SOIL FUNGISTASIS: NATURE OF THE INHIBITION OF NUTRIENT-INDEPENDENT PROPAGULES

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ABSTRACT

SOIL FUNGISTASIS: NATURE OF THE INHIBITION OF NUTRIENT-INDEPENDENT PROPAGULES

By

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Certain fungal propagules germinate to varying degrees in glass distilled water, but fail to do so on natural soil. Germination of these nutrient-independent yet soil fungistasis-sensitive propagules was also suppressed in a model system designed to imitate the microbial removal of exuded spore nutrients in soil. The model system imposed a nutrient stress by the continuous aqueous leaching of sand or glass microbeads upon which membrane filters bearing the propagules were incubated. Experiments were designed to gain a further understanding of the mechanism of inhibition of these propagules by the microbial nutrient sink in soil, and of the sink's significance in relation to possible volatile fungistatic factors.

Germination of Helminthosporium victoriae conidia in the model system with mineral salts solutions or subsoil extract as the leaching media was suppressed as effectively as with water. Conidia of this fungus and

Curvularia lunata, and sclerotia of Sclerotium cepivorum collected from cultures containing ^{14}C-glucose, initially lost about twice as much radioactivity on leached sand as on unleached sand. Over 90 percent of the radioactivity exuded by H. victoriae conidia was detected in the leachings after only 12 hr.

Conidia of H. sativum and H. victoriae lost the capability to germinate in buffer solution after 7 days of prior continuous incubation on Conover loam soil or in the model system. Viability of H. sativum decreased rapidly 1-2 days following loss of nutrient-independence, whereas significant decreases in viability of H. victoriae occurred only after another 21 days of nutrient deprivation.

Glucose and amino acids were among the substances present in conidial exudates. Conidia of H. victoriae, first converted to nutrient dependency by extended incubation on Conover loam soil or in the model system, accumulated radioactivity and germinated when incubated in exudate collected from ^{14}C-labelled spores. Germination was stimulated, over that occurring with buffer, when conidia of H. victoriae and C. lunata were leached with their own exudate or the exudate of the other fungus. A similar stimulation of germination occurred when a solution of glucose and casein hydrolysate was used as the leaching medium: the concentration of each

being equal to that in spore exudates.

Term tubes began to emerge from H. victoriae conidia after only 1.5 hr incubation on water-saturated sand. Germ tube formation was prevented by transferring conidia to conover loam soil or to the model system before they had completed 1.0 hr incubation on water-saturated sand. However, after 1.0 hr the conidia were irreversibly committed to producing germ tubes. In contrast, transfer of already committed spores to a clay loam soil from Colorado, which is known to produce a volatile fungistatic factor, prevented germ tube formation in about 50% of the committed spores.

Radioactive carbon dioxide was detected within 3 minutes after the addition of ^{14}C-glucose to either Conover loam or the Colorado clay loam soil. This demonstrates the very early establishment of a microbial nutrient sink in the soils. Exudates from ^{14}C-labelled H. victoriae conidia were also readily and quickly metabolized in both soils. Similarly, glucose loss from glucose-containing paper discs incubated on either soil was more rapid than when incubated on their respective sterile soil controls. The rates of $^{14}CO_2$ evolution and glucose loss were greater with Conover loam soil than with the Colorado clay loam soil. Moreover, bacteria isolated from these two soils accumulated ^{14}C-labelled materials exuded from spores.

metabolites of spore exudates. In the Colorado clay loam soil, where the microbial nutrient sink appeared to be less than in Conover loam soil, the volatile fungistatic factor may have played a major role in the inhibition of germination.

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To Libbie

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INTRODUCTION

Previous work from this laboratory has shown a highly significant direct correlation between the ability of fungal spores to germinate in glass distilled water and on soil (54). Conidia of four fungi, however, were exceptions in that they germinated to various degrees in water, but were completely inhibited on soil. Eleven other nutrient-independent yet fungistasis-sensitive fungal propagules were later identified (43). Germination of these propagules was suppressed when gently leached with water or dilute buffer solution in a model system thought to imitate the microbial nutrient sink of soil. Other work from this laboratory demonstrated that nutrients exuded from this type of propagule were rapidly utilized by soil microorganisms (59). Moreover, incubating conidia of Helminthosporium victoriae or Glomerella cingulata with washed cells of bacteria or actinomycetes inhibited germination (59). The reduction in germination was not accompanied by the production of detectable inhibitory substances. All these results supported the hypothesis that the removal of nutrients due to the microbial nutrient

sink prevented germination of nutrient-independent propagules.

My research was undertaken with two primary objectives, to gain a further understanding: a) of the mechanism of the inhibition by the microbial nutrient sink in soil, and b) of its significance in relation to recently reported volatile fungistatic factors (36). A clay loam soil from Colorado known to produce a volatile inhibitor was used to compare results with Conover loam soil, the soil used in all previous fungistasis studies from this laboratory.

LITERATURE REVIEW

Fungistasis and mycostasis are the descriptive terms given to the phenomenon whereby fungal propagules fail to germinate in soil under conditions considered favorable for germination. The phenomenon was first described just over twenty years ago by Dobbs and Hinson (22), and since then considerable research effort has been directed toward understanding its nature, not only because of its importance in soil ecology, but because of the implication for control of soil-borne plant pathogens. Fungistasis occurs in most soils throughout the world, and can be annulled by the following: i) sterilization of the soil, ii) the addition of nutrients, iii) the proximity of fresh organic matter, iv) the proximity of the rhizosphere or spermosphere of plants, or v) long periods of air drying the soil (63). There is agreement among researchers that widespread fungistasis is microbial in origin, and Dobbs termed the general phenomenon "microbial" fungistasis to differentiate it from the few soils where a permanent thermostable inhibition, that cannot be overcome by nutrients, exists: the latter being

called "residual" fungistasis (18). The subject was comprehensively reviewed by Lockwood in 1964 (63), while a recent interpretive, but less thorough review was made by Watson and Ford (91). The review presented here will consider literature concerning the mechanism of fungistasis, especially those papers directly related to research reported herein.

Nutrient Requirements for Germination and Their Availability in Soil

Before reviewing the various mechanisms proposed for the inhibition in soil, a brief consideration of the nutrient requirements for spore germination and the availability of such nutrients in soil might provide a framework upon which further discussion can be developed.

From the outset it must be assumed that any constituitive dormancy requirements have been fulfilled. Germination of fungal propagules is an active metabolic process which requires energy, and two extremes are found in fungi with respect to exogenous nutrients: a) those completely dependent upon, and b) those completely independent of them (54). A borderline character is exhibited by some species, in that within a population of spores a low percentage will germinate without an exogenous substrate. For many of the nutrient-dependent spores, glucose alone may satisfy

the energy requirements for germination, while others may need some specific carbon source or form of nitrogen (24,28,34,49,55,59).

Soil is characterized by an abundant and diverse microflora (4,28,32), which is normally inactive as indicated by the minimal respiration rates measured for fallow unamended soil (27,59). Even the addition of the fungicide pentachloronitrobenzene to unamended soil did not further reduce respiratory activity (27). In addition, the soil microflora may be responsible for only a portion of the total respiration measured, the balance owing to microfaunal activity. Gray and Williams (31) conclude, based on calculations of the yearly input of energy substrates to soil and the biomass which must be supported, that most microbes existing in soil are present as resting or survival forms. They and Babiuk and Paul (5) also point out that the amount of energy available in soil on an annual basis, taking into account that required for maintenance of existing populations, would allow cells to divide only a few times a year. Thus respiration in unamended soil likely represents maintenance metabolism, not growth and reproduction. The soil is thus, an environment in which energy yielding substances are in chronically short supply. This idea is further supported

by the observation that no nutrient-independent fungal propagules, which are also fungistasis-sensitive, have ever been identified. Propagules representing the other three possible combinations of these characters are abundant.

Nature of Soil Fungistasis

As early as 1949 it was suggested that the inability of most human and animal pathogens to survive for more than a few days in soil was due to their failure: a) to obtain proper nutrients, b) to compete with indigenous microorganisms, or c) to withstand antibiotics produced by soil microbes (72). Since the original report by Dobbs and Hinson numerous mechanisms have been proposed to explain the nature of fungistasis. Physical factors of the soil environment such as hydrogen ion concentration and oxidation-reduction potential have been ruled out as playing a significant role. There are at present two hypotheses regarding the nature of the phenomenon: i) the nutrient hypothesis where nutrients needed as an energy source for germination are deficient due to microbial activity in the soil, and ii) the inhibitor hypothesis which postulates the existence of inhibitory substances of microbial origin. The latter includes recently reported volatile fungistatic factors.

The inhibitor hypothesis has been championed by Dobbs and co-workers who proposed that the observed inhibition was caused by a soluble yet unstable substance present in the soil solution (22,23). However, Jackson (44) demonstrated that soils remained fungistatic even after extensive aqueous leaching, and believed that fungistasis was caused by resistant organic matter that leaching could not remove. Lingappa and Lockwood (58) found that decomposition products of lignin could inhibit spore germination, but they did not suggest that these compounds were responsible for the widespread inhibition. Lockwood (62) initially proposed that antibiotics produced by Streptomyces might be the inhibitory factor, but later discounted the idea since such substances could not be extracted from soil (57). Brian (12) after a thorough review of the literature on antibiotics in soil concluded, that they are not produced in unamended soil because of insufficient energy, and therefore probably are not responsible for fungistasis. Both degradation of resistant organic matter and synthesis of antibiotics assume active metabolism by appropriate microorganisms, but as already indicated the energy for such activity is not available. Moreover, Lingappa and Lockwood (57) point out that sites in soil where antibiotics are most likely to be synthesized

(i.e. adjacent to fresh organic debris or the rhizosphere) are microsites favorable for spore germination. Even sand without organic matter was found to be fungistatic (63).

Evidence has since been obtained to show that fungal propagules, notably those which do not require exogenous nutrients for germination, themselves provide energy for microbial activity in soil (29,59). Spores and spore washings markedly increase microbial populations, as well as oxygen uptake in soil (29). Gilbert and Linderman (29) report an increase in bacteria only, in the "mycosphere" of Sclerotium rolfsii sclerotia. However, there could have been a corresponding increase in the biomass of fungi and actinomycetes adjacent to sclerotia, as the result of germination, but before sporulation. They also reported a shift toward greater numbers of streptomycin-tolerant bacteria, and suggest that such a shift represents a response to a selection pressure because of elevated antibiotic concentrations in the "mycosphere" soil. No attempt, however, was made to directly demonstrate the presence of antibiotics; moreover, streptomycin is readily degraded and adsorbed in soil (12).

The similarity between some of the morphological changes in staled fungal cultures and on soil led Park (70) to suggest that staling products may cause

fungistasis. Staling products do exist in culture, but their presence in soil has not been shown (16,17,75). Moreover, the morphological changes observed by Park, also occurred when the fungi were deprived of nutrients (34,42). If staling products or antibiotics play a role in fungistasis, then reinfested sterilized soil should be an ideal environment for their production.

It is well documented that reinoculation of sterilized soil with a pinch of natural soil or any one of a number of microorganisms restores inhibitory properties to the sterile soil (33,64,84). Griffin (33) found that both microorganisms antagonistic and non-antagonistic in culture restored fungistasis, and concluded that the accumulation of staling products was responsible for the inhibition by the non-antagonists. Each of sixty-seven microorganisms tested by Lingappa and Lockwood (64), some of which produced antibiotics in culture, returned some level of inhibition to autoclaved soil, even when reinoculated into sterilized soil which was first thoroughly washed to remove nutrients which would be used for antibiotic or staling product synthesis. Filter-sterilized aqueous extracts from reinfested topsoil were not inhibitory, but some contained carbohydrates which can annul fungistasis; thus it was not until after extracts from reinfested subsoil were shown to be both non-toxic and nutrient-free

were Steiner and Lockwood (83) able to conclude that fungistatic substances were not involved. Their extensive reexamination of the mechanism of fungistasis in reinfested soil provided no evidence that inhibition was due to anything other than the depletion of nutrients released upon sterilization, and the maintenance of a nutrient-deprived environment by the reinoculated organism. They further pointed out that if inhibitors of microbial origin accumulated in reinoculated soils, then the rate of glucose utilization in soil should decrease with time of incubation. Their data and that of others (3) clearly showed that the rate actually increased.

Both volatile (17,75) and non-volatile (16) inhibitors from fungal cultures have been isolated and identified. However, if the concentrations necessary to inhibit fungal spore germination in vitro are the same in soil, then such compounds should have been easily extracted from reinfested sterilized soil, if not from natural soil. The same considerations apply to the work of Chinn (14), who found that sand amended with only 0.005% natural soil was still inhibitory, and stated that the fungistatic principle was over 200-times more concentrated than needed.

There are numerous reports of inhibition of spore germination in non-sterile extracts and diffusates (23,

34,76,86). However, the effect is negated partially or completely when those extracts are sterilized by heat or filtration (23,34,76,86). Brown (13), providing the first evidence of soil bacteriostasis, also showed that natural soil and non-sterile extracts retarded growth of bacteria, and that the inhibition was removed by filter sterilization. Despite this evidence to the contrary, Brown still proposed that bacteriostasis was caused by bacteriostatic factors of biological origin. By extracting garden and forest soils and bioassaying the extracts under nitrogen, Dobbs and Carter (20) were able to retain some of the inhibition in sterile extracts. Attempts to obtain inhibitory sterile extracts from other soils were unsuccessful (54,57,63,83). However, in a recent preliminary report, Basmith and Vaartaja (8) found that both sterile and non-sterile extracts of natural soil restricted growth of Pythium ultimum. The inhibition was overcome by heating or adding nutrients to the soil, or passing the extracts through filter paper. Interestingly, those extracts, inhibitory to P. ultimum, stimulated Fusarium oxysporum. When Vaartaja (90) fractionated aqueous soil extracts by molecular sieve chromatography, certain fractions inhibited while other fractions stimulated or had no effect on mycelial growth. Estimates of inhibitor and stimulator molecular weights ranged from 100 to 80,000

and 2,000 to 50,000, respectively. If inhibitory fractions were not contaminated and further research demonstrates the inhibition of germination of a wide range of fungal spores, it will be the first strong evidence for a non-volatile inhibitor in soils.

Agar discs incubated on soil often fail to support spore germination. This inhibition has been taken to represent the diffusion of a soluble inhibitor from soil into the agar. Using gel electrophoresis techniques on fungistatic agar, Weltzein found an inhibitory band migrating toward the anode (92). Such results may instead represent the migration of nutrients establishing a zone depleted of energy sources or the accumulation of ions directly or indirectly preventing germination. Griffin (34) incubated agar in contact with soil at 3 C to lower microbial activity and to reduce the loss of essential nutrients. However, Ko and Lockwood (54) clearly showed that nutrients diffuse into soil from agar rapidly at either 24 and 1 C. Moreover, inhibitors could not be extracted from agar previously in contact with soil (57).

The occurrence of residual fungistasis has been reported in a few soils. Dobbs and Gash (21) attributed the residual inhibition in calcareous dune sands and limestone subsoils to inorganic iron and $CaCO_3$, respectively. Ko and Hora (50,51) recently reported that an

aluminum ion was the fungitoxin in certain cultivated acidic soils in Hawaii. Liming these soils to a neutral pH annulled the fungicidal effect. Moreover, aqueous solution of Al from $AlCl_3$ or $Al_2(SO_4)_3$ at concentrations equal to that in soil inhibited germination of Neurospora tetrasperma ascospores, which are sensitive to the residual fungistasis but not to the more common microbial fungistasis.

Volatile substances have also been suggested as the cause of soil fungistasis (19): Lingappa and Lockwood (57) could not demonstrate their presence in soil. More recently, Kouyeas and Balis (7,48) provided indirect evidence that volatile fungistatic factors may be involved in the phenomenon. Remoistened air-dried soil was fungistatic when suspended over water, but only partially so when incubated over either silver nitrate or mercuric perchlorate solutions, which were thought to inactivate the volatile substance by binding with it at points of unsaturation. Toxic vapors from these solutions may have partially sterilized the surface of the soil, releasing nutrients stimulatory to spores of the test fungus. Balis (6) tentatively identified allyl alcohol as the volatile inhibitory factor found in water condensates collected from the head space over soil. This conforms to earlier assumptions that the volatile was a water soluble unsaturated carbon compound (7).

Allyl alcohol at 10 ppm inhibited conidial germination of Penicillium chrysogenum in vitro, and the inhibitory effect was overcome by nutrients.

Hora and Baker (36) provided the first direct evidence for the existence of a volatile factor in certain remoistened air-dried alkaline soils. The inhibitory effect of the volatile generally increased with increasing soil pH. When the sensitivity of the bioassay technique was improved, through exhaustive leaching of the test agar discs, significant volatile inhibition was detected from acidic soils as well, even 25 days after remoistening (77). Furthermore, the inhibition of Helminthosporium sativum conidia by the volatile factor was nearly equal to that when membrane filters bearing conidia were incubated directly on the soils.

The most inhibitory soil, one particular alkaline clay loam, was used in more extensive studies. Autoclaving did not eliminate the volatile factor (38,52), and spores incubated directly on the surface of either sterile or nutrient-amended soil failed to germinate, fulfilling the criterion for residual fungistasis (52). When this soil was treated with hydrochloric acid, it no longer released the volatile inhibitor (52). The volatile was inactiviated by passage through water and absorbed by activiated charcoal (76), however, the

presence of conidia were required in the water traps during volatile extraction to demonstrate the inhibition of conidial germination (37). This suggests that spores have a strong affinity for the inhibitor. Moreover, Hora and Baker (39) found that a volatile fungistatic factor can be generated non-biologically in soil, showing that its release was dependent upon alkaline pH's. Reinoculating the sterilized clay loam soil with actinomycetes increased volatile production, while bacteria and fungi removed its effect; when assayed 2 weeks after reinfestation (38). Furthermore, when this soil was amended with chitin, glucose, cellulose, or lignin, only chitin increased inhibition by a volatile factor. Interestingly, reinoculation of sterilized soil by microorganisms or the addition of an organic amendment increased the level of fungistasis compared with that in the natural clay loam soil, as determined by the direct method. This suggests that the microbial component of fungistasis was increased by all the treatments, even though their affect on volatile production differed. The stimulation of volatile generation by chitin amendment and the observation that they were only detected above those Streptomyces cultures with earthy odors, suggests that actinomycetes were involved in its production (38). They may be involved in reinoculated sterile soil and in chitin-amended soil,

but their involvement in natural unamended soil seems unlikely because of nutrient deficiency. Germination and growth of streptomycetes, like that of fungi and bacteria, is also suppressed on natural soil (61). To this reviewer these results suggest that the volatile inhibitor in the clay loam soil from Colorado is not of biological origin, but that its generation may in part be mediated by actinomycetes when sufficient energy for their activity is available.

Smith (79) recently reported that microbially synthesized ethylene was the main inhibitor of fungal propagule germination in soil. This volatile was the only material detected in fifty Australian soils at concentrations high enough to be biologically active. Such soils were, however, oven dried at 40 C for 24 hr prior to rewetting and subsequent sampling for ethylene. Ethylene concentrations equal to that detected in some of the rewetted soils inhibited germination of Sclerotium rolfsii sclerotia and Helminthosporium sativum conidia. Fungistasis was reduced when a water-saturated air stream was swept over the surface of these soils. He proposes that nutrients are the main stimulators in soil, and that the balance between them and ethylene determines whether soil is fungistatic at any given time.

If volatile inhibitors are as universal as Baker and co-workers, and Smith suggest, it is a wonder that Dobbs and Hinson did not detect them in their "gap traps" or on the margins of cellophane folds extending out beyond soil, which they used for control germination. In reference to ethylene, it has been demonstrated that this compound is only produced in soil by microbes when oxygen concentrations are less than 2 percent (1), and is rapidly degraded under aerobic conditions (80).

Lockwood (63) proposed a nutrient deficiency hypothesis to explain the widespread microbial fungistasis. Some of the evidence presented by Ko and Lockwood (54) to support the nutrient hypothesis appeared previously in this review. Analysis of sterile soil showed nutrients were present in sufficient quantities to support germination of nutrient-dependent spores, whereas those in natural soil were not. More importantly, they demonstrated that soil microorganisms can rapidly remove nutrients around spores, not only creating, but maintaining an environment deprived of energy substrates. Spores of nutrient-dependent fungi require an exposure to an energy source throughout the germination process in order to produce a germ tube (82,93). Thus, unless there is enough energy in the vicinity of the spore to satisfy both its germination requirements until germ tube emergence, as well as that

of the surrounding soil microbes, the spore fails to germinate. Hsu and Lockwood (41) also demonstrated that the inhibition of fungi by some streptomycetes in culture was due to nutrient deprivation.

The extension of the nutrient deprivation hypothesis to explain inhibition of nutrient-independent yet fungistasis-sensitive spores (54), has been confirmed by others. Germination of Verticillium albo-atrum microsclerotia was suppressed when the propagules were continuously leached with water on a modified leaching system, but not when leaching was discontinued (25). Similar results were reported by Adams et al. (3) for chlamydospores of Fusarium solani f. sp. phaseoli. These propagules germinated freely when leached with glucose or asparagine solutions. Germination of the nutrient-independent conidia of Botrytis cinerea was inhibited in the phyllosphere (10), and also in a model system (87) similar to that used by Ko and Lockwood (54). Suspensions of phyllosphere bacteria were inhibitory to conidia, while sterile filtrates of such mixtures were stimulatory.

Fungistasis can be thought of as a survival mechanism, in that it prevents the spontaneous germination of fungal propagules in the absence of a suitable substrate. Therefore, it seems unreasonable that a mechanism for survival based on the continuous

expenditure of energy for production of inhibitory substances by soil microorganisms, would have evolved in a highly competitive environment where energy-producing substrates are in chronically short supply. In an environment where conservation of limited energy reserves is necessary for survival, a passive rather than an active mechanism for dormancy seems more reasonable and consistent with reported observations.

METHODS AND MATERIALS

Maintenance of Fungi and Bacteria

The following fungi were maintained on potato-dextrose agar (PDA): Helminthosporium sativum Pam., King and Bakke, Helminthosporium victoriae Meehan and Murphy, Neurospora tetrasperma Shear and Dodge, Sclerotium cepivorum Berk., and Thielaviopsis basicola (Berk. and Br.) Ferr. Curvularia lunata (Wakker) Boedijn was kept on V-8 juice agar (per liter: 200 ml V-8 juice [Campbell Soup Co.], 2.0 g $CaCO_3$, 20 g agar).

Bacteria isolated from soil dilution plates on soil extract agar (71) were cultured on a nutrient agar (per liter: 5 g peptone, 5 g yeast extract, 1 g glucose, 5 g K_2HPO_4, 20 g agar).

Production, Collection, and Preparation of Propagules

Conidia of the helminthosporia were produced by Lukens' method (65). Shake cultures were grown in 25 ml modified Fries No. 3 basal medium (74) with glucose substituted for sucrose for 5-7 days. The mycelium produced was comminuted in a Servall Omnimixer for 2 minutes. The resulting hyphal fragments were washed

by suspending them in sterile 0.02 M phosphate buffer (pH 6.4) after each of three refrigerated centrifugations. A 2.5 ml aliquot of the final suspension was spread over sterile filter paper in a 9.0 cm diam petri dish. Sporulation commenced within several days under continuous fluorescent light (four, 40 watt, cool white, tubes). Conidia were not used until the filter paper was dry, and kept for up to 6 months.

Neurospora tetrasperma was cultured on plates of PDA and ascospores were obtained by washing the ejected spores from the inside surface of petri dish lids with sterile glass distilled water. Ascospores were separated from any conidia of the fungus by sedimentation in sterile water. After washing three times by centrifugation the spores were stored in sterile glass distilled water at 4 C. Ascospores were heat activated for use at 58 C for 20 min in a water bath (30).

Conidial suspensions of T. basicola were prepared by adding glass distilled water to PDA-slant cultures and agitating on a Vortex Genie mixer. Conidia of C. lunata and of the helminthosporia produced on petri plates of V-8 juice agar and filter paper, respectively, were rubbed free with a glass rod after irrigating the plates with glass-distilled water. C. lunata suspensions were then passed through a 325-mesh sieve (44 μ) to

remove hyphal fragments. The suspensions were washed 3 times by refrigerated centrifugation.

Sclerotia of S. cepivorum were collected from PDA plates covered with non-coated cellophane. The sclerotia were treated in 1% sodium hypochlorite for 1 min to kill attached hyphae and then washed as previously described.

When radioactive spores were required the culture medium was amended with the following quantities of ^{14}C-glucose (D-glucose-U-C-14, 20 hour grade, specific activity 50 mCi/mM, Calatomic, La Jolla, Calif.): a) H. victoriae, 1 µCi/ml Fries basal medium; b) T. basicola, 10 µCi/ml PDA or 50 µCi/slant; c) C. lunata, 25 µCi/plate; and d) S. cepivorum, 25 µCi/plate. The same procedures outlined above for collection, etc., were followed.

Unwashed conidia of H. victoriae were usually collected by rubbing a Millipore membrane filter or the dull side, not shiny, of a Nuclepore membrane filter on the surface of dry filter paper cultures of the fungus. When large quantities of unwashed conidia were required, they were collected with a sterile Tervet cyclone spore collector.

Spore Exudates

Conidia (5-70 X 10^3/ml) were suspended in 0.5-1 liter sterile glass distilled water and incubated at

25 ± 2 C with stirring for a period of time short of germination (3 hr for C. lunata, 1.5 hr for H. victoriae). Conidia of the nutrient-dependent fungus T. basicola were incubated similarly for 20 hr. Exudate was collected and sterilized by passage through a 0.22 μ Millipore membrane filter. One-half (ca. 1 liter) of the collected exudate was taken to dryness in a flash evaporator at 45 C and then redissolved in 10 ml sterile glass distilled water. The other half was used in germination experiments.

Carbohydrates in the concentrated exudates were determined using the anthrone reagent (69), while glucose was measured by the Glucostat reagent (Worthington Biochemicals Corp., Freehold, New Jersey). The ninhydrin method (68) was used to determine amino compounds.

Synthetic exudates were prepared from a mixture of glucose and casein hydrolysate (acid hydrolysed, vitamin free, Nutritional Biochemicals Corp., Cleveland, Ohio). The concentration of each was based on the respective glucose and glycine equivalents of total carbohydrates and amino acids in natural exudates.

Exudates collected from ^{14}C-labelled conidia were reduced to approximately 1/80 their original volume in a flash evaporator at 45 C before being filter-sterilized again, and stored until use at 4 C.

Source and Preparation of Soils

Conover loam soil collected from the Michigan State University Botany and Plant Pathology Farm possessed the following characteristics: pH 6.7, organic matter 3.8%, water holding capacity (WHC) 42.7%, clay 7.5%, silt 42.8%, and sand 49.7%. A clay loam soil from Colorado, reported to produce a volatile fungistatic factor (36), was supplied courtesy of Dr. Ralph Baker, Colorado State University. Hereafter, this soil will be referred to as the clay loam soil. The clay loam soil was collected from a low lying swale where water frequently collected then evaporated. Its characteristics are as follows: pH 8.6, organic matter 1.1%, lime 5.9%, NH_4^+-N 54.5 ppm, NO_3^--N 34.7 ppm, P_2O_5 140 lbs/acre, K_2O 865 lbs/acre, and conductivity (salts) 48.0 mhos/cm. All soils were screened through a 10-mesh sieve and stored air dry in plastic bags in the laboratory. Subsoil collected from beneath Conover loam soil was similarly screened but stored at ca. 8% moisture in plastic bags.

For use, the Conover loam and the clay loam soils were brought to 27 and 33% moisture levels, respectively, by thorough mixing with distilled water. These levels represented an equivalent moisture tension of 0.06 bars in each soil as measured with a tensiometer

(Soilmoisture Probe, Cat. No. 2100, Soilmoisture Equipment Corp., Santa Barbara, Calif.). In most experiments the moist soil was incubated for at least 24 hr prior to use. Soil plates were prepared by placing moist soil in petri dishes and smoothing the surface with a stainless steel spatula. Plates of sterile soil were made in glass petri dishes and autoclaved at 121 C (15 psi) for at least 30 min. Moisture content of sterile soil was returned to the level prior to autoclaving by the addition of sterile glass-distilled water, and then the surface was smoothed again with a sterile spatula.

Model System for Imitating the Microbial Nutrient Sink of Soil

A model system previously described (43) was used to impose a diffusion stress upon spores or agar and polyacrylamide gel discs incubated on an acid-washed silica sand or glass microbead bed (Figure 1). Discs and spores were separated from the bed by either a Nuclepore (0.4 μ) or Millipore (0.22 μ) membrane filter. For most experiments the model system was modified by removing the needle valve and connecting a 1/8 in O.D., 1/16 in I.D. plastic (Tygon) tube between the stem of the separatory funnel and the inlet tube of the leaching dish, then accurately metering the flow rate with a precision peristaltic pump (Manostat

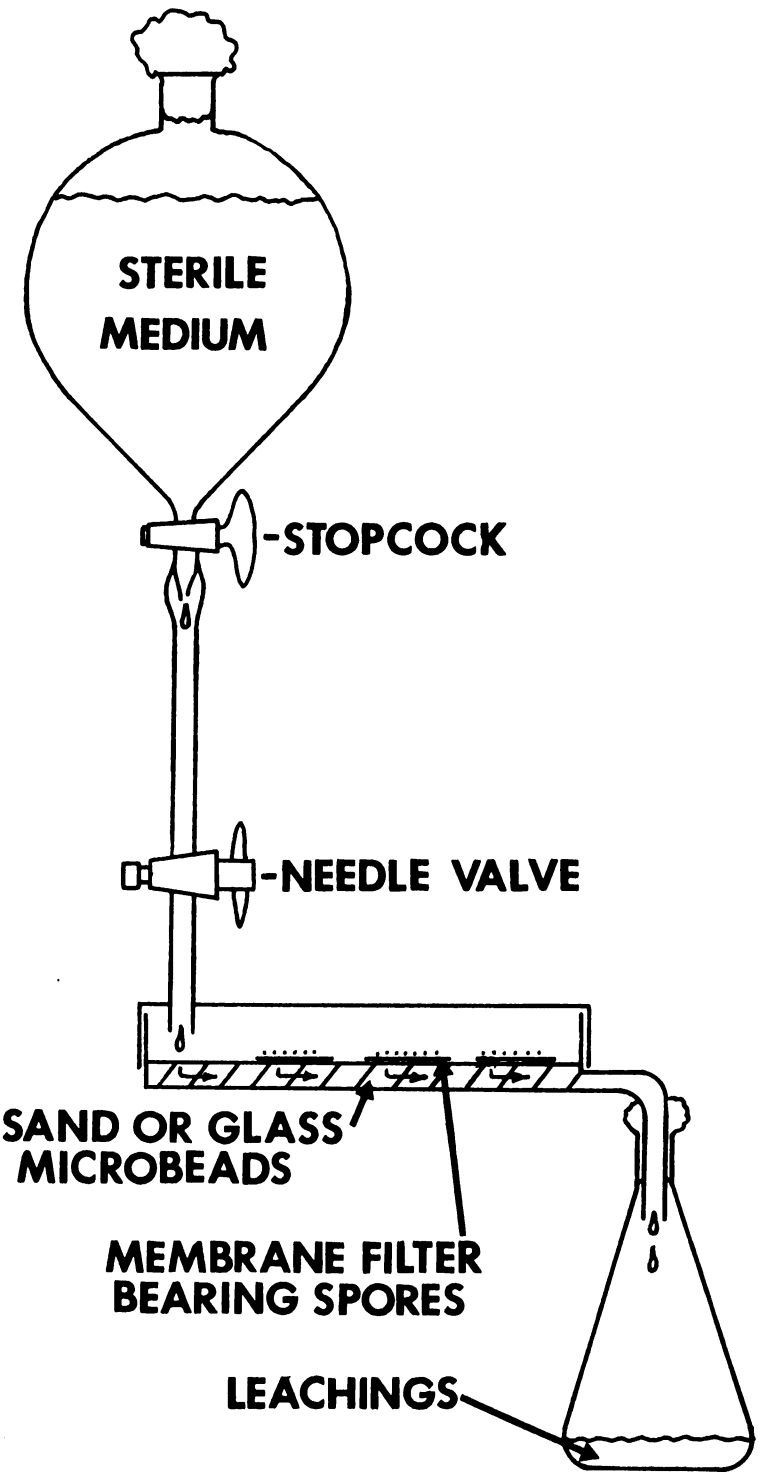


Figure 1. The model system used to impose a diffusion stress on fungal propagules and discs.

Cassette Pump, Manostat, New York, N.Y.). Sterile glass-distilled water or a dilute buffer solution (0.025 M potassium phosphate buffer, pH 7.0) were generally used as the leaching media. The term 'buffer solution' henceforth will refer to this buffer unless otherwise specified. Both natural and synthetic spore exudates were also used. In experiments designed to evaluate the effect of mineral nutrients on germination in the model system Pfeffer solution (87) without trace element supplement (per liter: 0.8 g $Ca(NO_3)_2$, 0.2 g $MgSO_4$, 0.2 g KNO_3, 0.1 g KCl, and 0.2 g K_2HPO_4) and subsoil extract were used. Subsoil extract was prepared as follows: 1.0 Kg natural subsoil was vigorously stirred with 1.0 liter distilled water for 30 min then filtered through Whatman No. 1 filter paper and finally filtered sterilized using a 0.22 μ Millipore membrane filter.

When non-leaching conditions were required as a control, silica sand or glass microbeads in a petri dish were saturated with the same aqueous medium that was used in the model system.

Metabolism of Glucose and Spore Exudates by Soil Microorganisms

Metabolism of ^{14}C-glucose was measured by two methods: a) moist soil (50.0 g) with a smooth surface was prepared in the bottom of a ground-glass weighing

dish (90 X 15 mm). The lid was fitted with inlet and outlet tubes, and moist air was passed over the soil surface at a rate of 3 liters per hr. Two µCi of ^{14}C-glucose was placed on the soil in ten 2 µl drops with a microsyringe. Air from the soil chamber passed through a drying tube of anhydrous calcium sulfate before respired carbon dioxide was trapped by bubbling, via a capillary pipette, into 2.0 ml ethanolamine solution (methyl cellosolve: ethanolamine, 7:3, v/v) contained in a scintillation vial. At predetermined intervals the pipette was transferred to additional vials containing ethanolamine solution. Fifteen ml modified Bray's liquid scintillation fluid (11) was added to each vial and the samples counted in a Packard Tricarb Spectrometer. b) Soil suspensions (1.0 g air-dried soil plus 1.0 ml H_2O) were incubated for 1 hr at 25 ± 2 C before 2.0 µCi ^{14}C-glucose and an additional 1.0 ml of water were added. Air was bubbled through the suspension using a capillary pipette, and the respired $^{14}CO_2$ was trapped and measured as described above. To measure respiration of concentrated radio-active exudate of H. victoriae conidia, 1.0 ml of the exudate was added to the soil suspensions after the 1 hr incubation. Application of ^{14}C-glucose to sterile soils or soil suspensions served as controls for both methods.

In experiments measuring the uptake of radioactive spore exudate by bacteria, the bacteria were grown in nutrient broth (nutrient agar minus the agar) for 3-4 days on a shaker. Before use the cells were washed by centrifugation in buffer solution and the concentration of cells in the final suspension adjusted to $A_{500} = 0.40$ in a B & L Spect 20. One ml of concentrated exudate from ^{14}C-labelled conidia of H. victoriae was added to an equal volume of the adjusted cell suspension. At zero time and specified time intervals, 0.30 ml was removed and placed on a Nuclepore membrane filter. The excess exudate was removed by suction and the cells were washed with 10 ml cold 0.01% Tergitol TMN (non-ionic surfactant) followed by 10 ml glass distilled water. Solutions without bacteria but similarly handled served as controls. Membranes and cells were placed in vials containing scintillation fluid and counted.

Paper Disc Technique for Assaying the Microbial Nutrient Sink of Soil

Paper antibiotic assay discs (½ in diam, Carl Schleicher and Schuell Co., No. 470-E) were amended with 90 µl 0.05, 0.005, and 0.0005% glucose solutions each containing 0.05 µCi ^{14}C-glucose/ml using a microsyringe fitted with a Chaney adapter. Amended discs were then

placed directly on the soil surface and incubated at 24 C for various periods of time. Upon removal, discs were immediately dried in an oven (100 C), and then combusted in a Nuclear-Chicago Semi-Automatic Sample Combustor (Model 3152, Nuclear-Chicago, Chicago, Ill.). To facilitate combusion discs were first wrapped in small pieces of Kimwipes tissue. Radioactive carbon dioxide was trapped in 15 ml ethanolamine solution (ethanol:ethanolamine, 2:1, v/v) and an aliquot was transferred to a scintillation vial containing 15 ml liquid scintillation fluid and counted. The loss of glucose on soil, expressed as the percent of radioactivity initially present in the discs, was plotted as a function of incubation time on the soil.

Fungistasis Assays

Various assays of fungal spore germination required the use of agar discs. These were prepared from 18 ml of 2% purified agar (Difco, Detroit, Mich.) poured into a 9.0 cm diam sterile plastic petri dish. Discs were cut from the 2.5 mm thick agar with a 9.0 mm diam sterile cork borer. Nutrient amendments to the agar were made prior to autoclaving, but sterile ^{14}C-glucose (usually 0.1 µCi) was added to the sterile molten agar using a microsyringe. When discs of various hydrogen ion concentrations were needed, 9.0 ml 4% molten

purified agar was mixed with 9.0 ml hot sterile 0.2 M potassium phosphate buffer solutions and after cooling discs were cut from plates of these mixtures.

In some experiments discs of polyacrylamide gel were used. A modified procedure of Bishop et al. (9) was used to prepare polyacrylamide gels. Eight ml of an aqueous solution of 15% acrylamide and 0.75% bisacrylamide were mixed with 8.45 ml glass distilled water and 8.33 ml 3E buffer (0.12 M Tris, 0.06 M sodium acetate, 0.003 M sodium EDTA adjusted to pH 7.8 with glacial acetic acid). Dissolved air was removed from this mixture by vacuum before 0.02 ml of N,N,N',N' tetramethylethylenediamine followed by 0.2 ml of a freshly prepared 10% ammonium persulfate solution were added. The final mixture was stirred quickly and 18.0 ml pipetted into a 9.0 cm diam plastic petri dish. Once polymerized 9.0 mm diam discs were cut from the gel with a cork borer and transferred to buffer solution in which they were soaked for at least 7 days with daily changes of buffer solution to completely remove toxic materials. Before use the gel discs were autoclaved and then collected in a sterile Buchner funnel.

To assay for fungistatic materials in the soil agar or gel discs were incubated on the soil surface, and for volatile inhibitors they were placed on sterile glass microscope slides separated from soil by glass rods

or suspended over soil on the inside surface of petri dish lids. After incubation the discs were bioassayed in situ or after transfer to an empty petri dish. Test fungi included conidia of H. victoriae and ascospores of N. tetrasperma. When conidia of T. basicola were used, 50 µl sterile glucose solution was added to each agar disc with a microsyringe 12 hr prior to inoculation with the test organism. Once inoculated, all discs were incubated for 12 hr at 24 C unless otherwise stated. Discs incubated on the model system, or on or above water-saturated sand served as controls.

An assay, henceforth called the radiochemical-biological (R-B) assay, was developed to separate that portion of the fungistasis phenomenon due to the microbially-induced nutrient sink in soil from any other fungistatic factor(s). Agar discs containing 0.1% glucose with and without ^{14}C-glucose label (ca. 3,000 cpm per disc) were incubated in the model system (10 ml water/hr) or on soil for periods of 0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hr. When they were removed from the model system and soil radioactive discs were dropped immediately into scintillation fluid and counted to determine the amount of glucose remaining. Non-radioactive discs were placed in a petri dish and each was inoculated with one drop (ca. 0.04 ml) of a washed conidial suspension of T. basicola. Inoculated discs

were incubated at 24 C for 12 hr before the conidia were strained and killed with phenolic rose bengal (per 100 ml distilled water: 1 g rose bengal, 5 g phenol, 0.01 g $CaCl_2$ and the solution clarified by passage through filter paper). Standard discs of known glucose concentrations were prepared from solutions ranging from 0.1 to 0.00001% glucose. Both germination and radioactivity were expressed as percent of controls without incubation on soil or in the model system.

In all fungistasis assay experiments discs incubated on soil or in the model system were kept sterile by a Nuclepore membrane filter (0.4 μ) placed between discs and the soil, or sand or glass bead bed. Maintenance of sterility was routinely checked by transferring some discs to PDA.

Radiochemical Purity of Glucose in Agar Discs Incubated on Soil

In the R-B assay it was important to know whether the radioactivity remaining in the discs represented ^{14}C-glucose or some labelled metabolite of the sugar. Radioactive agar discs containing 0.1% glucose were incubated on soil for the intervals of time used in the R-B assay. Each disc, initially, contained ca. 200,000 cpm instead of the 3,000 cpm per disc normally used in the R-B assay. After

removal from soil, 2 discs for each time interval were shaken in 1.5 ml sterile glass-distilled water at 45 C for 1 hr. Duplicate discs were also dissolved by adding 0.5 ml 4N HCl to the eluting water and incubating at 100 C for 20 min. One-tenth ml of each aqueous eluate was streaked in a narrow band across the center 1 in of a 2 in wide strip of Whatman No. 1 chromatography paper or spotted on Silica Gel GF thin layer chromatography (TLC) plates (250 μ, Analtech Inc., Newark, Delaware). The same volume of acid-dissolved discs were spotted on TLC plates only. Paper chromatograms were developed by ascending chromatography in n-butanol:n-propanol:water (4:1:1, v/v/v) with the radioactive bands being detected on a Packard Radiochromatograph Scanner (Model 7201) with a 5 mm aperture.

Triplicate TLC plates were run in three different solvent systems: a) t-butanol:n-propanol:water (4:1:1, v/v/v), b) n-butanol:acetone:water (4:5:1, v/v/v), and c) methyl ethyl ketone:acetic acid:water (3:1:1, v/v/v). Radioactive spots were detected by exposing the plates to No-screen Medical X-ray film (NS-2T, Eastman Kodak Corp., Rochester, New York) for 6 wks in the dark at 6 C. Exposed film was developed in total darkness for 15 min in the following developer at 21 C (per liter: 1 g p-methylaminophenol sulfate, 75 g anhydr. sodium sulfite, 9 g hydroquinone, 58 g sodium

carbonate·10 HOH, and 5 g potassium bromide). Developed films were rinsed in water, fixed in commercial fixer, for 15 min, and rinsed again before drying. Prior to fixing, the films were previewed under a 25 watt No. 2 dark red safety light to identify any spots. Radioactive spots were then compared with spots on the TLC plates that were developed by spraying with 50% H_2SO_4 and then charring at 120 C.

Enumeration of Soil Microorganisms

Microbial populations in Conover loam and the clay loam soil were estimated by the soil dilution plate technique. Ten grams of air dried soil were remoistened to previously mentioned soil moistures and incubated at 24 C for 1 day. Soil suspensions were prepared by adding 95 ml sterile 0.85% NaCl solution to the soil and shaking vigorously for 30 min on a Burrell wrist action shaker. All further dilutions were made in saline solution. One ml of the diluted soil suspensions was swirled in a petri dish with ca. 15 ml of molten (43 C) soil extract agar containing 25 ppm PCNB (26) and chitin agar (60) to determine numbers of bacteria and actinomycetes, respectively. For fungi, 0.5 ml of the diluted suspensions were spread on solidified plates of acidified NPX-PDA (84). All plates were incubated at

24 C for 2 wks before counts were made with a Quebec colony counter.

All experiments were run in duplicate and repeated at least once.

RESULTS

Effect of Washing Conidia, and of Different Leaching Media on Germination in the Model System

Nutrient-independent spores germinate readily in glass distilled water, while few do so when incubated on soil, or when leached with water or buffer solution in the model system. To test whether media more like the natural soil solution could also suppress germination, conidia of H. victoriae were leached in the model system with Pfeffer's solution and subsoil extract, as well as water and buffer solution. An extract of natural subsoil was used to approximate the mineral environment of the soil solution. It contained only a small fraction of the energy-yielding compounds present in extracts of topsoil. For comparison, conidia were also incubated on natural subsoil, Conover loam soil, and PDA. The various mineral salt solutions were neither more nor less effective in reducing germination than water; likewise the solutions stimulated no greater

germination than water in the non-leached controls (Table 1). Thus it appears that water alone and buffer impose stresses on the spore like those imposed by the soil solution.

Two common practices are to wash spores before use and to incubate them on membrane filters. Three membrane filters bearing both washed and unwashed conidia of H. victoriae were incubated under several conditions to determine the effects of each on germination. In the model system and on soil the unwashed conidia germinated better than the unwashed spores (Table 2). Germination on Nuclepore membranes and nylon mesh (28 μ) on soil was not unlike that of spores placed directly on the soil. Millipore membranes were somewhat stimulatory under the nutrient deprived conditions of soil and the model system. Where possible, unwashed conidia of H. victoriae were used in this work.

Exudation from Spores Incubated for Alternate Periods when Leached and not Leached

Materials are lost from fungal propagules, via exudation, prior to the formation of a germ tube (9,15,46,59). Ko and Lockwood (54) proposed that the inhibition of spores requiring no exogenous

Table 1. Germination of Helminthosporium victoriae conidia (unwashed) incubated in the model system leached with various media, in the corresponding non-leached controls, on natural soils, and on PDA.

Incubation time	Germination, %											
	Glass distilled water		Buffer solution		Subsoil extract		Pfeffer solution		Conover loam soil	Natural subsoil	PDA	$LSR_{0.05}$ [b]
	+[a]	-	+	-	+	-	+	-				
hr												
0	0	0	0	0	0	0	0	0	0	0	0	
3	23	56	28	60	30	51	25	64	6	32	48	13.9
6	26	74	26	69	31	60	22	66	15	41	70	16.3
12	27	77	20	70	40	81	29	80	12	54	83	15.8

[a] + = Model system leached with indicated solution at ca. 75 ml/hr;
- = Sand saturated with indicated solution.

[b] Least significant range (LSR) using Tukey's w-procedure (81). Comparisons are only made within a given time period.

Table 2. Germination of washed and unwashed Helminthosporium victoriae conidia incubated for 12 hr in the model system, on saturated sand, on natural Conover loam soil, and on PDA.

Treatment	Membrane filter	Germination, %					
		Glass distilled water		Buffer solution		Conover loam soil	PDA
		+[a]	-	+	-		
Washed	Nuclepore	0	88	2	82	0	
	Nylon mesh	0	84	2	86	0	
	Millipore	4	81	15	81	4	
	None					0	97
Unwashed	Nuclepore	9	89	10	92	1	
	Nylon mesh	5	94	12	87	0	
	Millipore	29	81	34	82	14	
	None					0	95

[a] + = Model system leached with indicated solution at ca. 45 ml/hr;
- = Sand saturated with indicated solution.

energy for germination may be due to the rapid utilization of exuded endogenous materials by microorganisms in the soil, or their physical removal from the spore in the model system. Alternatively, spores may be able to modify their pattern of exudation under nutrient stress conditions so as to conserve endogenous energy reserves.

To determine whether less materials are lost from spores under diffusion stress conditions, ^{14}C-labelled conidia of H. victoriae, C. lunata, and T. basicola on membrane filters were incubated alternately on glass beads leached with buffer solution and on buffer-saturated glass beads without leaching. Conidia of the latter fungus requires exogenous nutrients for germination. At various times the exudates and the washings of the glass bead beds were collected and taken to dryness, and the radioactivity measured. One ml of a 0.3% thimerosal solution was added to the exudates during drying to prevent microbial activity.

More radioactivity was lost from the spores when they were leached than when they were not leached. This indicates that increased loss occurs under diffusion stress conditions (Figure 2). Most of the radioactivity was collected during the early

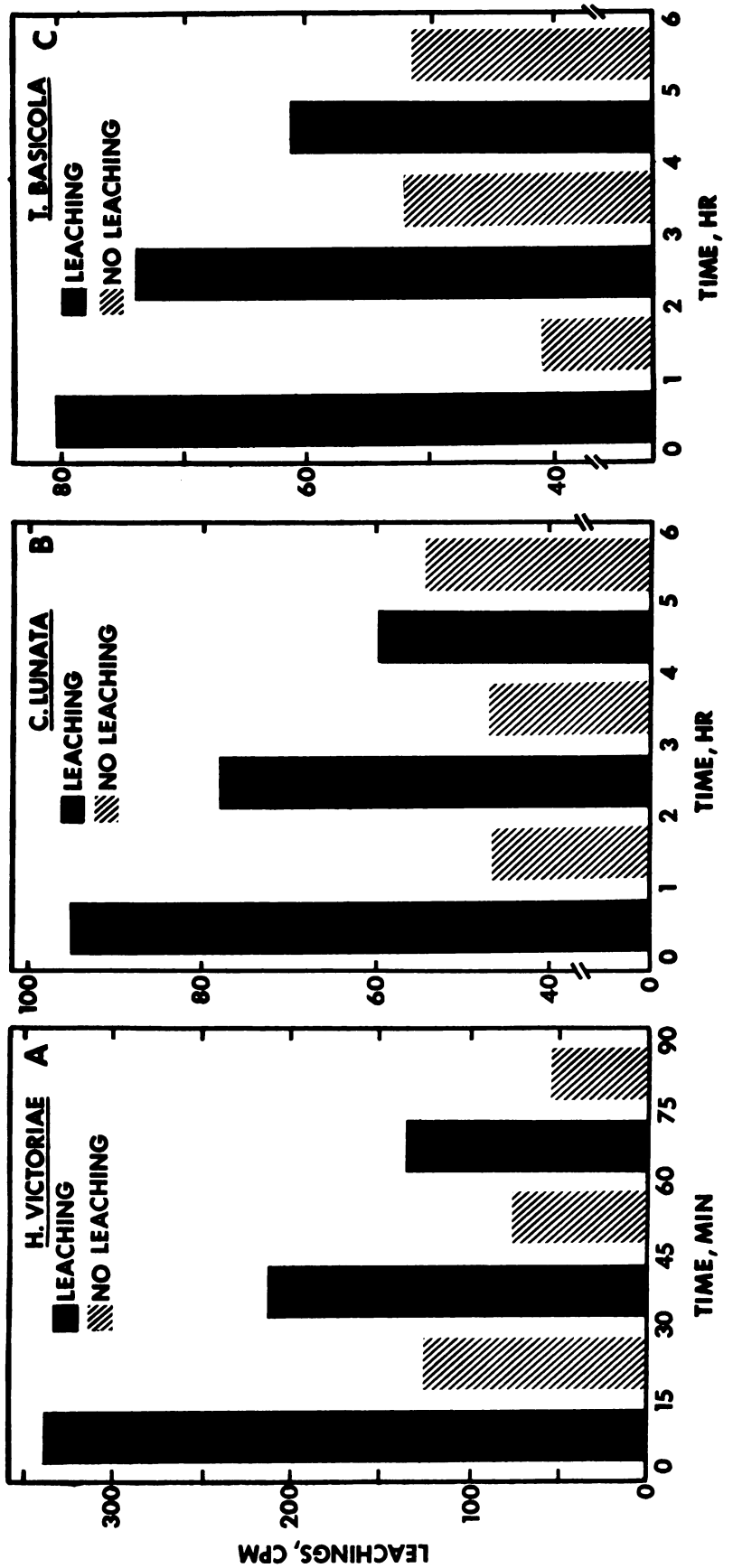


Figure 2. Exudation from ^{14}C-labelled conidia of A) Helminthosporium victoriae, B) Curvularia lunata, and C) Thielaviopsis basicola incubated for alternate periods in the model system (leaching) and on buffer-saturated glass beads (no leaching).

incubation periods. A similar pattern of exudation was obtained when sclerotia of S. cepivorum were incubated for alternate periods. Interestingly, the nutrient-dependent conidia of T. basicola exhibited the same type of exudation pattern as the three nutrient-independent propagules.

Attempts to measure exudation from spores on soil were unsuccessful as the variation in the amount of radioactivity in labelled spores applied to several replicate membrane filters was greater than the difference between untreated spores and those incubated on soil.

Exudation from H. victoriae Conidia Incubated Continuously in the Model System

To determine the pattern of exudation under continuous diffusion stress, ^{14}C-labelled conidia (unwashed) of H. victoriae were incubated in the model system leached with buffer solution for 96 hr. The leachings were collected in 10 ml fractions with a fraction collector (LKB Instruments Inc., Chicago, Ill.). Each fraction was transferred to a scintillation vial containing 1.0 ml 0.3% thimerosal solution and taken to dryness on a hot plate at 60 C, before the addition of scintillation

fluid, and then counted. After 12 hr several fractions were combined to obtain enough radioactivity to count.

Approximately 10% of the initial 65,558 cpm in the spores was lost after 96 hr. More than 90% of the 6,233 cpm lost was leached from the spores within the first 12 hr, and over 99% by 24 hr (Figure 3). Most of the exuded material was readily leached from the conidia and after its removal exudation continued at a lower, almost constant rate.

Similar results were obtained by passing ten 10 ml volumes of sterile glass distilled water, in rapid succession, through ^{14}C-labelled (unwashed) conidia of H. victoriae on a Millipore membrane filter (0.22 μ), and then determining radioactivity in each of the ten dried filtrates (Figure 4). Such treatment removed ca. 2.5% of the radioactivity from the conidia. Unwashed conidia of H. victoriae germinated ca. 10% when incubated in the model system (50 ml water/hr) for 12 hr, whereas conidia washed by centrifugation failed to germinate. Both washed and unwashed conidia germinated greater than 85% on water-saturated sand, and were completely inhibited directly on

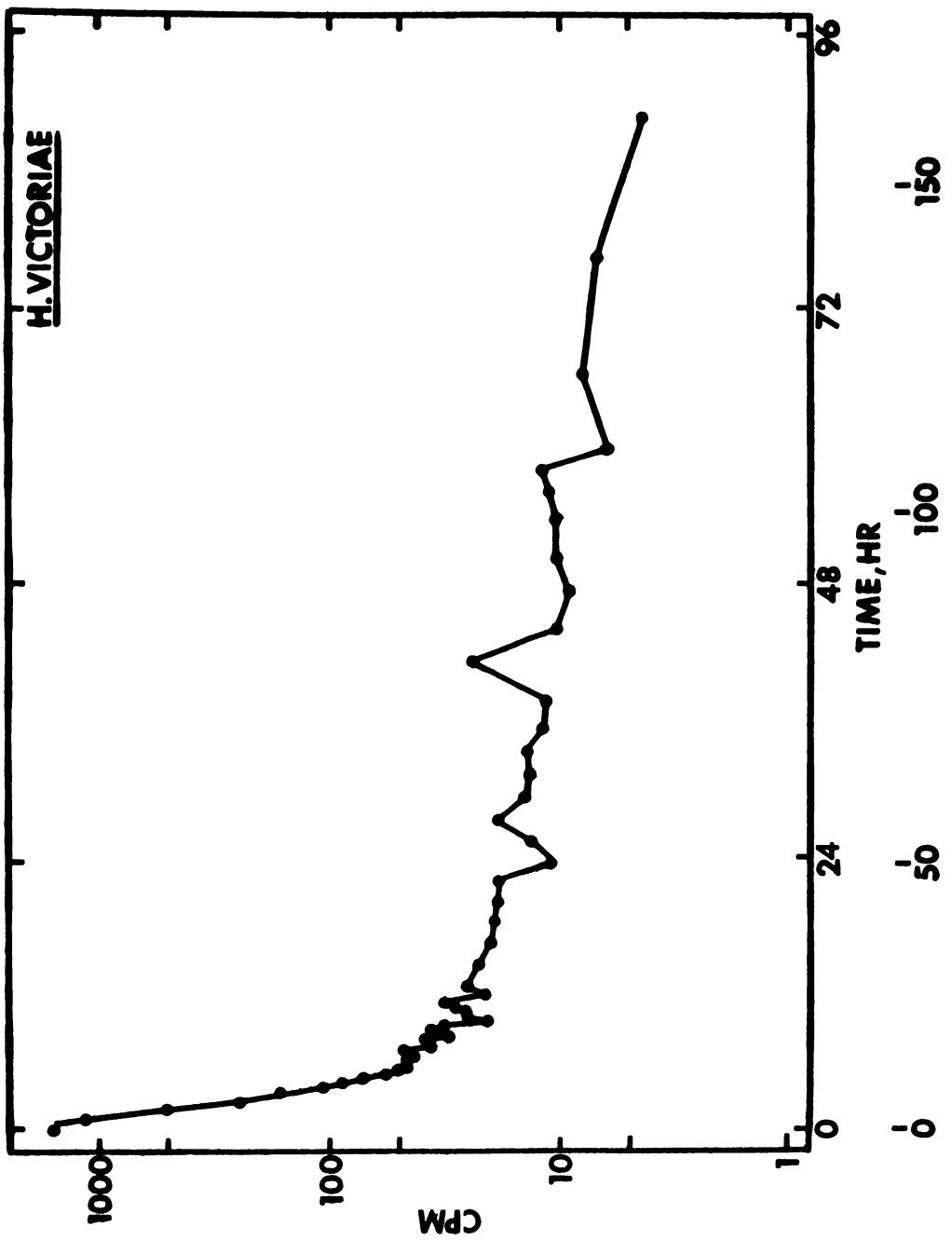


Figure 3. Exudation from ^{14}C-labelled conidia of Helminthosporium victoriae (unwashed) incubated in the model system for 96 hr.

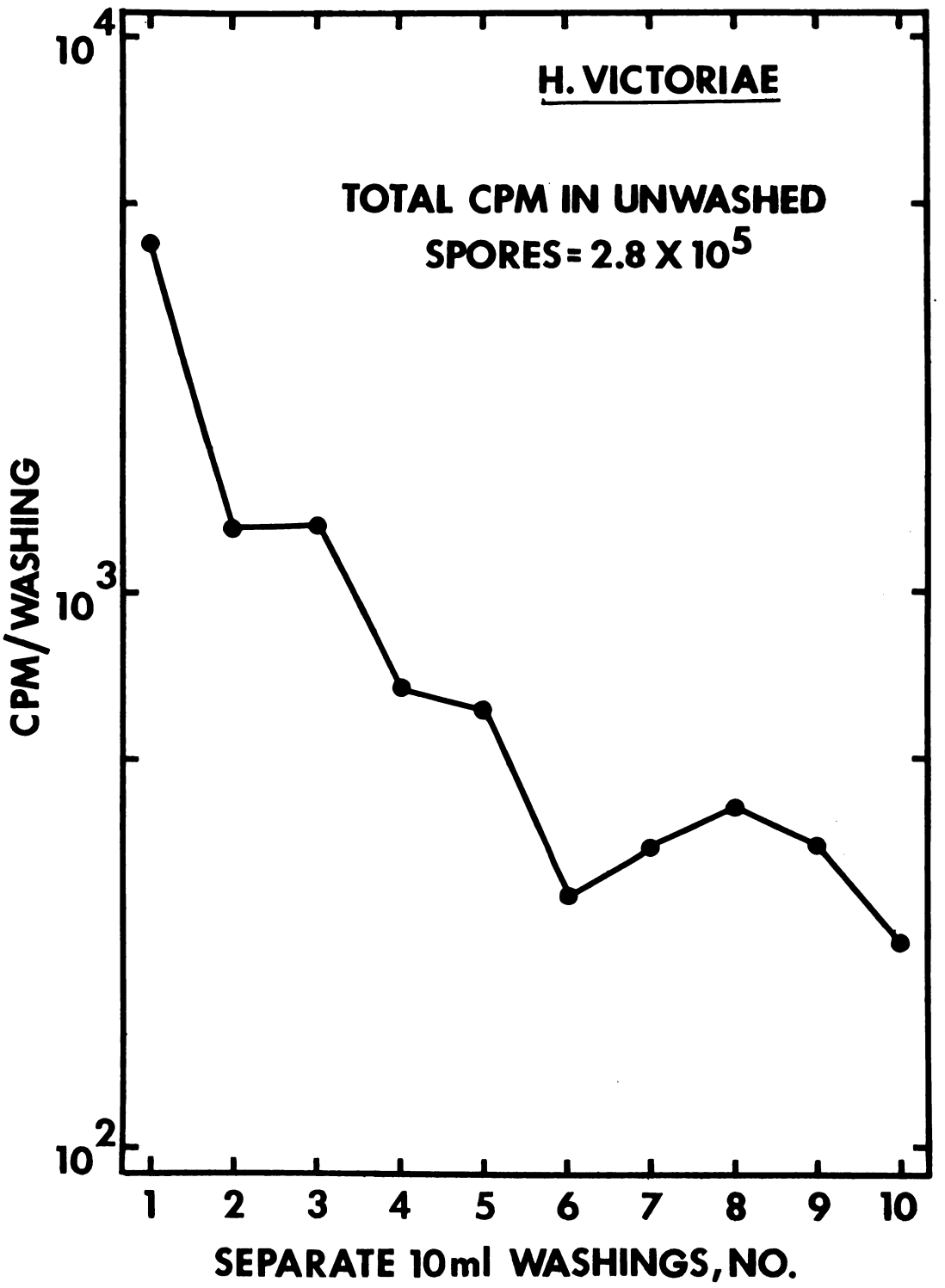


Figure 4. Radioactivity in each of ten successive 10 ml aqueous washings of ^{14}C-labelled, initially unwashed, Helminthosporium victoriae conidia.

natural Conover loam soil. The ability of washed spores to germinate in water suggests that spores still contain sufficient reserves.

Effect of Extended Incubation on Conover Loam Soil or in the Model System

Data presented in Figure 3 suggest that loss of materials from conidia of H. victoriae after 24 hr on the model system proceeds at a very low, but almost continuous level. This raised the question whether the endogenous energy reserves of the spore eventually become depleted to the extent that an exogenous source of nutrients is required for germination. Hsu and Lockwood (43) recently reported that the nutritionally independent propagules of five test fungi did not lose the ability to germinate in buffer solution after 24 hr exposure to either the model system or soil.

To further examine the possibility of such a conversion, conidia of H. victoriae and H. sativum were incubated for up to 56 days in the model system or on soil. At predetermined intervals membrane filters bearing the conidia were either stained and killed, or were transferred to sterile buffer solution or PDA for an additional incubation

of 12 hr to assess nutrient-independence and viability, respectively.

Germination of H. victoriae conidia in buffer decreased from 87 to 58% after only 12 hr incubation in the model system or on soil (Figure 5-A). By 7 days almost all the spores had become nutrient-dependent. Extending the incubation period in the buffer solution to 24 hr did not increase germination over that at 12 hr. Viability of conidia incubated in the model system began to decrease after 21 days and declined to 30% by 56 days. Viability declined more slowly for conidia incubated on soil. The loss of nutrient-independence by H. sativum conidia was as rapid as that by H. victoriae, but viability of H. sativum declined much more rapidly both on soil and in the model system (Figure 5-B).

Accumulation of Exudates by Spores

To test whether spore exudates may be utilized in the process of germination, radioactive conidia of C. lunata were incubated in the model system assembled so that their leachings passed directly into a second leaching dish containing non-radioactive ("cold") conidia (Figure 6). After 12 hr ca. 2% of the radioactivity (2,080 cpm) was leached from the ^{14}C-labelled

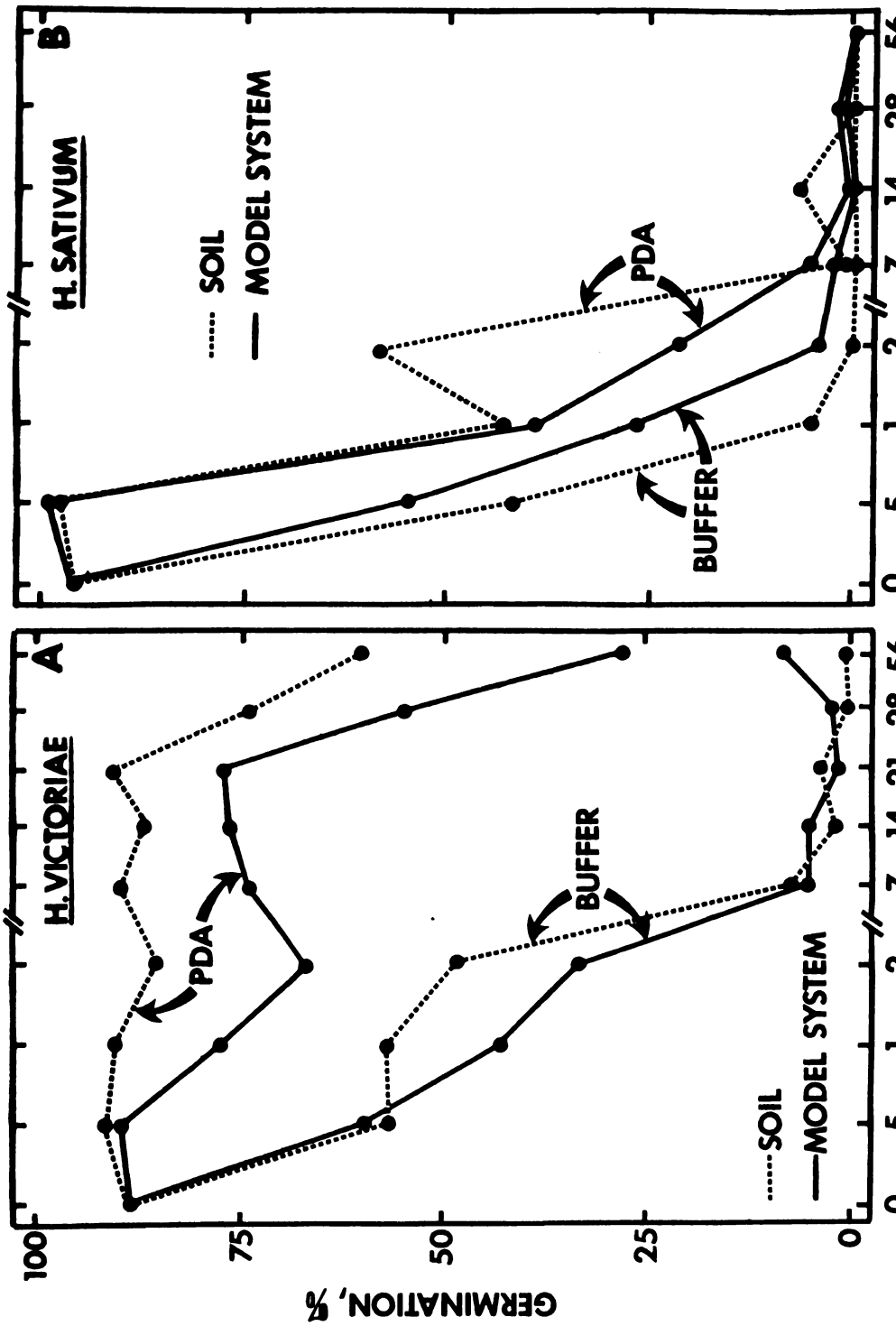


Figure 5. Conidial germination of A) Helminthosporium victoriae and B) Helminthosporium sativum incubated on natural Conover loam soil or in the model system (45 ml buffer/hr) for up to 56 days before transfer to either buffer solution or PDA for an additional 12 hr incubation.

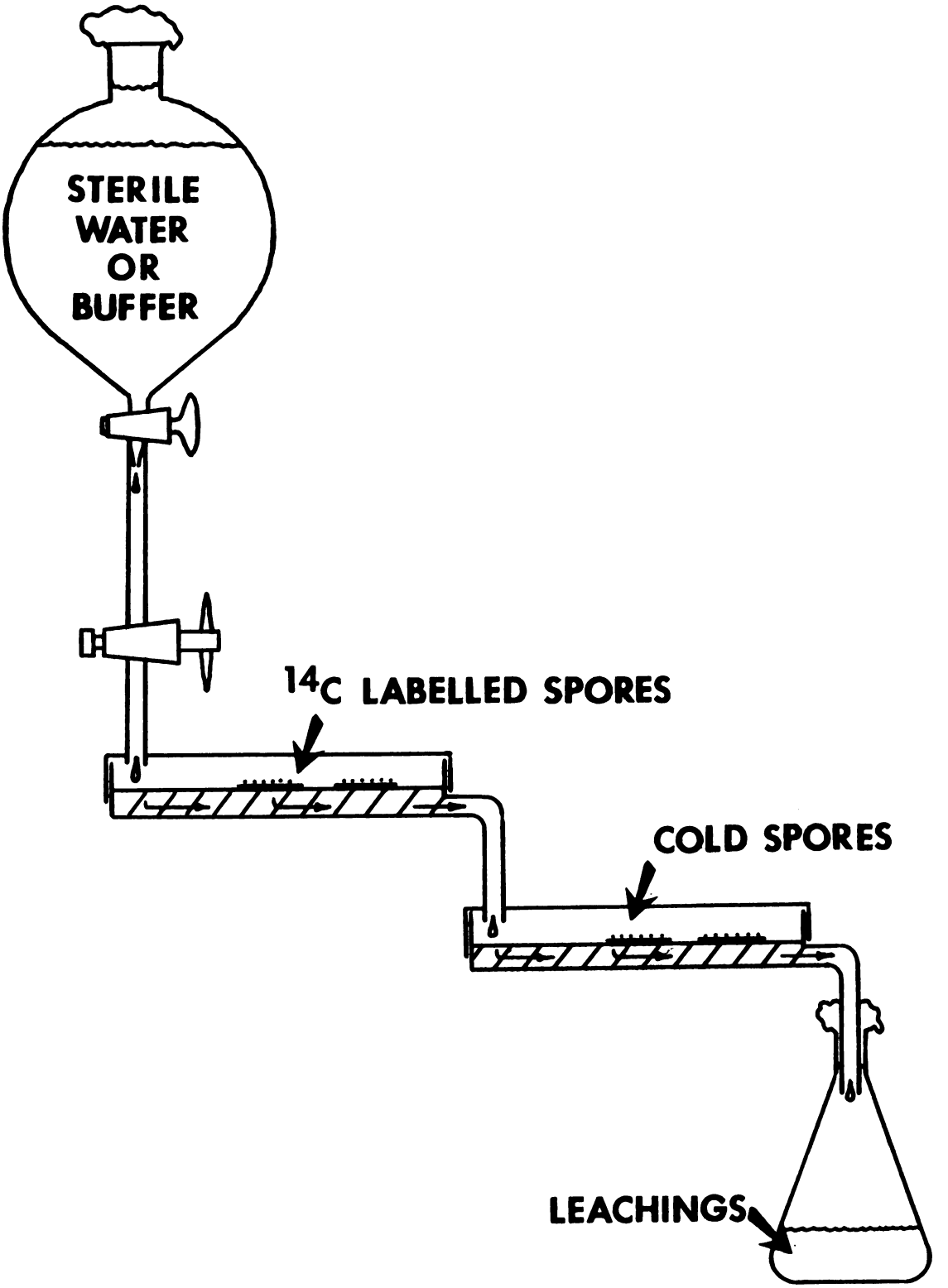


Figure 6. Diagram of the model system, modified to measure the uptake of ^{14}C-labelled spore exudates by non-radioactive spores.

conidia. Membrane filters with the "cold" conidia from the lower dish gave 38 cpm over membrane filters similarly incubated in the lower dish, but without spores. In absolute amounts the radioactivity taken up was low, but it should be considered that: a) the "cold" conidia covered only a small portion of the glass bead bed, b) the leachings entering the lower dish became diluted, and c) the "cold" spores were undergoing leaching at the same time they were taking up the labelled leachings from the upper dish.

In other experiments conidia of H. victoriae converted to a nutrient-dependent condition by extended incubation on soil or in the model system were tested for their ability to take up materials in spore exudates, and for stimulation of germination by the exudates. Membrane filters bearing such spores were floated for 12 hr on 1.0 ml concentrated ^{14}C-labelled exudate from H. victoriae conidia. Following incubation the conidia and membranes were washed with 30 ml glass distilled water on a Millipore filter holder. Membranes were scanned under the microscope to estimate germination before being placed into scintillation fluid for counting. Over 50% of the radioactivity available was found in the

spores after only 12 hr (Table 3), and more than one-half of the spores had germinated.

Table 3. Uptake of radioactivity by nutrient-dependent conidia of Helminthosporium victoriae incubated 12 and 24 hr on 1.0 ml of concentrated ^{14}C-labelled spore exudate.

Previous incubation of conidia		Radioactivity, % of total available[a]	
Treatment	Time	12 hr	24 hr
	days		
Model system	14	72.8	87.0
	21	51.5	90.0
Conover loam soil	14	70.6	86.2
	21	64.1	67.5

[a]Total available = 1050 cpm.

Stimulation of Germination by Spore Exudates

If the removal of exudates by leaching is important in the inhibition of germination of nutrient-independent spores, then the exuded materials should contain substances which when resupplied to spores stimulates germination. Exudates collected from conidia of H. victoriae, C. lunata, and T. basicola each contained amino compounds and carbohydrates,

including glucose (Table 4). Conidia of T. basicola exuded only 0.16 µg glucose equivalents of carbohydrate per million spores during 20 hours of incubation, while H. victoriae lost over 95 µg and C. lunata ca. 4.3 µg in 1.5 and 3.0 hr, respectively. Approximately 30% of the carbohydrates detected in the exuded materials of C. lunata and T. basicola were accounted for as glucose, while greater than 90% was measured for H. victoriae. Exudation of amino compounds was one-sixth to one-half that of the carbohydrates for each of the three fungi.

In order to determine directly whether substances present in conidial exudates would stimulate germination, conidia were leached in the model system with exudate from C. lunata and H. victoriae. To establish the role of nutrients present in natural spore exudate, conidia were also leached with a synthetic exudate, based on nutrient concentrations found in natural exudate. Synthetic exudate for H. victoriae contained 523.5 µg glucose and 83.5 µg glycine equivalents of casein hydrolysate per liter of glass distilled water, and that for C. lunata 319.0 and 124.0 µg per liter of the two nutrients, respectively.

Table 4. Quantities of nutrients exuded from conidia of Thielaviopsis basicola, Curvularia lunata, and Helminthosporium victoriae incubated in sterile glass distilled water for periods less than those required for germination.

Fungus	Preparation of conidia	Incubation time	µg/10^6 conidia		
			Glucose eq[a]	Glucose[b]	Glycine eq[c]
		hr			
T. basicola	washed	20	0.16	0.05	0.08
C. lunata	washed	3	4.23	1.03	1.65
H. victoriae	unwashed	1.5	95.22	88.79	15.00

[a] Anthrone reagent.

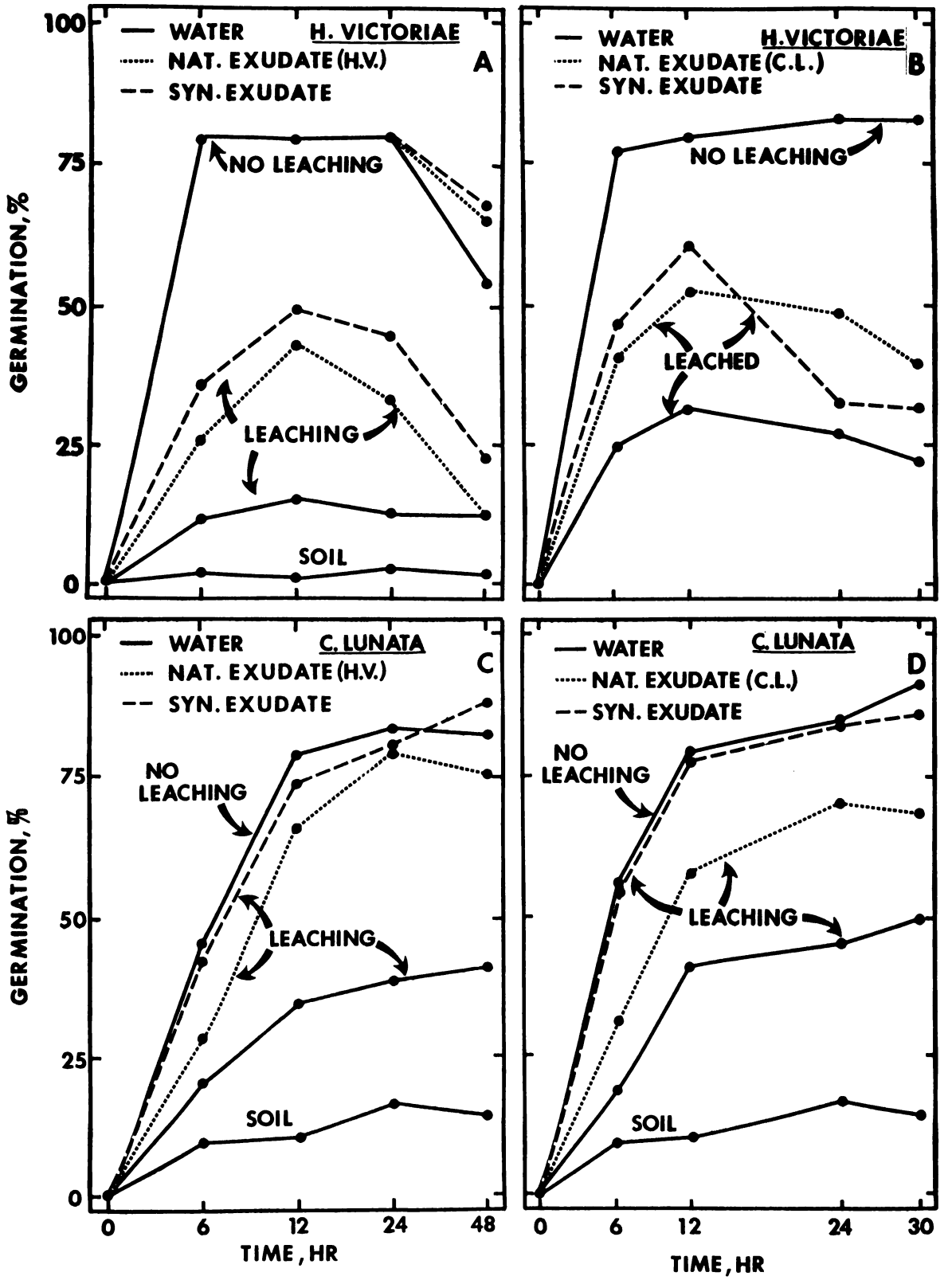
[b] Glucostat reagent.

[c] Ninhydrin reagent.

Exudate from conidia of H. victoriae increased the germination of its own conidia in the model system from 18% under leaching conditions with water to 45% with natural exudate and over 50% with synthetic exudate (Figure 7-A). The apparent decrease in germination after 12 hr leaching with natural and synthetic exudates (Figure 7-A and B) was the result of either germ tube lysis or the retraction of germ tube protoplasm into the spore proper. This observation suggests that the nutrients provided in the natural and synthetic exudates were sufficient to stimulate germination but not to maintain germ tube growth under the diffusion stress of the model system. Germination of C. lunata conidia was stimulated by its own natural and corresponding synthetic exudate and by that of H. victoriae (Figure 7-C and D). Similar trends were observed when conidia of H. sativum were leached with natural and synthetic exudates of C. lunata. Germination on sand saturated with the various exudates was never greater than with water. Germination of H. victoriae was never as great as in non-leached controls (75%), indicating that even though materials were being resupplied, the effect of the diffusion sink was still evident.

Figure 7. Germination of Helminthosporium victoriae (A and B) and Curvularia lunata (C and D) conidia incubated in the model system with either water, natural exudate from both fungi, or their corresponding synthetic exudates, and on sand saturated with each of the media.

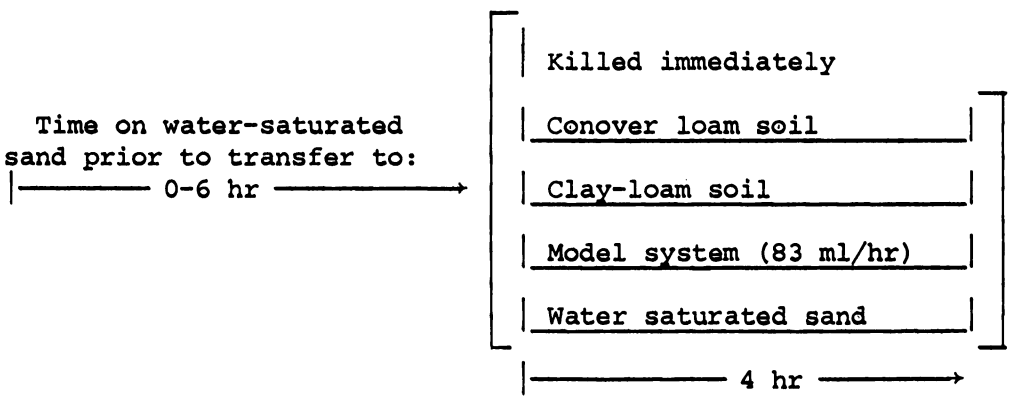
Figure 7.


WATER
NAT. EXUDATE (H.V.)
SYN. EXUDATE
H. VICTORIAE
A
NO LEACHING
LEACHING
SOIL
WATER
NAT. EXUDATE (C.L.)
SYN. EXUDATE
H. VICTORIAE
B
NO LEACHING
LEACHED
C. LUNATA
C
NO LEACHING
LEACHING
SOIL
C. LUNATA
D
NO LEACHING
LEACHING
SOIL
GERMINATION, %
TIME, HR
0
25
50
75
100
0
6
12
24
30
48

The Point in the Germination of H. victoriae at which Fungistasis Becomes Ineffective

Previous results have shown that conidia of H. victoriae germinate well over 50% in several hours when incubated on water-saturated sand. The question arose as to whether there was a point in the spore's metabolism, prior to the formation of a germ tube, beyond which the spore was no longer sensitive to fungistasis.

To answer this question Nuclepore membrane filters bearing unwashed conidia of H. victoriae were incubated on water-saturated sand for intervals up to 6 hr before transfer to one of the following for 4 hr additional incubation: a) Conover loam soil, b) the clay-loam soil, c) model system [83 ml water/hr], or d) returned to water-saturated sand. The following diagram illustrates the treatments:

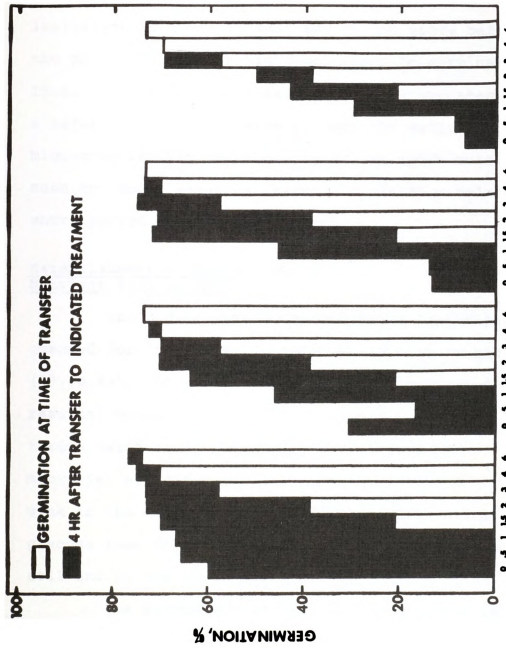


During the initial incubation on water-saturated sand germ tubes began to emerge by 1.5 hr (Figure 8). After only 1.0 hr some of the conidia were no longer sensitive to fungistasis: they had become irreversibly committed to the formation of a germ tube. Transfer of these spores to either the model system or natural Conover loam soil failed to stop their germination. All the conidia capable of germinating independent of exogenous nutrients were irreversibly committed to germinate after 3 hr incubation on water saturated sand with the exception of some of those transferred to the clay loam soil. This suggests that the clay loam soil is more fungistatic than Conover loam soil or the model system.

Germination of spores placed directly in the model system, without prior exposure to water-saturated sand was 30% compared with 18% for those incubated on water-saturated sand for 0.5 hr before transfer to the model system. It is not known why these conidia, having had initiated germination but not yet produced a germ tube, were more sensitive to the diffusion stress of the model system.

Figure 8. Germination of Helminthosporium victoriae conidia (unwashed) incubated on water-saturated sand for 0-6 hr prior to transfer to the following for 4 hr additional incubation: a) Conover loam soil, b) the clay loam soil, c) the model system, or d) water-saturated sand.

Figure 8.


GERMINATION AT TIME OF TRANSFER
4 HR AFTER TRANSFER TO INDICATED TREATMENT
GERMINATION, %
100
80
60
40
20
0
0 .5 1 1.5 2 3 4 6 0 .5 1 1.5 2 3 4 6 0 .5 1 1.5 2 3 4 6 0 .5 1 1.5 2 3 4 6
TIME ON WATER SATURATED SAND PRIOR TO TRANSFER, HR
WATER SATURATED SAND
MODEL SYSTEM
CONOVER LOAM SOIL
CLAY-LOAM SOIL

This experiment emphasizes the importance of time in the inhibition of nutrient-independent propagules. Whatever factor(s) is responsible for inhibition in soil, it must act on the spore before the point of irreversible committment to germination. Thus, if microbial utilization of spore exudates is a major cause of fungistasis, then the activity of microorganisms in the vicinity of the spore must be such to remove essential materials within a relatively short period of time.

Establishment of the Microbial Nutrient Sink in Soil

Rapid decreases in carbohydrate levels in amended soil have been reported by several researchers (27,40,83). More recently Lockwood (unpublished results) developed an assay to obtain a quantitative estimate of the energy sink caused by microbial activity in soil, and the diffusion sink in the model system by measuring the rate of glucose loss from amended paper discs incubated on soil and in the model system.

The microbial nutrient sink in Conover loam and the clay loam soil was assessed using a modification of the paper disc technique. Very small

quantities of ^{14}C-glucose were added to paper discs (90 µl of 0.05, 0.005, and 0.0005% glucose solutions, each containing 0.05 µCi/ml) that were incubated on the two soils. Loss of glucose was detected within 15 minutes (Figures 9 and 10). Moreover, less time was required for 50% of the radioactivity to be lost from discs the lower the initial concentration of glucose in the discs. This may be of particular importance in the region of the spore where very minute quantities of substrate are exuded. Utilization of such materials probably occurs quite rapidly, if not immediately.

To better determine the minimum time required for the expression of the microbial sink, evolution of radioactive carbon dioxide from metabolism of ^{14}C-glucose applied to the soil surface was monitored. Radioactive CO_2 was detected within 3 minutes from both Conover loam and the clay loam soils, with ca. 7 and 3% of the 2.0 µCi (72 µg glucose) applied to each soil, respectively, respired after just 4 hr (Figure 11). No $^{14}CO_2$ was respired when radioactive glucose was applied to sterile soils, nor when non-radioactive glucose was used.

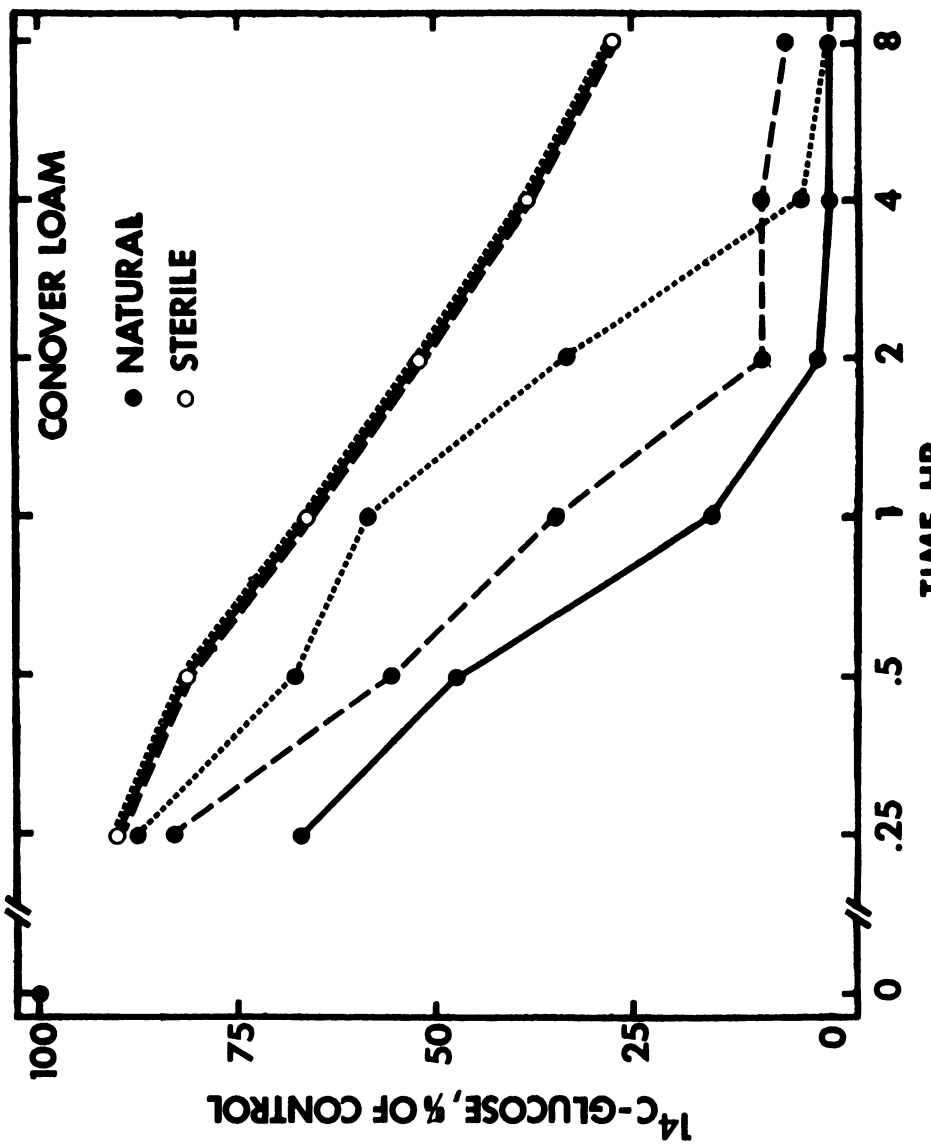


Figure 9. Loss of ^{14}C-glucose from amended paper discs incubated on sterile and natural Conover loam soil. Discs were amended with 90 µl 0.05 (•••), 0.005 (---), and 0.0005% (——) glucose solutions each containing 0.05 µCi/ml.

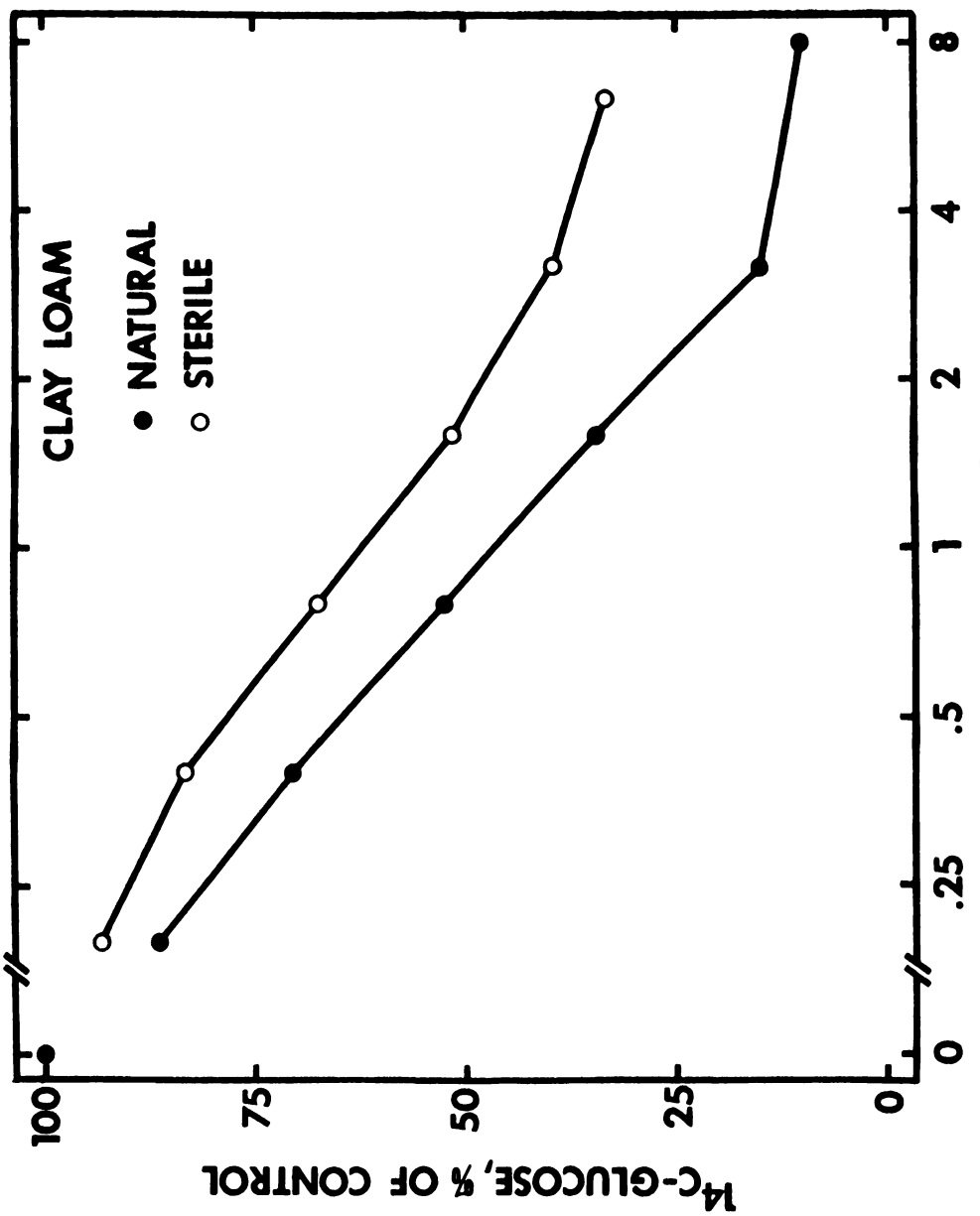


Figure 10. Loss of ^{14}C-glucose from paper discs amended with 90 µl 0.00005% glucose solution (0.05 µCi/ml) when incubated on the sterile and natural clay loam soil.

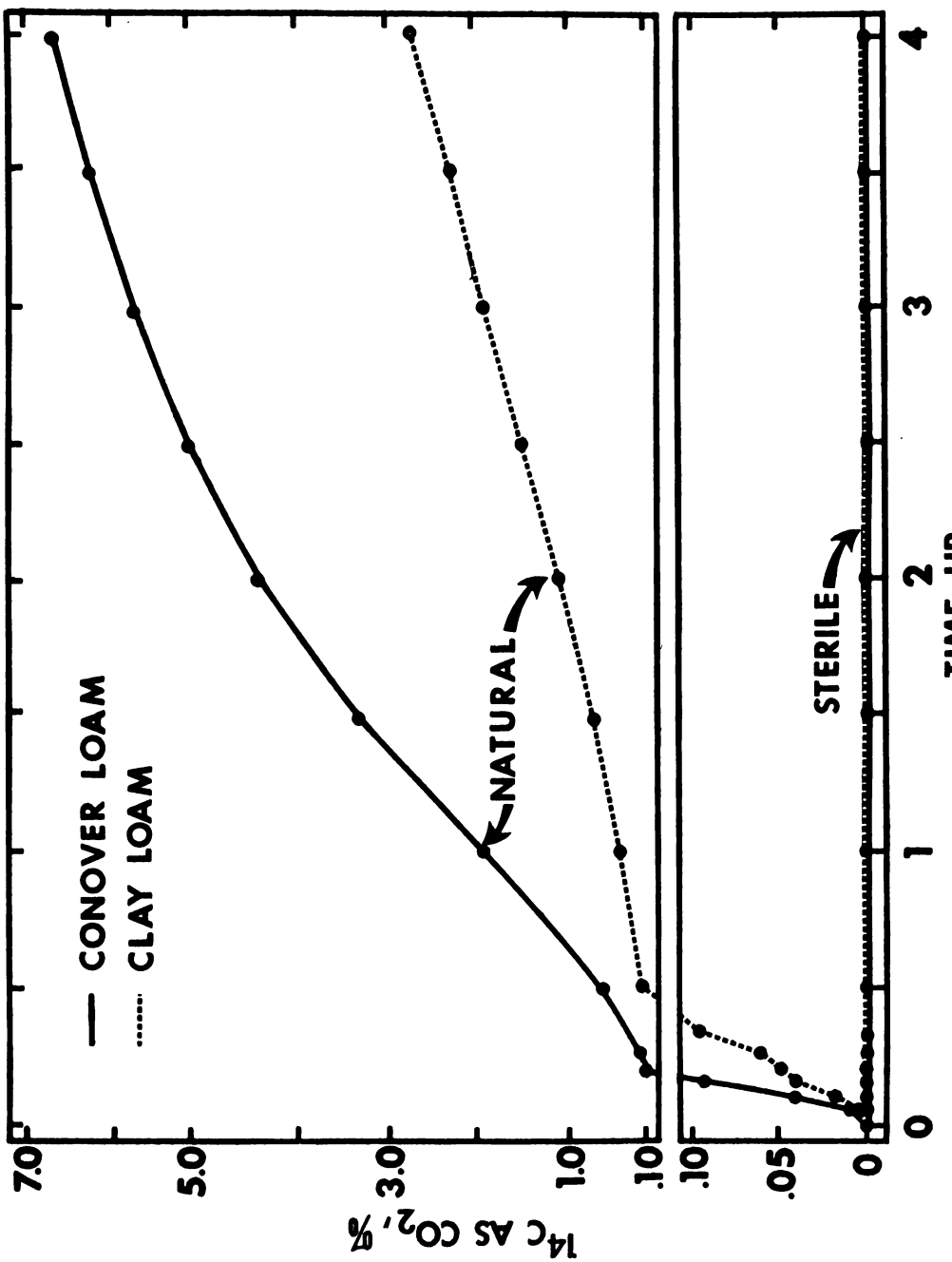


Figure 11. Evolution of $^{14}CO_2$ from natural and sterile Conover loam and the clay loam soil plates following application of 2 µCi ^{14}C-glucose (72 µg) to the soil surface.

When ^{14}C-labelled exudates from H. victoriae conidia were mixed with suspensions of either soil, $^{14}CO_2$ was respired within 15 minutes (Figure 12). After 4 hr 3% of the radioactivity added to the Conover loam soil suspension was respired, while only 1.4% was trapped from the clay loam soil. However during the first two hours, the rate of exudate metabolism in both soils was similar. Addition of ^{14}C-glucose to soil suspensions resulted in respiration of 20 and 11%, respectively, of the added radioactivity in 4 hr (Figure 13). The slower rate at which exudates were metabolized, compared with glucose, indicates that only a fraction of the exuded materials can be readily utilized by soil microorganisms.

Bacterial isolates obtained from soil dilution plates were tested directly for their ability to take up radioactive exudates collected from H. victoriae conidia. All four isolates, two each from Conover loam and the clay loam soil, accumulated radioactivity within 15 minutes and two of the isolates contained ca. 6% of the initial amount of radioactivity after 60 minutes incubation (Figure 14).

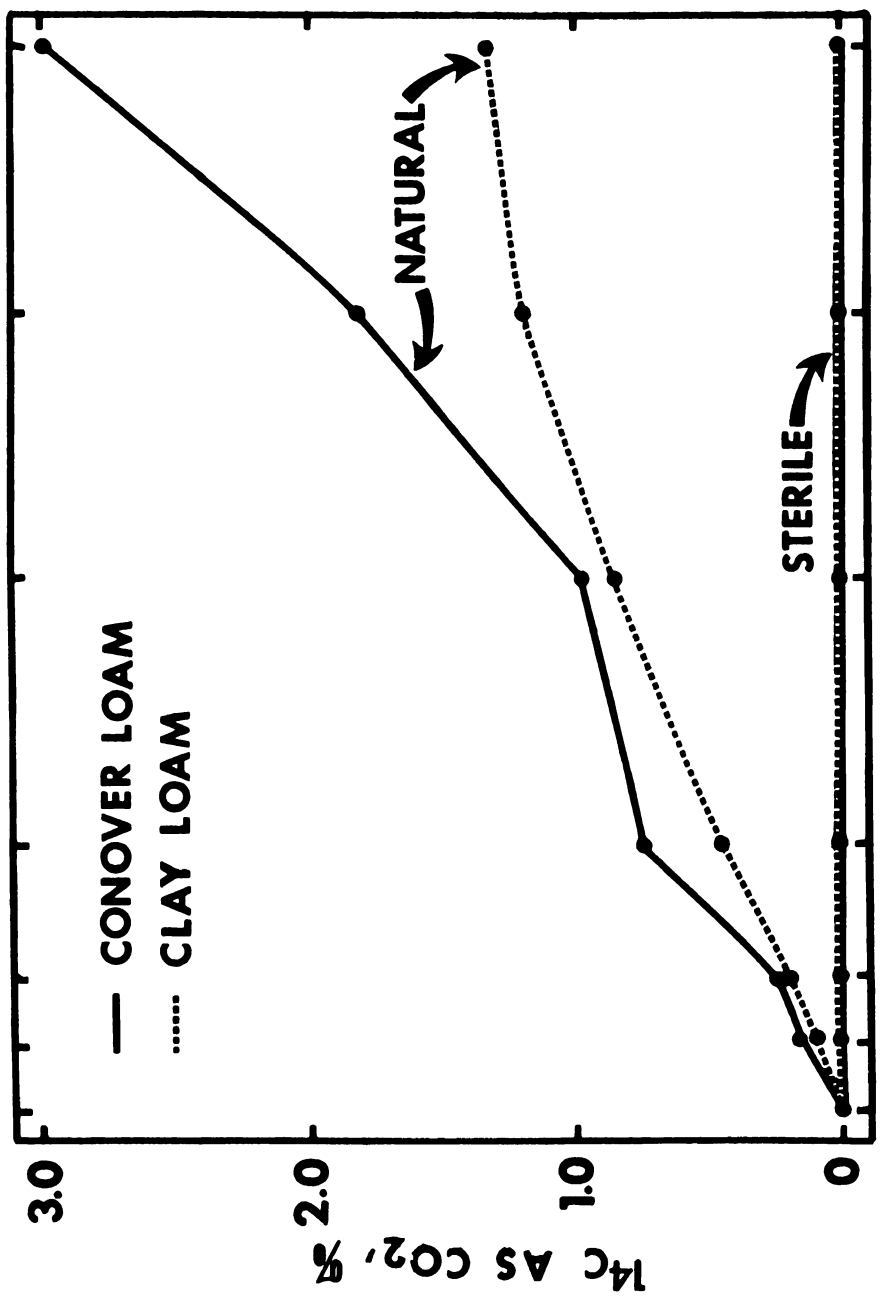


Figure 12. Evolution of $^{14}CO_2$ from Conover loam and the clay loam soil suspensions (soil:water, 1:2, w/v), amended with concentrated exudate from ^{14}C-labelled conidia of Helminthosporium victoriae.

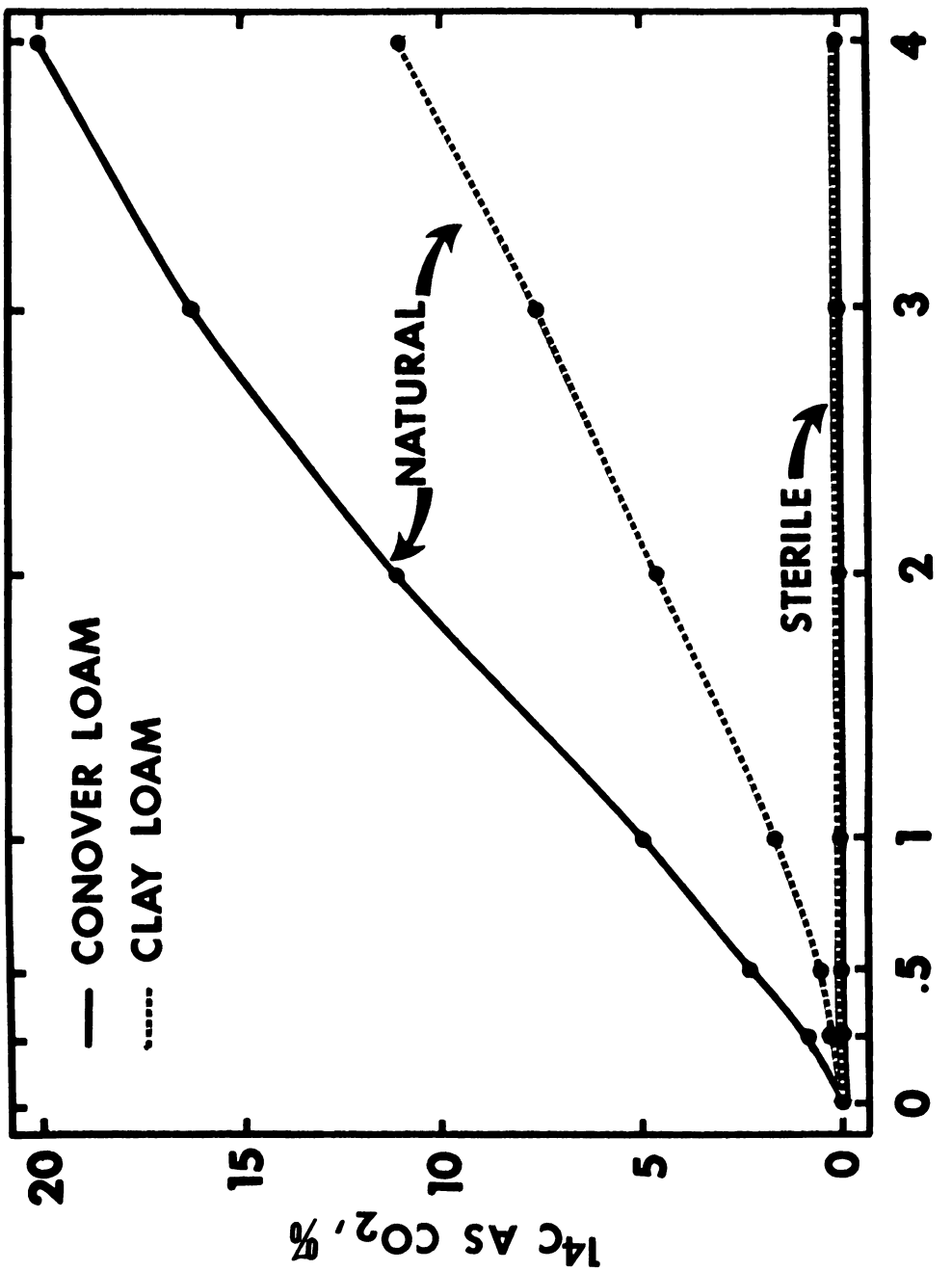


Figure 13. Evolution of $^{14}CO_2$ from Conover loam and the clay loam soil suspensions (soil:water, 1:2, w/v), amended with 0.05 µCi ^{14}C-glucose.

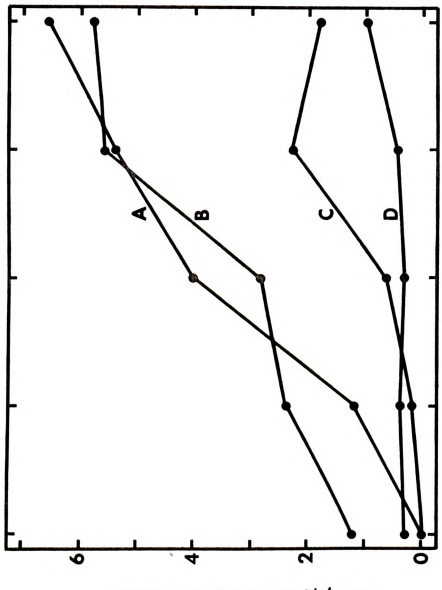


Figure 14. Accumulation of radioactive exudates from ^{14}C-labelled Helminthosporium victoriae conidia by bacterial isolates from Conover loam soil (A and B) and the clay loam soil (C and D).

The difference in the two soils used might be due to differences in microbial populations and/or their activities. The number of bacteria and actinomycetes in the two soils was quite similar. However, the fungal population in the clay loam was less than 0.03 that in Conover loam soil (Table 5). This was not surprising as a volatile fungistatic factor has been reported for the clay loam soil (36). The lower fungal populations and reduced microbial activity, possibly due to the volatile inhibitor may account for the less active sink in the clay loam soil.

Table 5. Populations of fungi, bacteria, and actinomycetes in Conover loam and the clay loam soil 24 hr after remoistening the air-dried soils.

Soil	Propagules per g of air-dried soil[a]		
	Fungi	Bacteria	Actinomycetes
Conover loam	6.96×10^4	5.75×10^6	8.60×10^6
Clay loam	2.10×10^3	7.89×10^6	6.21×10^6

[a]Fungi were enumerated on acidified PDA plus detergent (84), bacteria on soil extract agar (26), and actinomycetes on chitin agar (60).

Role of the Microbial Nutrient Sink of Soil in Fungistasis

In previous experiments more H. victoriae conidia germinated in the model system than on soil. Also the rate of glucose loss from paper discs on the model system, operated at flow rates capable of inhibiting spore germination, was greater than that on soil (Lockwood, unpublished results). This was confirmed by incubating agar discs amended with glucose on Conover loam and the clay loam soils, and the model system, even though agar discs were far less sensitive than the paper discs in measuring the loss of glucose (Figure 15).

A radiochemical-biological (R-B) assay was developed to determine whether the microbial nutrient sink accounted for all the inhibition observed in soil. The assay was designed to experimentally separate the role of the microbial nutrient sink from any other mechanism of inhibition in soil. Purified agar discs amended with 0.1% glucose were incubated on the two soils, in the model system, and on water-saturated sand.

Germination of T. basicola on discs removed at intervals from either the model system or water-saturated sand corresponded with that on standard

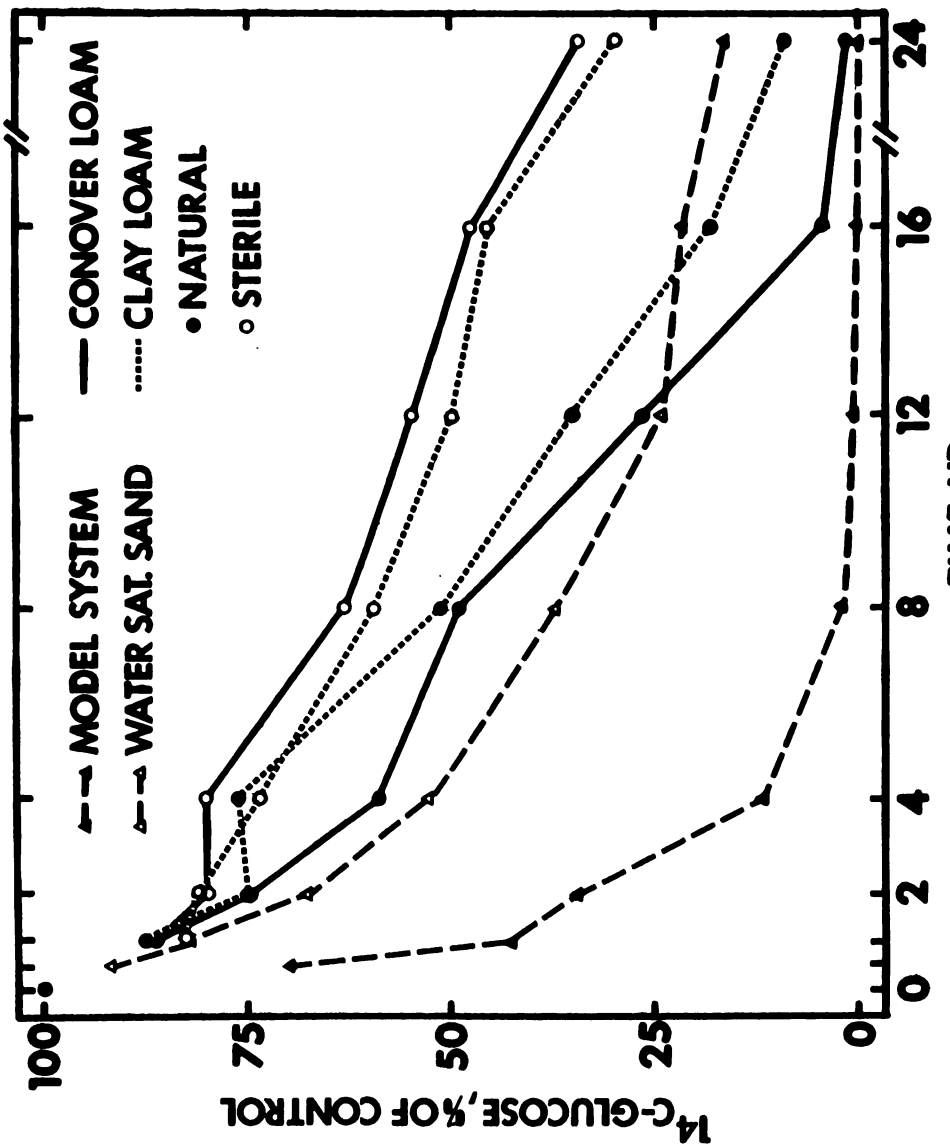


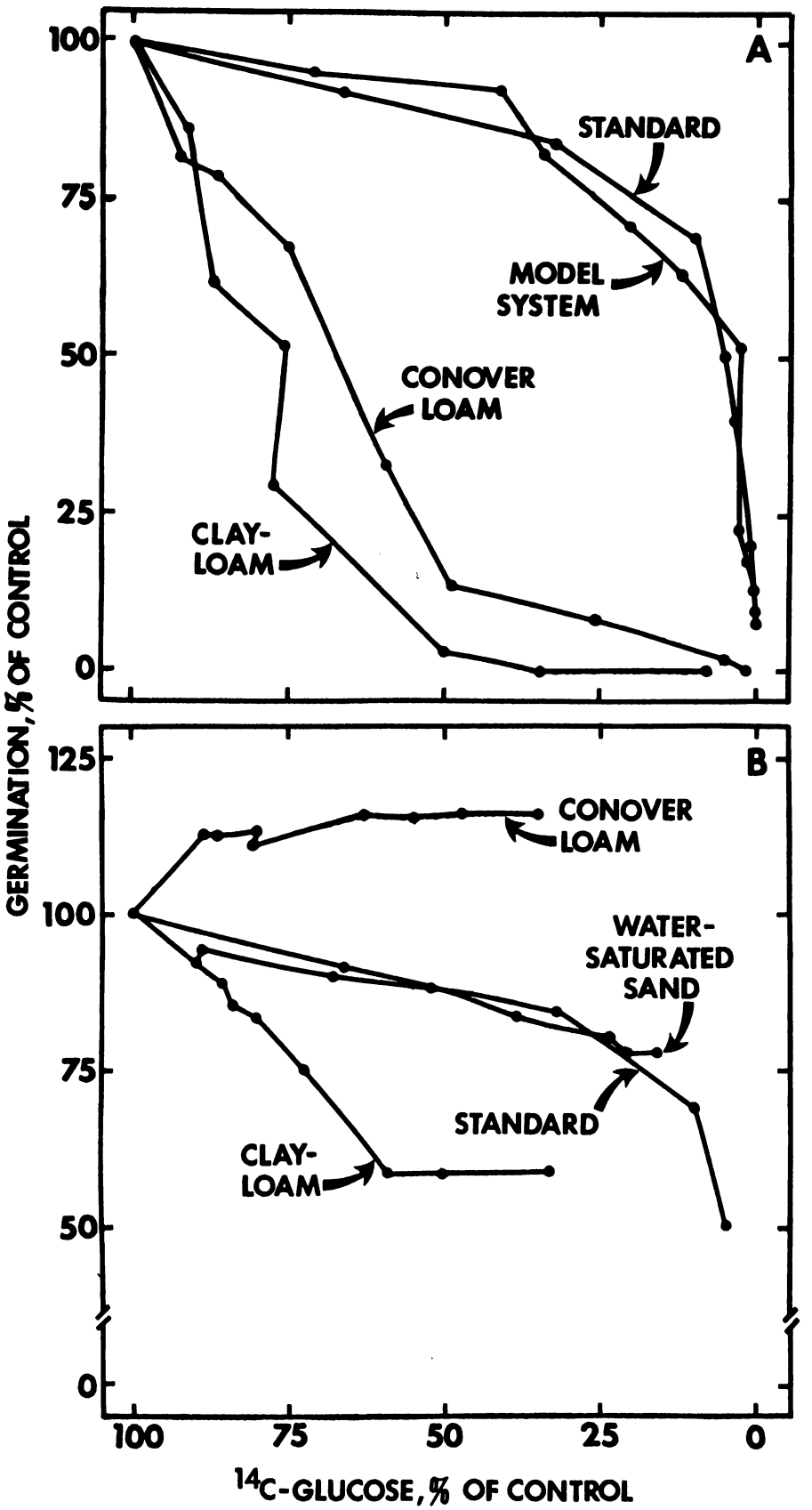
Figure 15. Loss of ^{14}C-glucose from amended agar discs incubated on water-saturated sand, in the model system (10 ml water/hr), or on sterile and natural Conover loam and the clay loam soil.

discs prepared with known glucose concentrations (Figure 16A and B). In contrast, germination on discs which had been incubated on natural Conover loam soil or the clay loam soil was markedly reduced. For example, conidial germination was ca. 15 and 5% of the controls (percent germination in the controls was ca. 80%) on discs previously incubated for 8 hr on Conover loam and the clay loam soil, respectively, whereas discs from the model system containing the same amount of glucose supported over 90%. Inhibition was recorded for discs incubated for only 0.5 hr on either soil. Discs assayed after removal from the clay loam soil were more inhibitory than those from Conover loam soil. Thus in the R-B assay, removal of glucose from amended discs through microbial activity in the two soils did not account for all the inhibition.

A stimulatory effect was recorded when the R-B assay was performed using sterile Conover loam soil, whereas fungistasis in the clay loam soil was only partially annulled by sterilization (Figure 16B), even though the nutrients released during autoclaving were approximately equal to or greater than those released in Conover loam soil (Table 6). Autoclaving

Figure 16. Conidial germination of Thielaviopsis basicola on discs, initially amended to contain 0.1% glucose, after incubation for up to 24 hr A) in the model system (10 ml water/hr), and on natural Conover loam and the clay loam soil, and B) on water-saturated sand and sterile soils. Germination is expressed as the percent of that on untreated glucose-amended discs. Amended agar discs containing ^{14}C-glucose (ca. 3,000 cpm) were incubated exactly as non-radioactive discs to determine the percent glucose remaining.

Figure 16.


A
STANDARD
MODEL SYSTEM
CONOVER LOAM
CLAY-LOAM
B
CONOVER LOAM
WATER-SATURATED SAND
STANDARD
CLAY-LOAM
GERMINATION, % OF CONTROL
100
75
50
25
0
125
100
75
50
0
100
75
50
25
0
14C-GLUCOSE, % OF CONTROL

may have reduced the concentration of the heat-stable volatile inhibitor from the clay loam soil. These observations agree with those of Ko and Hora (52) who demonstrated the inhibitory nature of this particular clay loam soil following autoclaving or amendment of nutrients.

Table 6. Carbohydrates and amino compounds in natural and sterile Conover loam and the clay loam soil 1 day after remoistening the air dried soil.

Soil	Condition	µg/g air dried soil	
		Carbohydrate[a]	Amino Compounds[b]
		glucose eq	glycine eq
Conover loam	Natural	8.5	1.9
	Sterile[c]	352.9	45.8
Clay-loam	Natural	44.0	85.6
	Sterile	297.4	107.3

[a]Anthrone reagent.

[b]Ninhydrin reagent.

[c]Autoclaved at 121 C (15 psi) for 60 min.

Radiochemical purity of the ^{14}C-glucose remaining in amended agar discs incubated in the various treatments was determined to make certain that all radioactivity measured represented glucose.

No radioactive peaks or spots other than glucose were identified on paper or thin layer chromatograms, respectively. Moreover, no additional spots other than those present from untreated discs, were observed on TLC plates sprayed with sulfuric acid.

In the R-B assay, discs were inoculated immediately after removal from the various treatments. To test whether the inhibition was due to unstable or volatile materials, as reported for the clay loam soil, discs were incubated in a petri dish for 12 hr at 24 C prior to inoculation. The inhibition was not diminished by delaying inoculation for any of the treatments.

Cellulose-amended soil, 1-2 weeks after amendment, required addition of greater amounts of glucose than unamended soil in order to achieve the same level of germination of Fusarium solani f. sp. pisi chlamydospores (2). The elevated level of fungistasis was related to an increased rate of microbial utilization of glucose, but the possibility of greater amounts of inhibitory materials in the amended soil was not ruled out (3). Conover loam soil was amended with 2% (w/w) alfalfa hay particles (<40 mesh, 420 μ), incubated for two weeks, then assayed for the rate of glucose utilization and the

presence of inhibitory compounds using agar discs. Glucose was lost more rapidly from agar discs incubated on amended than unamended soil. On amended soil 4.0 hr were required for 50% (half-life) of the glucose to be lost, compared with 7.0 hr on unamended soil. The half-life of glucose was ca. 12.5 hr on sterile soil with and without amendment. The R-B assay was used to test whether the level of inhibitory compounds increased in soil amended with the plant residue. There was no significant difference between the levels of inhibition in amended and non-amended soils, indicating that alfalfa hay amendment did not stimulate production of greater amounts of inhibitory substances. This suggests that increased microbial activity was the major cause for elevated fungistasis in cellulose-amended soil reported by Adams et al. (3).

Fungistatic Substances in Soil

The metabolism of glucose lost from amended agar discs in the R-B assay may lead to microbial synthesis of fungistatic materials in soil, even though the radiochemical purity tests did not indicate the involvement of glucose metabolites in

the observed inhibition. To test for the existence of a preformed fungistatic substances in unamended soil, purified agar discs, first leached on the model system with glass distilled water (flow rate 60 ml/hr) for 24 hr to render them as nutrient-free as possible, were used. The discs were incubated on or over the surface of soil to trap non-volatile and volatile materials, respectively.

No substances, volatile or non-volatile, were detected in discs from Conover loam soil assayed with H. victoriae conidia or N. tetrasperma ascospores. Germination was reduced when discs were left on soil following inoculation with H. victoriae, but not when transferred to an empty petri dish. This reduction appeared to be caused by the microbial nutrient sink below the disc, as germination on discs similarly treated on the model system (10 ml water/hr) was correspondingly reduced. Conidia of H. victoriae germinated ca. 90% on discs removed after 12 hr incubation on sterile soil, compared with 75% on the untreated discs.

Agar discs incubated on or over the natural clay loam soil, then removed, inhibited germination of H. victoriae (Figure 17). Further decreases in germination occurred when the discs were left in situ

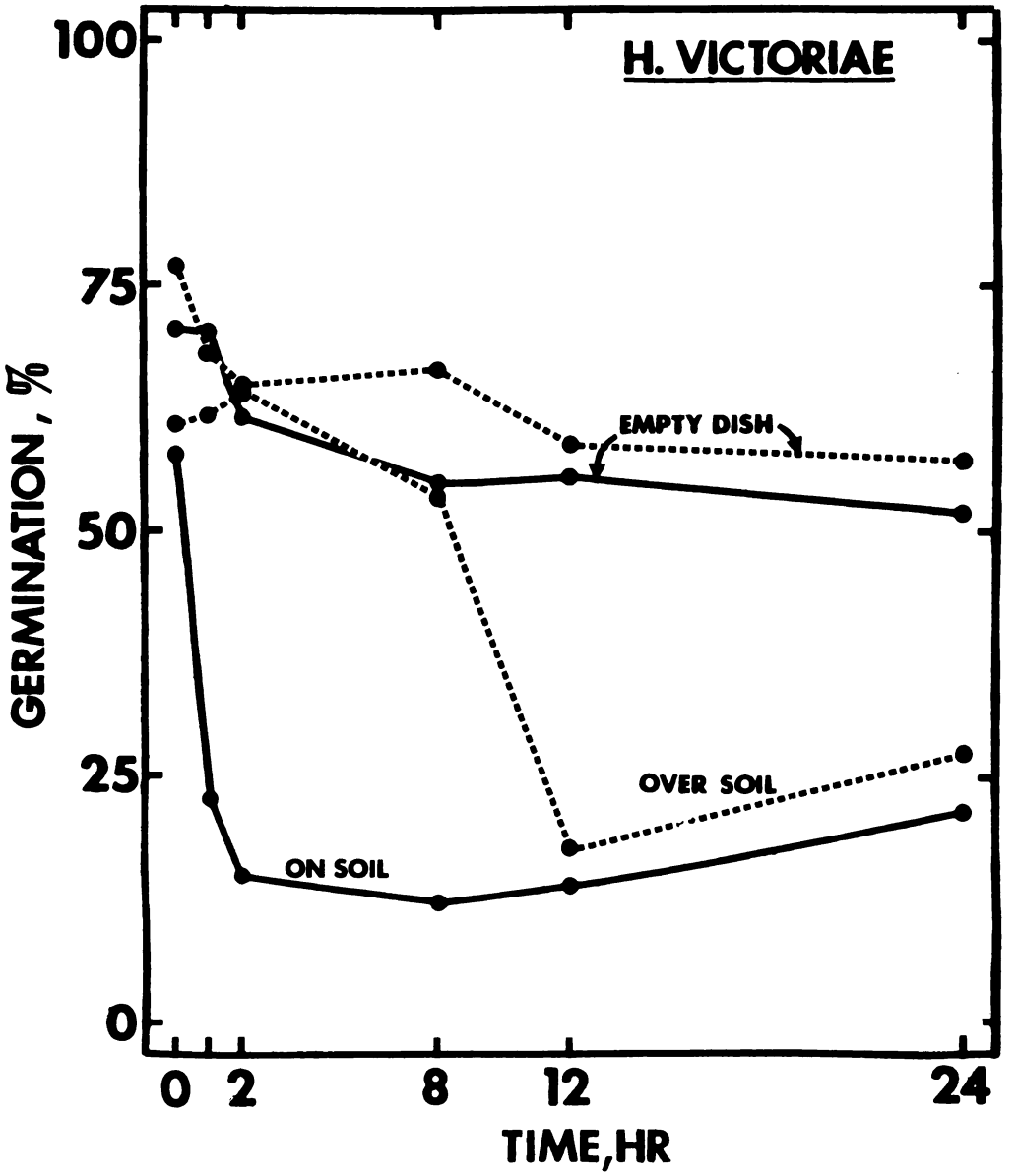


Figure 17. Germination of Helminthosporium victoriae conidia on purified agar discs that were incubated on (——) or over (•••) the natural clay loam soil for up to 24 hr before inoculation, and then either left on soil (on soil) or transferred to a petri dish (empty dish) for an additional 8 hr.

after inoculation. A volatile fungistatic factor was detected in the sterile as well as the natural clay loam soil. As in the R-B assay, discs incubated on the natural clay loam soil were more inhibitory than those from sterile soil. Similar results were obtained with discs inoculated with ascospores of N. tetrasperma.

For germination of T. basicola, the test organism in the R-B assay, glucose had to be added to the discs following their removal from soil or the model system. Discs were finally inoculated 12 hr after the addition of glucose, to test for the presence and stability of any inhibitor. Discs incubated on the clay loam soil then amended to contain 0.1 or 0.005% glucose inhibited germination, while discs on Conover loam soil or in the model system did not (Figure 18A), except that at 0.005% glucose a marked decrease in the germination of T. basicola conidia was noted at 24 but not 12 hr or earlier (Figure 18B). There was about a 50% reduction in germ tube lengths on discs incubated for 24 hr on Conover loam soil then assayed at 0.1% glucose.

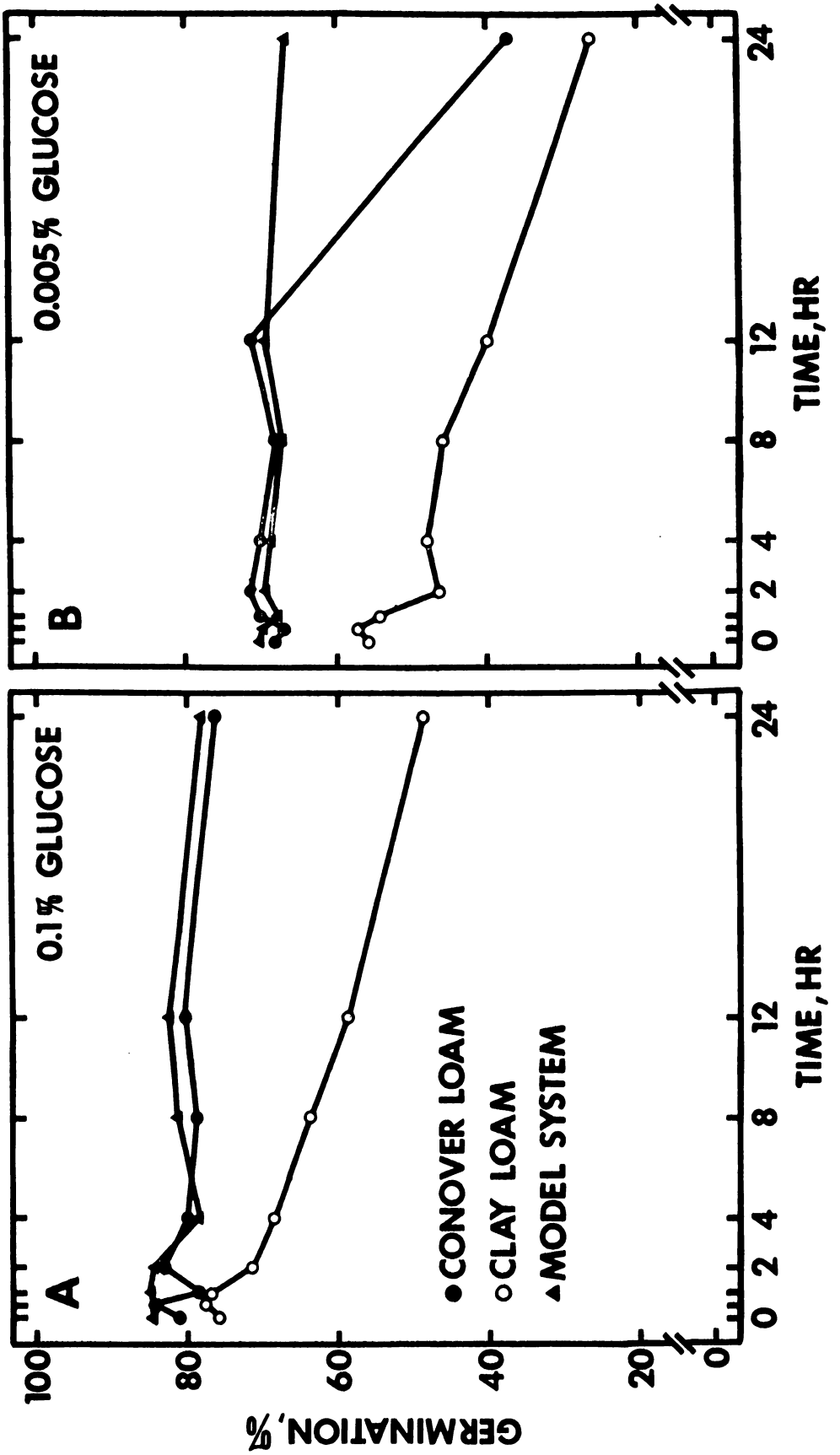


Figure 18. Germination of Thielaviopsis basiccla conidia on purified agar discs incubated in the model system (10 ml water/hr), on Conover loam soil, and on the clay loam soil, then removed and amended to contain A) 0.1% or B) 0.005% glucose before inoculation.

It was still possible that the reduction in germination and germ tube growth on Conover loam soil was due to microbially synthesized inhibitors in the agar, as the leached discs may contain traces of nutrients and/or the soil microorganisms may be able to utilize the agar itself. To avoid any microbial stimulation from materials in agar discs, nutritionally inert discs, prepared from polyacrylamide gel (PAG), were used in similar experiments. Germination of N. tetrasperma ascospores, which were completely insensitive to fungistasis in Conover loam soil, steadily decreased the longer PAG discs were incubated on the natural clay loam soil (Figure 19A). Further reductions in germination were recorded on discs first incubated on soil, then left in situ or placed over soil on a glass microscope slide after inoculation. Similar reductions occurred on discs bioassayed with H. victoriae conidia (Figure 19B). No inhibition of either test fungus occurred when PAG discs were treated in the same manner in sterile clay loam soil. As with agar discs, germination of H. victoriae conidia was not reduced by any treatment on Conover loam soil, except where discs were incubated on natural soil after inoculation (Figure

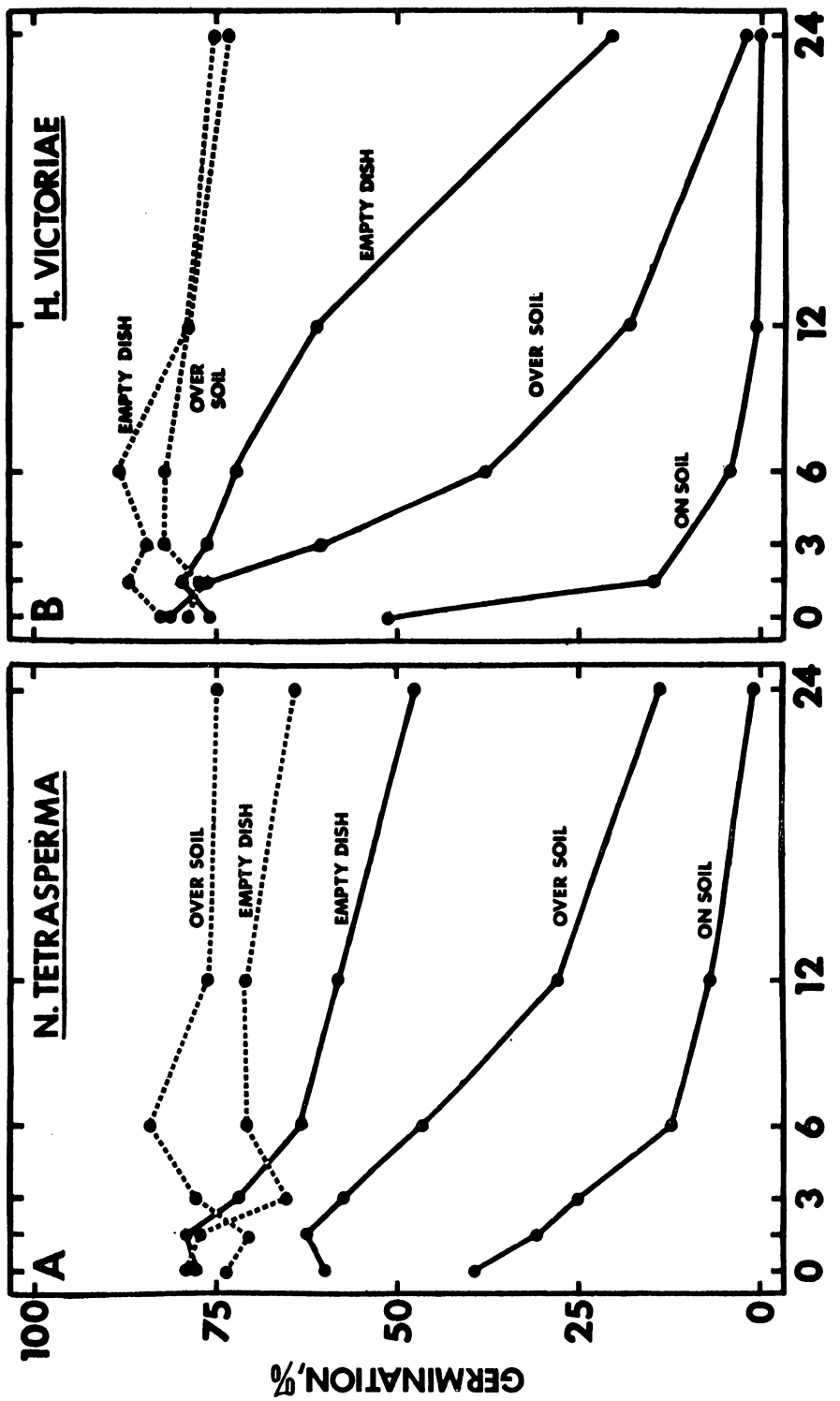


Figure 19. Germination of A) Neurospora tetrasperma ascospores and B) Helminthosporium victoriae conidia on polyacrylamide gel discs that were incubated on (——) or over (•••) natural clay loam soil for up to 24 hr before inoculation, and then either left on soil (on soil), placed on a glass microscope slide over soil (over soil), or transferred to a petri dish (empty dish) for an additional 8 hr.

20). Results using both agar and PAG discs demonstrated the presence of volatile and non-volatile fungistatic substances, which may be one in the same, in the clay loam soil, but provided little evidence for such in Conover loam soil. The inhibition of germination and germ tube growth of T. basicola on agar discs only after 24 hr incubation on Conover loam soil suggests that the fungistatic factor was synthesized to late, or if in a preformed state may be in too low a concentration to be effective in retarding germination of nutrient-independent spores.

There were, however, several differences in the results obtained with the two kinds of discs. For example, PAG discs were unable to trap the volatile inhibitor from the clay loam and they did not show the stimulatory effect of sterile Conover loam soil. Because of these differences in results, it seemed possible that the PAG discs were less permeable than the agar discs. Therefore, diffusion of glucose through discs of each type was compared. Two PAG discs were stacked one on top of the other, with purified agar discs handled similarly. A purified agar disc containing 0.1% ^{14}C-glucose (ca. 3,000 cpm), the donor disc, was placed atop

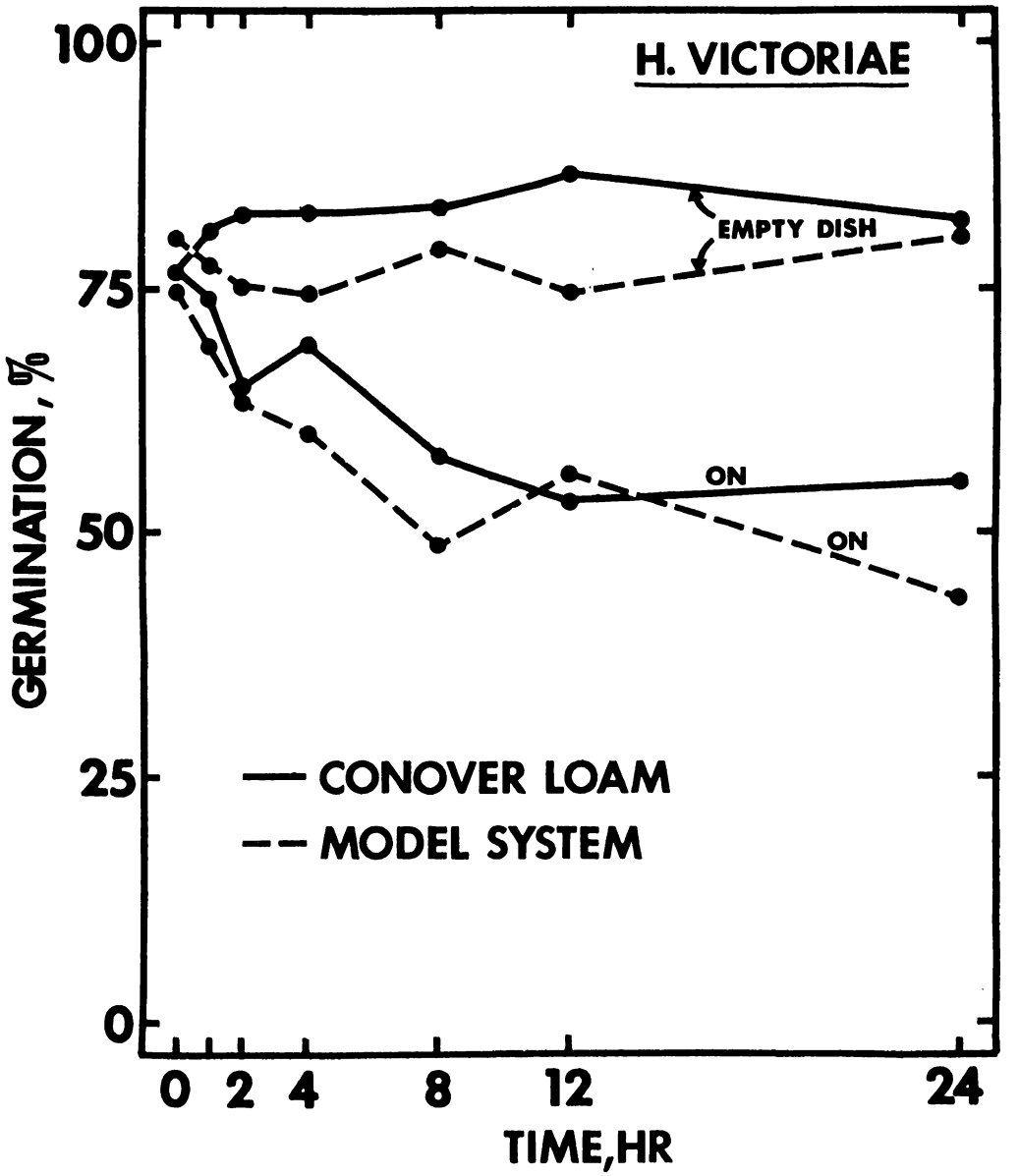


Figure 20. Germination of Helminthosporium victoriae conidia on polyacrylamide gel discs that were incubated on natural Conover loam soil (——) or in the model system (---) for up to 24 hr before inoculation, and then either left in place (on) or transferred to a petri dish (empty dish) for an additional 8 hr.

each stack. Each disc was separated one from the other by a Nuclepore membrane filter (0.4 μ). At predetermined intervals, the radioactivity in each disc was measured by liquid scintillation. Glucose was completely excluded from the PAG discs, even that disc adjacent to the donor. By contrast, an equal amount of radioactivity was found in all three agar discs after only 12 hr. This may explain the lack of germination stimulation from sterile Conover loam soil.

The rapid reduction in germination on agar and PAG discs incubated on the clay loam soil may reflect a direct effect of the alkaline pH of that soil. The pH of purified agar discs incubated on the surface of both soils was measured indirectly. After removing the discs from the soils, 50 μl of glass distilled water was incubated on the surface of each disc for 1 hr, then the pH of the water was determined with pH indicator paper. The pH of discs previously incubated on Conover loam soil did not change, whereas that of discs from the clay loam soil increased the longer the discs remained on that soil (Figure 21). Romine and Baker (77) reported only a slight decrease in the germination

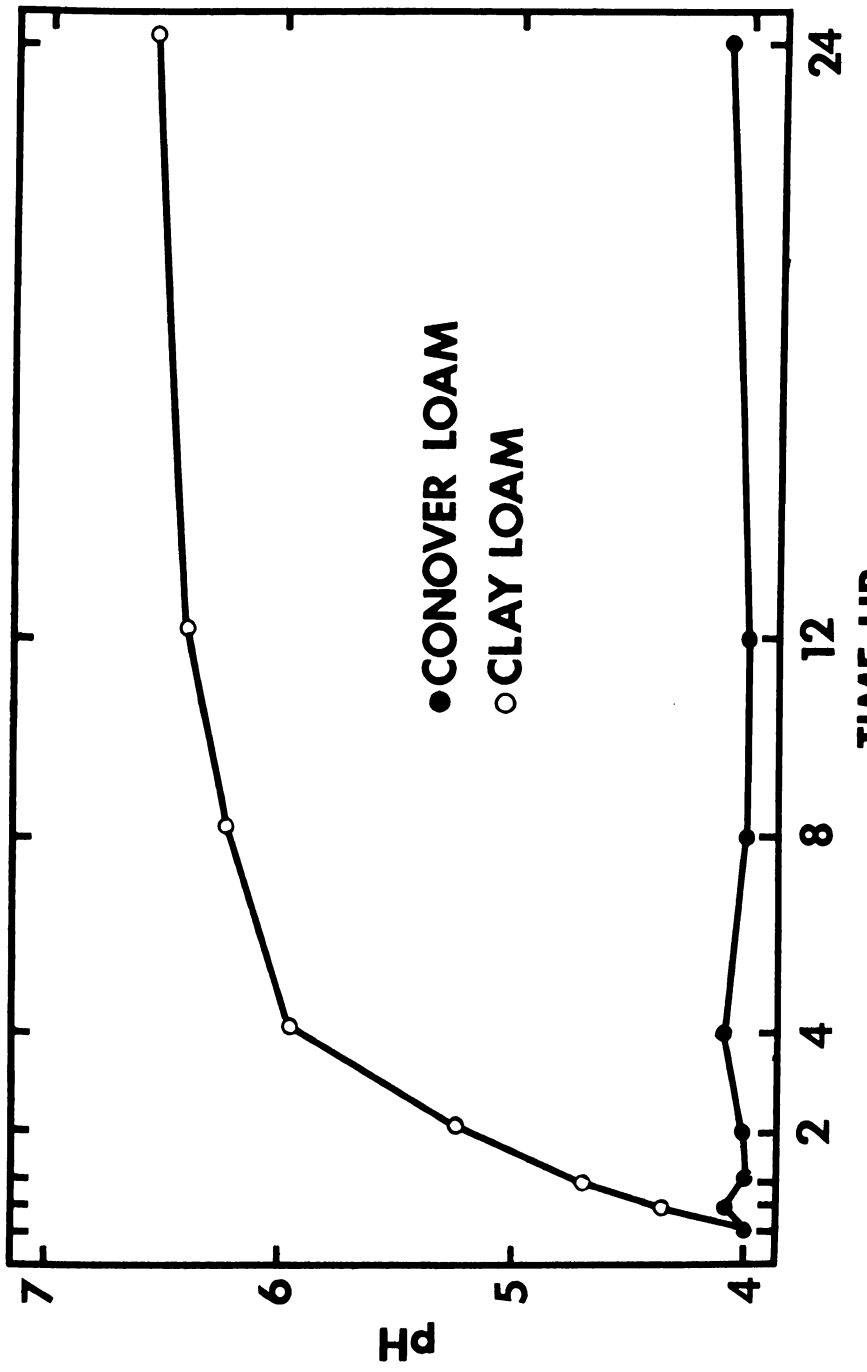


Figure 21. Determination of pH, with pH indicator paper, of 50 µl glass distilled water incubated for 1 hr on purified agar discs which were previously incubated for 24 hr on Conover loam soil and the clay loam soil.

of H. sativum conidia on buffered agar discs and in solutions with pH values similar to the pH 8.6 of the clay loam soil. Similarly, buffered agar discs and solutions adjusted to various pH values from 5.0-8.0 did not affect germination of either H. victoriae (Figure 22) and T. basicola (Figure 23) conidia. This lack of inhibition strongly suggests that hydrogen ion concentration per se, within this range, does not account for the inhibition observed on discs incubated on the clay loam soil. It is possible that metallic cations from this soil, which has a high salt content (77), could cause the high pH and be the inhibitory factor.

The presence of glucose did not completely annul either the non-volatile (Figure 16) or the volatile (52) fungistatic substances from the clay loam soil. Thus, to test the ability of nutrients to overcome the effect of the volatile inhibitor, purified agar discs amended with up to 0.1% glucose were incubated over both soils (200 g) and water-saturated sand for 24 hr before being inoculated in situ with conidia of T. basicola. Inoculated discs were incubated over the treatments for an additional 12 hr. The volatile inhibitor emanated from the clay loam soil reduced germination on discs

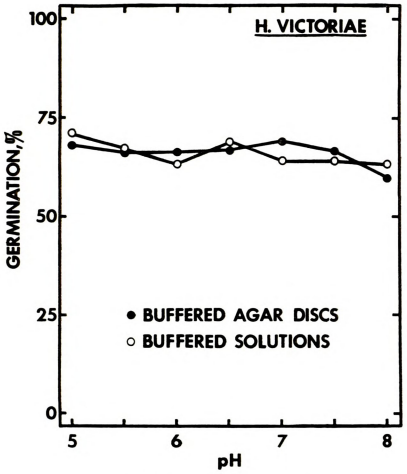


Figure 22. Germination of Helminthosporium victoriae conidia in solutions and on purified agar discs containing 0.1 M potassium phosphate buffers at various pH values.

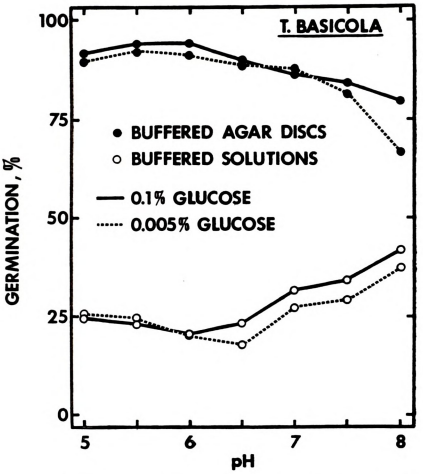


Figure 23. Germination of Thielaviopsis basicola conidia in solutions and on purified agar discs containing glucose and 0.1 M potassium phosphate buffers at various pH values.

at all nutrient concentrations (Figure 24), whereas germination on discs from over Conover loam soil corresponded with that on discs incubated over water-saturated sand.

Numerous attempts to negate fungistasis by the removal of volatile fungistatic substances, which may have been present, failed. When moist air was passed over either soil at a rate of ca. 75 ml/min conidia of H. victoriae on the soil surface did not germinate, although those on water-saturated sand treated identically germinated over 75% in 4 hr. Treating remoistened air-dried Conover loam and the clay loam soil in such a manner for one month did not allow any mold growth, and sclerotia of Sclerotium rolfsii picked from the surface of PDA-slant cultures, remained dormant on the surface of the aerated soils. Sclerotia incubated on water-saturated sand controls germinated within 3 days. Passing moist air over the surface of Australian soils allowed both copious mold growth and germination of S. rolfsii sclerotia (79).

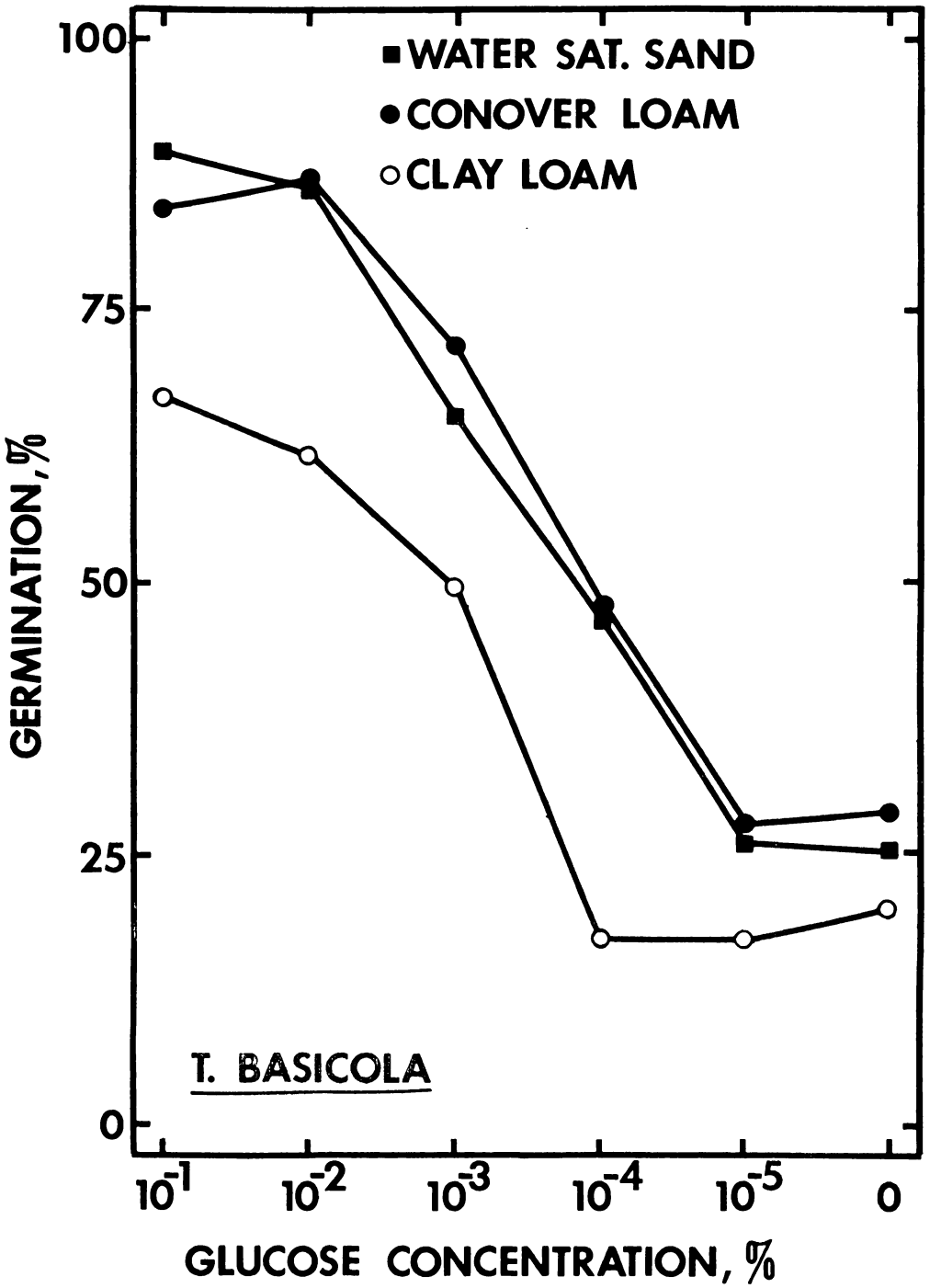


Figure 24. Germination of Thielaviopsis basicola conidia on glucose amended agar discs incubated over Conover loam soil, the clay loam soil, and water-saturated sand.

DISCUSSION

The research reported in this thesis confirmed the findings of others in this laboratory (43,54) and elsewhere (25,45,87), that propagules which will germinate in distilled water, fail to germinate when exposed to conditions of greater diffusion stress. This research also established the importance of nutrients in spore exudates in initiating germination, and of their loss in preventing it. Spore materials, including carbohydrates and amino acids, were exuded more rapidly during incubation on leached glass microbeads than without leaching. Losses occurring in non-leaching conditions were not sufficient to prevent germination, but when the diffusion gradient away from the spore was steep enough germination was inhibited. The bulk of the exuded materials were lost from H. victoriae conidia within twelve hours when spores were incubated in the model system, or when washed with successive aliquots of water in a membrane filter holder. Similarly, Jackson and Knight (45)

found that most of the glucose lost from unwashed conidia of H. sativum occurred within several minutes when washed with water in a membrane filter holder: they ruled out the source being traces of adhering media. When they washed conidia for four days the pattern of exudation was nearly identical to that found in the present research for H. victoriae (Figure 2). Interestingly, the nutrient-dependent conidia of T. basicola also exuded more materials under leaching than non-leaching conditions. This may explain why most conidia of this fungus remain viable for such a short time in natural soil (56,78,88). It is thought that continuous washing or leaching in a membrane filter holder or the model system, respectively, imitates the diffusion stress of the nutrient sink created by micro-organisms around the spore in soil. These findings support the proposal (54) that elevated microbial activity in the vicinity of the spore, stimulated by exuded energy yielding compounds, increases the diffusion gradient away from the spore, removing constituents normally available for germ tube synthesis. Blakeman (personal communication) suggested that the spore's energy reserves are insoluble and for germination they must first be

transformed into a soluble form, which may occur at a constant rate, and that it is the loss of these soluble materials at a rate faster than replacement that prevents germination.

Germination of H. victoriae and H. sativum conidia, as well as other nutrient-independent propagules (43), was inhibited in the model system when leached for periods up to 24 hr, but germinated freely when subsequently transferred to water- or buffer-saturated sand. In contrast, extended incubation in the model system or on soil converted conidia of H. victoriae and H. sativum to a nutrient-dependent condition; some of the conidia in the population were converted in only 12 hr. Apparently the endogenous energy supply required for germination in the absence of exogenous nutrients was depleted by such treatment. Further incubation for up to 28 days reduced the viability of H. sativum but not of H. victoriae. Several days incubation on either soil or in the model system were required to convert all the thick-walled spores of these two fungi, whereas thin-walled conidia may become nutrient-dependent more quickly. Ko and Lockwood (54) found that almost all conidia of Neurospora tetrasperma become dependent after only

12 hr exposure on soil or in the model system, and that the conversion was correlated with the loss of carbohydrates and amino acids. Viability data, however, was not provided in their report. Sztejnberg and Blakeman (87) reported the conversion of Botrytis cinerea conidia within 24 hr by leaching. Other nutrient-independent propagules may not undergo conversion, or are converted at a slower rate in a diffusion stress environment. For example, chlamydospores of T. basicola were still capable of germinating in water after extensive leaching (35,89).

The bulk of the exuded materials was not required to maintain the nutrient-independent status of H. victoriae conidia. This was also true for conidia of H. sativum (47). The rapidly lost materials may have been substances exuded during the formation of the spore and retained in the spore wall matrix. Exudation after the initial flush could represent the gradual depletion of the reserves which confer nutrient-independence, or the loss of soluble conversion products from an insoluble reserve, as suggested by Sztejnberg and Blakeman (87). Continued loss of this energy reserve, not only through exudation but also by

the spores own maintenance metabolism, would result in the conversion to nutrient dependence and finally loss of spore viability. Once spores are converted they behave like other nutrient-dependent propagules and germinate only upon the addition of an exogenous energy source. One mechanism which may prevent such rapid conversion and possible loss of viability would be the formation of chlamydospores within the conidia (67). Chlamydospore formation, however, was not observed in this study.

The importance of spore nutrients in germination was emphasized by the results of several experiments. The stimulation of germination of conidia resupplied with their own exudates in the model system strongly suggests that loss of such materials is responsible for the lack of germination where the diffusion gradient is steep enough to induce a sufficient degree of depletion. Stimulation by a very dilute solution of glucose and casein hydrolysate, or by exudates from another nutrient-independent spore implies that the germination stimulants are non-specific and may well be energy yielding compounds. Furthermore, nutrient-independent conidia took-up and metabolized some of their own exudates during germination under non-stress

conditions. The accumulation of exudates by H. victoriae conidia and their germination after conversion to nutrient dependency, clearly demonstrates the importance of exuded materials in germination. Likewise, Daly et al. (15) demonstrated the ability of Puccinia graminis f. sp. tritici uredospores to utilize exuded carbohydrates such as trehalose and mannitol during germination.

This research also provides further insight into the various mechanisms proposed to account for soil fungistasis. Whatever the mechanism for inhibition of nutrient-independent spores it must operate before the spores are irreversibly committed to producing a germ tube. Conidia of H. victoriae became insensitive to fungistasis in the model system and Conover loam soil by prior incubation for only 0.75-3.0 hr on water-saturated sand. By contrast, those spores which require an exogenous source of nutrients do not appear to have a point beyond which germination is insured (82,93). When the nutrient supply to nutrient-dependent spores was interrupted, by transfer to the model system or soil, progress toward formation of a germ tube ceased. Extended incubation in the model system or on soil resulted in reversal of the progress

made during the exposure to nutrients. Yoder and Lockwood (93) also found that incubation on Conover loam soil or water-saturated sand allowed nutrient-independent as well as nutrient-dependent spores to partially progress toward germination; such progress required only water and was irreversible. For instance, conidia of H. sativum required only 1.0 hr on sterile soil to germinate 50 percent after a one day exposure to natural soil, compared with 1.6 hr without prior incubation on soil.

Evidence obtained from this work and that of others supports the assumption in quantitative terms, that the model system imitates the microbial nutrient sink of soil; the evidence is as follows: i) The rates at which conidia of H. victoriae and H. sativum were converted to nutrient dependence in the model system and on Conover loam soil were similar. ii) Yoder and Lockwood (93) found that incubation of Penicillium frequentans conidia on each reversed to the same extent the progress made toward germination by previous exposure to nutrients. iii) They also found that ca. 50 percent of the ^{14}C-label in conidia, pulsed for 1 hr with radioactive glucose, was lost during subsequent incubation on either Conover loam soil or in the model system for

six days. iv) Both systems were unable to prevent germ tube formation in H. victoriae conidia in which germination had progressed to the point where they were no longer sensitive to soil fungistasis. v) Germination of ascospores of N. tetrasperma was not inhibited on either Conover loam soil or in the model system (54).

Not all results support the quantitative similarity between Conover loam soil and the model system. Glucose-amended paper (Lockwood, unpublished results) and agar discs lost glucose more rapidly on the model system than on Conover loam or the clay loam soil. In some experiments the diffusion of glucose from agar discs incubated on water-saturated sand, where nutrient-independent spores readily germinate, was greater than on natural soil. This indicates the loss of materials from spores may differ in rate from loss from various kinds of inaminate carriers. The disc assays might be useful in obtaining comparative estimates of the microbial sink in various soils, as presently used, but may not be valid for calibrating the model system. Data for the latter purpose may best be acquired by the use of propagules themselves.

Further work is necessary to more accurately measure the diffusion stress created by each. Such assays will have to be qualitative, as well as quantitative, as the diffusion sink in soil may be more specific for certain substances than the model system. However, from the limited biological data available, the diffusion sinks in soil and the model system may be more parallel than the disc assays indicate.

If the loss of exudates from spores in the model system equalled or exceeded that on Conover loam soil, it is difficult to explain the greater inhibition occurring on soil solely in terms of nutrient deprivation. The R-B assay provided evidence that the microbial nutrient sink may not be the only fungistatic mechanism operative in Conover loam soil. Correlations between the decrease in nutrients because of microbial utilization and decreasing fungal spore germination have been reported previously (3,27,54,83), but the R-B assay was the first attempt to quantitatively assess germination with respect to the available nutrients. The assay indicated that conidial germination of T. basicola on glucose-amended agar discs incubated on soil was much less than expected for the concentration of glucose present at the

time of bioassay. However, results obtained with the R-B assay are subject to their own interpretive problems. Inputs of large amounts of glucose to the soil were unavoidable in the R-B assay: these amendments could have provided the energy needed to stimulate microbial synthesis of fungistatic substances which might not be produced from spore exudates. However, the inability to detect radioactive metabolites of glucose in discs from the R-B assay suggests that such compounds were not produced, or were present in biologically active but chemically undetectible concentrations. The radiochemical purity test may have overlooked volatile inhibitors.

An attractive explanation for fungistasis of nutrient-independent spores is that their exudates provide the energy required for the production of antibiotics adjacent to the spore. Lingappa and Lockwood (59) were unable to find inhibitory microbial metabolites of exudates from H. victoriae or Glomerella cingulata conidia. It seems unlikely that soil fungi could contribute to antibiosis in the sporosphere, in that the propagules of such producers must themselves germinate rapidly within the sporosphere of the nutrient-independent spore.

Soil bacteria and actinomycetes could synthesize inhibitors, but even under the most favorable conditions, these microorganisms have a lag period before antibiotics can be detected (73). Moreover, the production of inhibitory substances in any quantity requires large amounts of energy which are generally not available in soil, thus their continuous synthesis in soil is unlikely.

Alternatively, the exudation of readily metabolized materials may, through "priming action," allow soil microbes to degrade more resistant organic matter such as humus or lignin. Decomposition products of lignicolous materials have been shown to inhibit spore germination (58). Even if spore exudates stimulate microorganisms in the sporosphere capable of producing antibiotics or of degrading resistant organic matter to toxic subunits, the question still remains, whether such substances are produced in sufficient quantity to act before nutrient-independent spores become committed to germination. However, once conidia are converted to nutrient-dependency, microbially synthesized inhibitors would not be necessary, as dormancy would be maintained by the lack of an energy

substrate. Concurrent with conversion, reduced exudation would markedly lower the energy available for the synthesis of inhibitors.

The use of exhaustively leached agar discs and polyacrylamide gel discs on soil was intended to eliminate the possibility of microbial synthesis of inhibitors by removing the energy source for their production. Using these discs, no volatile or non-volatile substances inhibitory to H. victoriae conidia or N. tetrasperma ascospores were detected in Conover loam soil. The inhibition of T. basicola on unamended discs occurring after 24 hr incubation on Conover loam soil, but not earlier, may be due to microbial metabolism of the agar itself to produce toxic materials in sufficient concentration to affect this fungus, but not the other two test organisms.

The clay loam soil was used in this study for comparative purposes because it contains a known volatile fungistatic factor (36). In various experiments this soil was shown to be more inhibitory than the Conover loam soil. For example, it was able to prevent germination of N. tetrasperma ascospores, which are insensitive to microbial fungistasis (54).

Also, R-B assay discs from this soil were more inhibitory than those from Conover loam, even though the microbially induced nutrient sink in the clay loam soil was less. Inhibition by both volative and non-volatile substances was obtained with the clay loam soil. The inhibitory material, however, may have been one in the same. Low hydrogen ion concentration per se in this soil was not responsible for the non-volatile inhibition, although various basic cationic species which could account for the alkalinity of this high salt content soil may have been involved. Ko and Hora (51) submitted that an aluminum ion appears to be the fungitoxin in some acid Hawaiian soils, however, this seems unlikely for the clay loam soil where the high pH would precipitate aluminum ions as aluminum hydroxide. Dobbs and Gash (21) reported that the cause of residual fungistasis in certain alkaline dune sands may be iron ions, the effect of which was partially annulled by lowering the pH to allow chelation with organic matter.

Explanations for the inhibition based on alkaline cations would not extend to the volatile fungistatic factor. It seems possible that ammonia might be the volatile compound, as it is extremely

toxic to fungal spores at very low concentrations (66), and would be readily emanated from alkaline soils. Lewis (personal communication) has found that ammonia liberated from decomposing crop residues in soil deformed Rhizoctonia solani hyphae. Ko and Hora (53, and personal communication) have identified ammonia produced from ammonium chloride as the volatile fungistatic substance in an alkaline Hawaiian soil.

Although no volatile fungistatic factor was detected in Conover loam soil, using methods capable of detecting such materials in the clay loam soil, Baker and his co-workers have found a volatile inhibitor in the Conover loam soil (personal communication).

The microbial nutrient sink alone may account for complete inhibition of nutrient-independent propagules. The microbially induced nutrient sink was established almost immediately which points out that the microorganisms are 'poised' and ready to metabolize an energy source as soon as it becomes available. Sztejnberg and Blakeman (85), submit that bacteria may increase the concentration gradient away from spores by the accumulation of exuded spore materials into their polysaccharide sheathes. The

passive establishment of a diffusion sink by this mechanism in addition to that provided by microbial activity might increase the diffusion stress on the spore. The loss of nitrogenous compounds, in addition to the exudation of carbohydrates, may accelerate the latter's rate of utilization by microbes. Glucose was found to be metabolized at a faster rate in soil when nitrogen and phosphorus were added (85). Fungistasis of Botrytis cinerea conidia on leaf surfaces was though to be due to removal of spore exudates by bacteria (10). Droplets containing spores previously incubated on leaves were fungistatic only when the bacteria were present in the droplet. Furthermore, droplet filtrates were stimulatory rather than inhibitory, demonstrating the absence of inhibitors from bacteria or the leaf. Conidial germination of B. cinerea was likewise inhibited in a model system similar to that used in the present research (87). Calculations made from the data of Blakeman and Fraser (10) indicate that fewer than one bacterium per conidium prevented germination. Similar calculations from the data of Lingappa and Lockwood (59) showed that 1-4 X 10^3 bacteria per conidium completely inhibited germination of H. victoriae and G. cingulata, while only one-tenth

that number were needed to inhibit some of the conidia of the two test fungi. Filtrates of the bacteria-spore suspensions were not inhibitory to either fungus.

Germination and the production of inhibitors of microorganisms are controlled by the availability of nutrients, which in soil are in chronically short supply. The introduction of a nutrient-independent spore, or a dependent one for that matter, into soil may provide a temporary source of energy for microbial activity around that spore, stimulating an increase in the nutrient sink, followed by the production of inhibitors. It seems unlikely that microbially produced inhibitors, especially volatile inhibitors, are involved in fungistasis in unamended soil, because the energy required for synthesis on a continuous basis to maintain inhibitory concentrations is not available (31). Moreover, diffusion rates of gaseous compounds in aerobic soils are high enough to prevent their accumulation (Tiedje, personal communication).

Thus, for germination to occur sufficient exogenous nutrients must be added to soil, not only to meet the requirements of the spore but also to overcome any fungistatic factors. Fungistasis may be

of significant survival value in that nutrient-independent propagules would only germinate when sufficient substrate was available for colonization or to support production of new propagules.

In summary, the main cause for inhibition of nutrient-independent propagules in a majority of soils is most probably due to exudation of propagule constituents beyond a critical rate. The critical rate is maintained in those soils by a steep diffusion gradient away from the spore as a result of intense microbial competition for exuded nutrient materials. Fungistasis in Conover loam soil seems to be mainly caused by such a microbial nutrient sink, however, some microbially produced inhibitors synthesized from the nutrients in spore exudates may play a role in some instances. The latter providing a possible explanation for the less complete inhibition in the model system compared with that on soil. In contrast, the residual fungistatic factor in the clay loam soil from Colorado, and possibly other alkaline soils, likely accounts for a significant portion of the inhibition in such soils. Moreover, the toxic nature and the volatility of this factor may also

reduce the activity of all soil microorganisms, thus decreasing the contribution to fungistasis made by the microbial nutrient sink in the clay loam soil.

APPENDIX

APPENDIX

A permanent record of the results from experiments which a) led to the modification of the paper disc technique used to estimate the microbial nutrient sink in soil, and b) further defined parameters of the model system are made in this appendix.

Modification of the Paper Disc Technique for Assaying the Microbial Nutrient Sink of Soil

As the assay was originally developed by Lockwood (unpublished results) Whatman filter paper antibiotic assay discs (1/2 in diam), each amended with 90 µl of a 0.05% glucose solution were used. The glucose remaining in the discs after incubation on soil was determined with the Glucostat reagent, and was expressed as the percent of that initially in the discs. The increased rate of loss on natural soil as compared with that on sterile soil was thought to be due to an increased diffusion gradient

due to microbial activity beneath the discs. In an attempt to increase the proportion of glucose lost by microbial activity to that lost by diffusion in the absence of the microbial nutrient sink the initial amount was decreased. Reducing the initial amount to 90 µl of a 0.0005% glucose solution markedly increased the sensitivity of the assay as shown in Figure 9. Since each disc contained only 0.45 µg glucose, a quantity well below the range of the Glucostat color test, radioactive (^{14}C) glucose was used.

Various methods of measuring the radioactivity remaining in the discs were found useful. A) To use the gas-flow counter, discs upon removal from soil, were first dried in a stainless steel planchet on a hot plate at 60 C, with that side formerly in contact with the soil down. Acceptable results were obtained even though counts represented only a small fraction of the ^{14}C-glucose remaining in the discs. Most of the Beta particles were absorbed by the paper. Considerable variation occurred when the side originally in contact with the soil was counted. B) Liquid scintillation counting in which dried discs were placed into vials of scintillation fluid was less variable than

gas-flow methods. Over 80 percent of the radioactivity in the discs was detected. C) The most accurate method, although the most time consuming, was the determination of ^{14}C-glucose indirectly by measuring the $^{14}CO_2$ produced upon combustion of amended discs.

Whatman discs are not available in the United States. However, Whatman discs, kindly supplied by S.D. Garrett, were compared with similar discs made by Schleicher and Schuell (S & S). Glucose was lost more rapidly from the S & S than from Whatman discs incubated on either natural or sterile Conover loam soil (Figure 25). In a similar experiment, after only one minute on soil, approximately 18% of the glucose had already diffused from the S & S discs, while essentially all the glucose remained in the Whatman discs. S & S discs, although the same dimensions as the Whatman discs, weighed only two-thirds as much. The greater bulk of the Whatman discs may account for the better retention of the glucose solutions. It is important therefore, to use discs from a single source.

Another carrier for glucose was Millipore membrane filters (13 mm diam, 0.22 μ). Each membrane

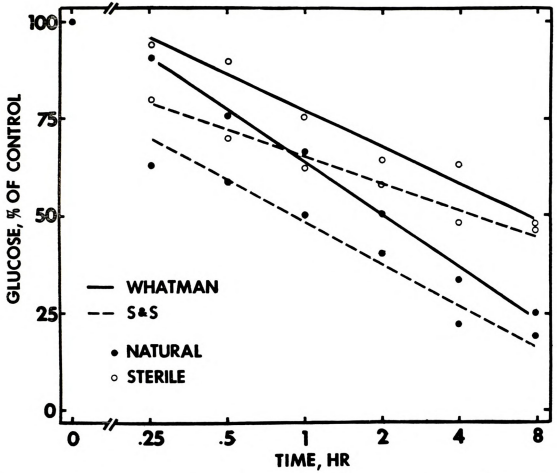


Figure 25. Loss of glucose from Whatman and Schleicher and Schuell (S & S) paper discs incubated on natural and sterile Conover loam soil. Each disc initially contained 90 µl of a 0.05% glucose solution, with the amount remaining determined by the Glucostat reagent.

filter was amended with 15 µl of a 0.005% ^{14}C-glucose solution (5.0 µCi/ml). The glucose remaining in each disc was determined by either the gas-flow counter, or dissolving the membrane filter in scintillation fluid and counting. Glucose was lost very rapidly from membranes incubated on the soil, and the activity of the microbial nutrient sink was detected within minutes (Figure 26).

Characteristics of the Model System

The rates of glucose loss from agar discs on soil and in the model system were compared. Agar, rather than paper discs, was used as a carrier for glucose because the former were used in the R-B assay. The diffusion sink in the model system exceeded that in natural Conover loam or the clay loam soil (Figure 15). For example, the half-life of glucose in the model system was ca. 1.0 hr compared with 7.5 and 9.0 hr for natural Conover loam and the clay loam soil, respectively. Thus, several alterations in the model system were tested in an attempt to make the rate of loss of glucose more closely imitate that of natural soil.

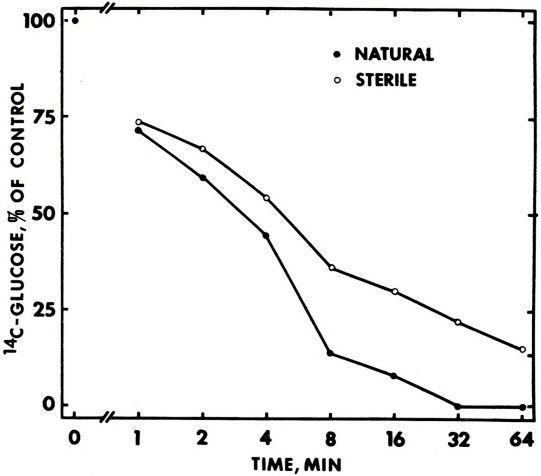


Figure 26. Loss of glucose from Millipore membrane filters (13 mm diam, 0.22 μ) to which 15 μl of a 0.005% ^{14}C-glucose solution (5.0 μCi/ml) had been added prior to incubation on natural and sterile Conover loam soil. Discs were dissolved in scintillation fluid and counted.

Three model systems were operated simultaneously at different flow rates to determine whether this factor would alter the rate of loss. The half-life of glucose was about equal for discs leached with water at flow rates of 8.9, 18.1 and 28.4 ml/hr. Leaching with buffer solution at 18.1 ml/hr resulted in a half-life similar to water at the same flow rate.

Reducing the diameter of the glass beads in the bed of the leaching dish increased the half-life of glucose only slightly. Sand, or glass beads of 470, 200 and 100 μ diam gave half-lives of ca. 70, 110, 100, and 120 min, respectively. Even this slight increase in half-life did not approach that for natural soil.

A change in either the flow rate or the size of the particles in the bed had little effect on the leaching characteristics of the model system with respect to loss of glucose from agar discs. It appears that any movement of the leaching medium at flow rates faster than ca. 9 ml/hr must keep this area directly below the disc devoid of glucose, thus maintaining a maximum diffusion gradient against distilled water. Using the paper

disc technique, Lockwood (unpublished results) also found that flow rates had a minimal effect on the rate of glucose loss as measured by the slope of the line; calculated for readings made between 0.5 and 4 hr, but had a greater effect on half-life. The half-life for natural soil was approximated when paper discs were leached in the model system with buffer at ca. 10 ml/hr.

LIST OF REFERENCES

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1. Abeles, F. B., L. E. Craker, L. E. Florrence, and G. R. Leather. 1971. Fate of air pollutants: Removal of ethylene, sulfur dioxide, and nitrogen dioxide by soil. Science 173:914-916.

2. Adams, P. B., G. C. Papavizas, and J. A. Lewis. 1968. Survival of root-infecting fungi in soil. III. The effect of cellulose amendment on chlamydospore germination of Fusarium solani f. sp. phaseoli in soil. Phytopathology 58:373-377.

3. Adams, P. B., J. A. Lewis, and G. C. Papavizas. 1968. Survival of root-infecting fungi in soil. IV. The nature of fungistasis in natural and cellulose-amended soil on chlamydospores of Fusarium solani f. sp. phaseoli. Phytopathology 58:378-383.

4. Alexander, M. 1961. Introduction to soil microbiology. Wiley, New York. p. 472.

5. Babiuk, L. A. and E. A. Paul. 1969. The use of fluorescin isothiocyanate in the determination of the bacterial biomass of grassland soil. Can. J. Microbiol. 16:57-62.

6. Balis, C. 1973. The nature of volatile inhibitors involved in soil fungistasis. Abstracts of Papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

7. Balis, C. and V. Kouyeas. 1968. Volatile inhibitors involved in soil mycostasis. Ann. Inst. Phytopath. Benaki, N. S. 8:145-149.

8. Basmith, M. and O. Vaartaja. 1973. Inhibition of Pythium ultimum by extracts from soils. Abstracts of Papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

9. Bishop, D. H. L., J. R. Claybrook, S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. J. Mol. Biol. 26:373-387.

10. Blakeman, J. P. and A. K. Fraser. 1971. Inhibition of Botrytis cinerea spores by bacteria on the surface of chrysanthemum leaves. Physiol. Pl. Path. 1:45-54.

11. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279-285.

12. Brian, P. W. 1960. Antagonistic and competitive mechanisms limiting survival and activity of fungi in soil. Pages 115-129 in D. Parkinson and J. S. Waid, eds. The ecology of soil fungi. Liverpool Univ. Press, Liverpool.

13. Brown, Margaret E. 1973. Soil bacteriostasis: limitation in growth of soil and rhizosphere bacteria. Can. J. Microbiol. 19:195-199.

14. Chinn, S. H. F. 1967. Differences in fungistasis in some Saskatchewan soils with special reference to Cochliobolus sativus. Phytopathology 57:224-226.

15. Daly, J. M., H. W. Knoche, and M. V. Wiese. 1967. Carbohydrate and lipid metabolism during germination of uredospores of Puccinia graminis tritici. Plant Physiol. 42:1633-1642.

16. Dennis, C. and J. Webster. 1971. Antagonistic properties of species-groups of Trichoderma. I. Production of non-volatile antibiotics. Trans. Brit. Mycol. Soc. 57:25-39.

17. Dennis, C. and J. Webster. 1971. Antagonistic properties of species-groups of Trichoderma. II. Production of volatile antibiotics. Trans. Brit. Mycol. Soc. 57:41-48.

18. Dobbs, G. C. 1966. Soil-spores of the fungi. Proc. Welsh Soils Discussion Group No. 7 p. 4-13.

19. Dobbs, G. C. and Joan Bywater. 1957. Studies in soil mycology I. Grt. Brit. Forestry Comm., Rept. on Forest Res. p. 92-94.

20. Dobbs, G. C. and Nancy C. Carter. 1963. Studies in soil mycology VI. Mycostasis in soil. Grt. Brit. Forestry Comm., Rept. on Forest Res. p. 103-112.

21. Dobbs, G. C. and M. J. Gash. 1965. Microbial and residual mycostasis in soils. Nature 207:1354-1356.

22. Dobbs, G. C. and W. H. Hinson. 1953. A widespread fungistasis in soils. Nature 172:197-199.

23. Dobbs, G. C., W. H. Hinson and Joan Bywater. 1960. Inhibition of fungal growth in soils. Pages 130-143 in D. Parkinson and J. S. Waid, eds. The ecology of soil fungi. Liverpool Univ. Press, Liverpool.

24. Doran, W. L. 1922. Effect of external and internal factors on germination of fungous spores. Bull. Torrey Botan. Club 49:313-340.

25. Ematty, D. A. and R. J. Green, Jr. 1969. Fungistasis and the behavior of microsclerotia of Verticillium albo-atrum in soil. Phytopathology 59:1590-1595.

26. Farley, J. D. and J. L. Lockwood. 1968. The suppression of actinomycetes by PCNB in culture media used for enumerating soil bacteria. Phytopathology 58:714-715.

27. Farley, J. D. and J. L. Lockwood. 1969. Reduced nutrient competition by soil microorganisms as a possible mechanism for pentachloronitrobenzene-induced disease accentuation. Phytopathology 59:718-724.

28. Garrett, S. D. 1970. Pathogenic root-infecting fungi. Cambridge Univ. Press, Cambridge. p. 294.

29. Gilbert, R. G. and R. G. Linderman. 1971. Increased activity of soil microorganisms near sclerotia of Sclerotium rolfsii in soil. Can. J. Microbiol. 17:557-562.

30. Goddard, D. R. 1935. The reversible heat activation inducing germination and increased respiration in the ascospores of Neurospora tetrasperma. J. Gen. Physiol. 19:45-69.

31. Gray, T. R. G. and S. T. Williams. 1971. Microbial productivity in soil. Pages 255-287. in D. E. Hughes and A. H. Rose, eds. Symposia of the Society for General Microbiology. No. XXI. Microbes and biological productivity. Cambridge Univ. Press, Cambridge.

32. Gray, T. R. G. and S. T. Williams. 1971. Soil Micro-organisms. Hafner, New York. p. 240.

33. Griffin, G. J. 1962. Production of fungistatic effect by soil microflora in autoclaved soil. Phytopathology 52:90-91.

34. Griffin, G. J. 1964. Long-term influence of soil amendments on germination of conidia. Can. J. Microbiol. 10:605-612.

35. Hawthorne, B. T. and P. H. Tsao. 1969. Inadequacy of the nutrient hypothesis to explain soil fungistasis in relation to chlamydospores of Thielaviopsis basicola. Phytopathology 59:1030 (abstr.).

36. Hora, T. S. and R. Baker. 1970. A volatile factor in soil fungistasis. Nature 225:1071-1072.

37. Hora, T. S. and R. Baker. 1972. Extraction of a volatile factor from soil-inducing fungistasis. Phytopathology 62:1475-1476.

38. Hora, T. S. and R. Baker. 1972. Soil fungistasis: microflora producing a volatile inhibitor. Trans. Brit. Mycol. Soc. 59:491-500.

39. Hora, T. S. and R. Baker. 1973. Non-biological generation of a volatile fungistatic factor in soil. Abstracts of Papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

40. Hsu, S. C. 1971. Sensitivity of fungal hyphae to soil fungistasis. Ph.D. Thesis, Michigan State University. p. 79.

41. Hsu, S. C. and J. L. Lockwood. 1969. Mechanisms of inhibition of fungi in agar by streptomycetes. J. Gen. Microbiol. 57:149-158.

42. Hsu, S. C. and J. L. Lockwood. 1973. Chlamydospore formation in Fusarium in sterile salt solutions. Phytopathology 63:597-602.

43. Hsu, S. C. and J. L. Lockwood. 1973. Soil fungistasis: Behavior of nutrient-independent spores and sclerotia in a model system. Phytopathology 63:334-337.

44. Jackson, R. M. 1958. An investigation of fungistasis in Nigerian soils. J. Gen. Microbiol. 18:248-258.

45. Jackson, R. M. and R. A. Knight. 1973. Conidial germination of Cochliobolus sativus on soil and under artificial nutrient stress. Abstracts of Papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

46. Jones, J. P. and J. P. Snow. 1965. Amino acids released during germination of ^{35}S-labelled crown rust spores. Phytopathology 55:499. (abstr.).

47. Knight, R. A. 1971. Ph.D. Thesis, University of Surrey, Guildford, Surrey, England.

48. Kouyeas, V. and C. Balis. 1968. Influence of moisture on the restoration of mycostasis in air-dried soils. Annales de l'Institut Phytopathologique Benaki N. S. 8:123-144.

49. Ko, W. H. 1966. Mechanism of Soil Fungistasis. Ph.D. Thesis, Michigan State University. p. 66.

50. Ko, W. H. and F. K. Hora. 1971. Fungitoxicity of certain Hawaiian soils. Soil Science 112:276-279.

51. Ko, W. H. and F. K. Hora. 1972. Identification of an Al ion as a soil fungitoxin. Soil Science 113:42-45.

52. Ko, W. H. and F. K. Hora. 1972. The nature of a volatile inhibitor from certain alkaline soils. Phytopathology 62:573-575.

53. Ko, W. H. and F. K. Hora. 1973. Characteristics of a volatile inhibitor isolated from an alkaline soil. Abstracts of Papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

54. Ko, W. H. and J. L. Lockwood. 1967. Soil fungistasis: Relation to fungal spore germination. Phytopathology 57:894-901.

55. Ledingham, R. J. and S. H. F. Chinn. 1955. A flotation method for obtaining spores of Helminthosporium sativum from soil. Can. J. Bot. 33:298-303.

56. Linderman, R. G. and T. A. Toussoun. 1967. Behavior of chlamydospores and endoconidia of Thielaviopsis basicola in nonsterilized soil. Phytopathology 57:729-731.

57. Lingappa, B. T. and J. L. Lockwood. 1961. The nature of the widespread soil fungistasis. J. Gen. Microbiol. 26:473-485.

58. Lingappa, B. T. and J. L. Lockwood. 1962. Fungitoxicity of lignin monomers, model substances, and decomposition products. Phytopathology 52:295-299.

59. Lingappa, B. T. and J. L. Lockwood. 1964. Activation of soil microflora by fungus spores in relation to soil fungistasis. J. Gen. Microbiol. 35:215-227.

60. Lingappa, Y. and J. L. Lockwood. 1961. Chitin media for selective isolation and culture of Actinomycetes. Phytopathology 52:317-323.

61. Lloyd, A. B. 1969. Behavior of Streptomycetes in soil. J. Gen. Microbiol. 56:165-170.

62. Lockwood, J. L. 1959. Streptomycin as a cause of natural fungitoxicity of soils. Phytopathology 49:327-331.

63. Lockwood, J. L. 1964. Soil fungistasis. Ann. Rev. Phytopathology 2:341-362.

64. Lockwood, J. L. and B. T. Lingappa. 1963. Fungitoxicity of sterilized soil inoculated with soil microflora. Phytopathology 53:917-920.

65. Lukens, R. J. 1960. Conidial production from filter paper cultures of Helminthosporium vagans and Alternaria solani. Phytopathology 50:867-868.

66. McCallan, S. E. A. and T. R. Weedon. 1940. Toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulfide, and sulfur dioxide gases. II. Fungi and bacteria. Contrib. Boyce Thomp. Inst. 11:331-342.

67. Meronuck, R. A. and E. H. Pepper. 1968. Chlamydospore formation in conidia of Helminthosporium sativum. Phytophathology 58:866-867.

68. Moore, S. and W. H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211:907-913.

69. Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 107:254-255.

70. Park, D. 1961. Morphogenesis, fungistasis, and cultural staling in Fusarium oxysporum Snyder and Hansen. Trans. Brit. Mycol. Soc. 44:377-390.

71. Pramer, D. and E. L. Schmidt. 1964. Experimental soil microbiology. Burgess, Minneapolis. p. 106.

72. Pratt, R. and J. Dufrenoy. 1949. Antibiotics. Lippincott, Philadelphia. p. 255.

73. Prescott, S. C. and C. G. Dunn. 1959. Industrial microbiology. McGraw-Hill, New York. p. 945.

74. Pringle, R. B. and R. P. Scheffer. 1963. Purification of the selective toxin of Periconia circinata. Phytopathology 53:785-787.

75. Robinson, P. M. and M. K. Garrett. 1969. Identification of volatile sporostatic factors from cultures of Fusarium oxysporum. Trans. Brit. Myco. Soc. 52:293-299.

76. Romine, M. and R. Baker. 1972. Properties of a volatile fungistatic factor in soil. Phytopathology 62:602-605.

77. Romine, M. and R. Baker. 1973. Soil fungistasis: evidence for an inhibitory factor. Phytopathology 63:756-759.

78. Schippers, B. 1970. Survival of endoconidia of Thielaviopsis basicola in soil. Neth. J. Pl. Path. 76:206-211.

79. Smith, A. M. 1973. Ethylene as a cause of soil fungistasis. Nature 246:311-313.

80. Smith, K. A., J. M. Bremner, and M. A. Tabatabi. 1973. Sorption of gaseous atmospheric pollutants by soils. Soil Science 116:313-319.

81. Sokal, R. R. and F. J. Rohlf. 1969. Biometry. Freeman, San Francisco. p. 776.

82. Steiner, G. W. and J. L. Lockwood. 1969. Soil fungistasis: Sensitivity of spores in relation to germination time and size. Phytopathology 59:1084-1092.

83. Steiner, G. W. and J. L. Lockwood. 1970. Soil fungistasis: Mechanism in sterilized, reinoculated soil. Phytopathology 60:89-91.

84. Steiner, G. W. and R. D. Watson. 1965. Use of surfactants in the soil dilution and plate count method. Phytopathology 56:728-730.

85. Stotzky, G. and A. G. Norman. 1961. Factors limiting microbial activities in soil. I. The level of substrate, nitrogen, phosphorus. Arch. Mikrobiol. 40:341-369.

86. Stover, R. H. 1958. Studies on Fusarium wilt of bananas. III. Influences of fungitoxin on behavior of Fusarium oxysporum f. cubense in soil extracts and diffusates. Can. J. Botany 36:439-453.

87. Sztejnberg, A. and J. P. Blakeman. 1973. Studies on leaching of Botrytis cinerea conidia and dye absorption by bacteria in relation to competition for nutrients on leaves. J. Gen. Microbiol. 78:15-22.

88. Tsao, P. H. and J. L. Brinker. 1966. Chlamydospores of Thielaviopsis basicola as surviving propagules in natural soils. Phytopathology 56:1012-1014.

89. Tsao, P. H. and B. T. Hawthorne. 1970. Soil fungistasis, soil amendments, lysis and biological control of Thielaviopsis basicola. Proc. Seventh Int. Cong. of Plant Protection. Paris. p. 534-535.

90. Vaartaja, O. 1973. Inhibition of Pythium ultimum in different molecular fractions from gel filtration of soil extracts. Abstracts of papers, Second Int'l. Cong. Plant Path., Univ. of Minnesota, Minneapolis.

91. Watson, A. G. and E. J. Ford. 1972. Soil fungistasis - A reaprassial. Ann. Rev. Phytopathology 10:327-348.

92. Weltzien, H. C. 1963. Untersuchungen über die Ursachen der Kermhemmung von Pilzsporen in Boden. Zbl Bakt. II. 116:131-170.

93. Yoder, D. L. and J. L. Lockwood. 1973. Fungal spore germination on natural and sterile soil. J. Gen. Microbiol. 74:107-117.