients were depleted very rapidly from soil and in general, small spores required a longer time to germinate than did large spores. Therefore, if nutrients are available for only a short time in soil, large spores would have a better chance to germinate. Large spores are less sensitive to soil fungistasis than small spores, probably because they can germinate before nutrients are depleted from the spore environment. Further evidence for the importance of germination time was the fact that germination time on soil was reduced by incubating spores in a nutrient solution prior to placing them on soil surfaces. As a result, sensitivity to soil fungistasis was also reduced.

The effects of intermittent nutrient sources on germination of fungal spores was also studied. Both in liquid media and in soil it was demonstrated that the germination process was irreversible and cumulative. Spores germinated only after a sufficient concentration of nutrients, supplied continually or intermittently, were available to the fungal spores for a time equal to the germination time requirement. In soil, small amounts of nutrients may periodically be released and reutilized due to alternate drying and wetting, thawing and freezing, animal activity, lysis of microorganisms, death of roots, etc. (32). These may allow limited growth of fungi if advantage can be taken of the released nutrients. It is conceivable that in a natural population in soil fungal spores would be in different stages of readiness to germinate. This would have the effect of dispersing the spores in time, and could have significant survival value to the fungus in soil. These considerations might explain the results of Caldwell (5), who found about 1% of Trichoderma viride conidia in soil germinating at any of several sampling times throughout a year or more.

The fact that spores were more sensitive than their corresponding mycelia to soil fungistasis, could also endow the fungus with increased survival ability in soil. The fact that spores require a higher concentration of nutrients to germinate than mycelia need to grow, increases the chances that mycelia will be exposed to a sufficient concentration of nutrients to allow growth from previously germinated spores. As nutrients become limiting for mycelial growth, sporulation or the formation of resting structures characteristically take place. It is suggested that one reason mycelia are less sensitive than spores to fungistasis is that mycelia have nothing equivalent to a germination time requirement. Thus, mycelia can take advantage of nutrients immediately upon exposure to them.

The range of sensitivities to soil fungistasis for mycelia was not as great as for spores. However, spores which were relatively sensitive to fungistasis also had more sensitive mycelia. No data are available to explain differences in sensitivity of mycelia of various fungi. Two possibilities for study which are suggested by results with spores, are diameter of mycelia and linear growth rate.

The reason why large spores germinate rapidly and small spores germinate slowly is not known. Perhaps large spores have large nutrient reserves and require less exogenous nutrients, or perhaps their large surface areas enable them to take up nutrients faster and thus, germinate in less time than small spores. Gregory (22) has suggested that the spores of foliar pathogens of grasses are large because of selection pressure. Large spores will impact on a grass leaf more efficiently than will small spores. Selection pressure might also be involved in the fact that spores of these same foliar pathogens germinate rapidly. The availability of optimum temperature and moisture conditions could be of short duration on foliage, and a short germination time would be advantageous to the fungus. These kinds of selection pressures would not be imposed in soil since temperature and moisture conditions would not change rapidly (3), and impaction is not an important means of spore deposit.

The results from this research indicate possible characteristics contributing to the success of _Fusarium_ species as soil-borne organisms. Chlamydospores, mycelia and conidia of _Fusarium_ species were all extremely insensitive to soil fungistasis. They required very small amounts of nutrients to be able to germinate and grow. The highly insensitive nature of *Fusarium* spores to soil fungistasis coupled with the possibility that they can be dispersed in time by intermittent exposures to nutrients could be features related to the success of these species in soil.

Some of the results from this research aid in understanding the nature of soil fungistasis. Partially germinated spores added to Millipore filters and placed on natural soil, were completely inhibited from further germination. Spores added to filters resting on washed, sterilized sand through which a nutrient-free buffer solution was flowing, were likewise inhibited. Results also showed that germination of conidia could be stopped and started several times before germination was completed merely by alternately withholding or providing essential nutrients. The results strongly suggest that the presence or absence of nutrients alone controls germination, growth and quiescence of fungi in soil, thus confirming the nutrient deficiency hypothesis for soil fungistasis suggested by Ko and Lockwood (31).

Differences in sensitivity of fungal spores to soil fungistasis as determined using different mixtures of sterile and natural soil also can be explained in terms of nutrients. Sterilized soil increases the nutrient level and natural soil provides microbes which rapidly use the available nutrients. Spores requiring a longer exposure to nutrients for germination would be expected to be more sensitive to fungistasis since more of the available nutrients would be utilized by the microbes from the natural soil. Results of other workers (13, 27, 28) using fungal spores on nutrient agar disks placed on soil to measure sensitivity to soil fungistasis can be explained in the same terms. Ko and Lockwood (31) showed that nutrients from agar disks are quickly lost to soil. Spores requiring a long germination period would have less nutrients available for germination in the disks as a result of the nutrient loss and thus, be considered more sensitive to soil fungistasis.

Restoration of fungistasis to sterilized topsoil by reinoculation with any of several fungi, bacteria or actinomycetes, singly or in combinations, has been demonstrated many times (36, 38, 43, 46, 56). Subsoil, amended with glucose and mineral salts and inoculated with specific organisms, provided a suitable system in which to follow nutrient loss from soil and the establishment of fungistasis in the soil. The loss of glucose from inoculated subsoil was clearly correlated with a decrease in conidial germination. No antibiotic activity could be demonstrated in extracts of these soils. Further, if staling products or self-inhibitors were present in the soil the rate of glucose loss would be expected to decrease with increasing time. However, the rate increased sharply with increasing time.

In glucose-amended and reinoculated subsoil the amount of glucose for a given percent germination of H. sativum was consistently somewhat higher than that normally required for germination of conidia of this fungus in sterile solutions of glucose. A possible explanation for this might be the fact that germination time for H. sativum as well as several other fungi is longer when nutrients are at a low level than when a high concentration is used. If the germination time is extended, less and less glucose would be available during the germination process since it is constantly being removed. Another explanation might be that glucose is more readily available to the spores in solution than from soil.

From these results it can be concluded that fungal germination and growth in soil is largely dependent on available utilizable carbon sources, length of time they are available and the germination time of the spores. There is no need to postulate the presence of inhibitors in soil to explain any of these results.

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