ENVIRONMENTAL FACTORS AFFECTING GERMINATION OF *HELMINTHOSPORIUM VICTORIAE* CONIDIA IN STERILE AND NON-STERILE CONDITIONS

By

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A DISSERTATION

Submitted to Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1982

ABSTRACT

ENVIRONMENTAL FACTORS AFFECTING GERMINATION OF *HELMINTHOSPORIUM VICTORIAE* CONIDIA IN STERILE AND NON-STERILE CONDITIONS

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Fungistasis in soil could be caused by the loss of a germination-regulating exudate from propogules. This hypothesis was evaluated using *Helminthosporium victoriae* conidia incubated on a sandy clay loam soil or on two aseptic "leaching systems" that apparently modeled soil fungistasis. ^{14}C-labelled *H. victoriae* conidia released a glucose-rich exudate, primarily during the first 30 min after wetting. There was no evidence that this exudate was a required substrate for germination. Conidia first incubated in a germination-suppressive environment for 2-8 hr, then transferred to germination-conducive conditions, germinated as rapidly as conidia held constantly in a conducive environment. There was also no evidence that the exudate, once released, was exogenously involved in germination; exudate did not consistently stimulate germination, uptake of ^{14}C-labelled exudate was not detected before germ tubes emerged, germination was not stimulated by exposure to nutrients during the period of greatest exudate release, and exogenous glucose oxidase did not affect germination. Moreover, conidia incubated on

the model "leaching system" germinated when temperature (30 C), pH (5.0) and osmotic potential (-1 bar) were optimal for spore germination, regardless of constant removal of exudate; germination was only suppressed when conditions were sub-optimal for germination. In comparison to the model systems, soil fungistasis was relatively unaffected by temperature, pH, and osmotic potential. These results suggested that the initial exudate was not involved in soil fungistasis.

To study the microorganisms which induced fungistasis, 49 isolates of bacteria and actinomyucetes which differed in colony morphology were obtained on several culture media from either fungistatic (soil extract) or non-fungistatic (non-soil) environments. Isolates were inoculated individually into a sterile soil extract which was used to saturate sand. Most of the bacterial isolates inhibited germination of H. victoriae conidia. The medium used for initial isolation did not affect the isolates ability to support the fungistasis. Prior colonization of the sand substratum was not required for suppression of conidial germination by the fungistasis-inducing isolates. The results indicated that fungistasis is caused nonspecifically by microorganisms. There was no evidence that antibiotics or inhibitory volatile or non-volatile compounds caused fungistasis of H. victoriae conidia incubated in a sandy clay loam soil.

ACKNOWLEDGEMENTS

I want to thank John L. Lockwood, chairman of my graduate committee, for his consistent interest and advice on my research and his conscientious help in the preparation of this manuscript. I also want to thank the other members of my committee for their review of this dissertation and to express my gratitude individually to Derek Lamport, for opening his laboratory to me, Norman Good for his counsel, Gene Safir, for serving as my interim advisor and Robert Scheffer for his assistance on short notice.

Thanks are also due to Lisa Purtill for aiding in the laboratory and Paula Moan for typing.

Throughout my program, I was fortunate to have had the encouragement and support of family and friends, for which I am most grateful. A special thanks goes to Martha Tilmann.

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CHAPTER I

THE ROLE OF EXUDATION IN SPORE GERMINATION: A LITERATURE REVIEW

1.1. THE ROLE OF FUNGAL SPORE EXUDATION IN SOIL FUNGISTASIS

1.1.1. Introduction. Available carbon in the vast bulk of soil is too low to support sustained growth and reproduction of the microbiota located in this environment; therefore, most soil microbes must be in a quiescent or dormant state for most of their life cycles (Lockwood, 1977). This quiescence of unfed spores is termed fungistasis, and is a key to the survival of heterotrophic organisms faced with an intermittent food supply. The term "soil fungistasis" is generally defined as a state of inhibition of both fungal germination and growth. However in this paper, fungistasis will refer only to an inhibition of germination.

There appears to be little debate that fungistasis is 1) a widespread phenomenon in soil 2) generally, but not always, due to the presence of living microbes and 3) annulled by the addition of nutrients (Lockwood, 1964, 1977; Watson and Ford, 1972). Direct contact between the fungus and surrounding microbiota, or actual parasitism of the fungus, are not required in order for fungistasis to occur; propagules will not germinate in soil, even if the

spores are separated from the soil by a membrane that excludes bacteria. Fungistasis which is caused by microbiota has been called "microbial fungistasis" (Dobbs and Gash, 1965). In some cases, fungistasis is not of microbial origin; it is not annulled by sterilization or addition of nutrients. This had been termed "residual fungistasis" (Dobbs and Gash, 1965) and will not be discussed here.

Microbial fungistasis of some fungi can be explained simply; soils generally fail to provide the food requirement needed for spore germination, since little of the soil organic matter is actually available for microbial metabolism (Alexander, 1977). Fungal propagules which will not germinate in either sterile or non-sterile environments, except in the presence of food, are termed "nutrient dependent" (Lockwood, 1977). Propagules which will germinate in sterile conditions in the absence of any organic or inorganic compounds are termed "nutrient independent." Many nutrient independent spores will not germinate in nonsterile conditions unless a carbonaceous energy source becomes available. Since the deficiency of nutrients in soil can not account for fungistasis of nutrient independent propagules, other mechanisms have been proposed.

Two general theories could account for fungistasis: 1) The surrounding microbes could produce a germination-inhibiting "signal" or 2) the spores could lose a

germination-promoting signal to the surrounding microflora. If the former is true, than either nutrients inhibit the formation of the microbial signal or annul the effect of the signal. Similarly, if the latter is true, then nutrients either inhibit the loss of the signal or render the loss of the signal inconsequential.

There have been numerous attempts to isolate a germination inhibiting compound from soil (Lockwood, 1977). While some fungistatic compounds have been isolated from soils, the results thus far tend to indicate that inhibitory compounds may be limited to specific habitats. If there are substances in soil which cause fungistasis, current research indicates that volatile compounds are the most likely candidates (Lockwood, 1977; Balis and Kouyeas, 1978; Pavlica et al., 1978). Watson and Ford (1972) and Hora et al. (1977) have argued that soil fungistasis is based on an interaction between inhibitors and stimulators in the soil.

1.1.2. Nutrient deprivation hypothesis. Lockwood and his co-workers (1977, 1981) have proposed that spores lose germination promoting nutrients to the environment. Soil microflora serve as a "nutrient sink" which constantly drains the spores of soluble metabolic reserves, thereby preventing germination. Losses may eventually occur to such an extent that the spores may lose the ability to germinate without exogenous energy. This hypothesis has been referred to as the "nutrient deprivation hypothesis."

Evidence in support of the hypothesis is based primarily on the observation that many nutrient-independent spores will not germinate in a sterile model system in which a sand substratum is continuously leached with either water, phosphate buffer, or a dilute salt solution (Hsu and Lockwood, 1973). There is generally a correlation between germination behavior (i.e., sensitivity to fungistasis) on the sterile system and in the soil (Ko and Lockwood, 1967). Ascospores of Neurospora tetrasperma will germinate on soil as well as on the leaching system. In the leaching system, germination is inversely proportional to the flow rate of leaching solution (Lockwood, 1975; Sneh and Lockwood, 1976). Similarly, when Cochliobolus victoriae (Helminthosporium victoriae) conidia were floated on distilled water, germination decreased as the volume of water increased (Sneh and Lockwood, 1976). In the leaching system, inhibition of germination was inversely correlated with the loss of ^{14}C label from the spores (Sneh and Lockwood, 1976; Filonow and Lockwood 1979). Loss of ^{14}C from labelled non-germinating spores on the leaching system was less than ^{14}C loss from spores incubated on soil (Sneh and Lockwood, 1976; Filonow and Lockwood, 1979). Germination was partially restored when the spores were incubated with ^{14}C-labelled compounds leached from the same or other spores (Bristow and Lockwood, 1975a). These data suggest that the loss of the ^{14}C-labelled compounds inhibits germination (Lockwood, 1977).

Lockwood and co-workers have examined the kinetics of exudation from H. victoriae conidia. There was no apparent lag time in the release of ^{14}C-labelled compounds (Bristow and Lockwood, 1975a; Sneh and Lockwood, 1976) and a plot of the logarithm of the loss of ^{14}C from the spores was roughly linear with time (Bristow and Lockwood, 1975a). During a 96 hr period of leaching, H. victoriae spores exuded nearly 10% of the total ^{14}C contained in the spores; over 90% of the ^{14}C exudate was released during the first 12 hr. Similarly, H. victoriae exuded only slightly more ^{14}C after 2 or 4 hrs than after 0.5 hr (Filonow and Lockwood, 1979). Other workers have confirmed that other spores exude in a similar pattern; at the initiation of the leaching process, exudation occurred without lag and most exudate was released within 0.5 hr. Within the first ten minutes of leaching, H. sativum conidia lost approximately half of the total ^{14}C removed during a 31.5 hr experiment (Knight, 1970). After 31.5 hrs, the conidia had lost 11.4% of their original ^{14}C content. Conidia of Drechslera rostrata suspended in water also rapidly released compounds; the conductivity of the water remained at approximately the same level between 1 and 31 min (Dix and Christie, 1974). ^{14}C-labelled conidia of Botrytis cinerea suspended in water also rapidly exuded ^{14}C (Brodie and Blackman, 1975).

The H. victoriae exudate contained anthrone- and ninhydrin-positive compounds (Bristow and Lockwood, 1975a).

More than 90% of the carbohydrate was glucose. Exudate from Curvularia lunata and Thielaviopsis basicola also contained ninhydrin- and anthrone-positive compounds, although in somewhat different quantities and proportions. Fungistasis of H. victoriae conidia incubated on the leaching system was partially annulled by a nutrient solution with glucose and casein hydrolysate, in concentrations which approximated the carbohydrate and amino compound composition of H. victoriae exudate. Neither exudate nor the defined nutrient solution affected germination of unleached spores, but both increased germination of leached spores to a similar extent.

There was a high degree of correlation between germination of spores incubated on soil and on the leaching system (Ko and Lockwood, 1967; Bristow and Lockwood, 1975b; Lockwood, 1977). Of 17 species of nutrient independent propagules, 14 germinated similarly in soil and on the leaching system. In addition, the rates of the following processes were similar on both soil and the leaching system: 1) the conversion of H. victoriae and H. sativum from nutrient independence to nutrient dependence (Bristow and Lockwood, 1975a), 2) the germination of H. victoriae conidia that were preincubated in a nonfungistatic environment before incubation on either system (Bristow and Lockwood, 1975a) and 3) the inhibition of germination of Penicillium frequentans conidia that were alternately

incubated on a germination conducive and suppressive environment (Yoder and Lockwood, 1973).

1.1.3. Exudation and germination. Despite the results discussed above, there are experimental results which do not appear to support the nutrient deprivation hypothesis. The relatively large initial loss of ^{14}C material from spores incubated in a germination-suppressive environment does not usually have any obvious effects on the spore's ability to germinate in a germination-conducive environment. After the initial loss of ^{14}C, spores incubated on the leaching system will generally commence germination as soon as the leaching is stopped. For example, five fungi with nutrient independent spores germinated to a high degree after termination of a 24 hr treatment on soil or the leaching system (Hsu and Lockwood, 1973). Leached conidia of Drechslera sorokiniana and D. teres exuded three sugars and five amino acids; after the leaching treatment, the conidia were still nutrient independent (Yadav and Mandahar, 1981). Similarly, repeated washing of the conidia of H. sativum did not affect the germinability of the spores (Knight, 1970). Conidia of Botrytis cinerea also leaked when placed in sterile water; however, germination was not impaired (Fraser, 1971). Mature sclerotia from ten species of fungi were reported to exude compounds upon hydration (Coley-Smith, 1976). However, the removal of exudate via washing prior to burial in soil generally had no

deleterious effect on survival (Smith, 1972b; Coley-Smith, 1976; Imolehin and Grogan, 1980). In fact, Smith (1972) noted that removal of exudate actually increased sclerotial survival; when exudate remained in the vicinity of the propagule, the incidence of rotten sclerotia increased. In contrast, there are a few reports that a relatively short period of washing increased a nutrient requirment for germination (Oku, 1960; Cochrane, 1966). In addition, sclerotia exposed to cycles of wetting and drying exuded deleteriously large quantities of nutrients (Smith, 1972a; Gladders and Coley-Smith, 1980), although this was not always the case (Papavizas, 1972).

According to the nutrient deprivation hypothesis, the mechanism of inhibition of germination of propagules in soil is simply the reverse of the mechanism of the annulment of the inhibition. If this is true, we can predict that spores that endured a greater loss of exudate would also require more nutrients to stimulate germination. Whether this occurs in a sterile environment is unknown; however, this pattern does not occur in soil. H. victoriae spores incubated on coarse-textured soils lost more exudate than spores incubated on fine-textured soils (Filonow and Lockwood, 1979). But, more nutrients were required to annul fungistasis of spores incubated on fine-textured soils than on coarse-textured soils.

There is some evidence that the first stage of germination is insensitive to soil fungistasis; this period

roughly coincides with the time of greatest loss of ^{14}C from nutrient independent propagules incubated on a leaching system or on soil. Conidia of H. sativum incubated for 1 day on nonsterile soil, and then transferred to sterile soil, germinated in 38% less time than conidia which were incubated only on sterile soil. (Yoder and Lockwood, 1973). Preincubation of the nutrient dependent conidia of Aspergillus ustus and Penicillium frequentans for 6 days on nonsterile soil also reduced subsequent germination time on sterilized soil. (On the contrary, germination time of two of three isolates of Fusarium chlamydospores was increased when preincubated for 6 days on nonsterile soil.) With the conidia of Penicillium frequetans, the first stage of germination was irreversibly initiated on nonsterile soil, required only water without any nutrients, was partially inhibited by incubation at 1C, and accounted for approximately 20% of the total germination time.

As stated previously, after the initial period in the germination process where the logarithm of the ^{14}C lost from the spores was linear with time, secondary exudation continued at a fairly constant and extremely low rate (Bristow and Lockwood, 1975a). For example, ^{14}C lost from H. victoriae spores continued at only 2-10 cpm or 7-33% above the background counts. It is possible that the change in the rate of release of ^{14}C-compounds indicates a change in the composition of the exudate. The

leaching system could inhibit germination by removing germination-stimulating compounds which are regulatory in nature and active in low concentrations. These compounds could also conceivably be unlabelled, since spores were produced on media in which ^{14}C-glucose was incorporated with other (unlabelled) carbon sources. However, since the ^{14}C levels in secondary exudate were so low, it is also possible the ^{14}C is present in fungal waste products, toxins, enzymes, dying or germinating spores, or bits of broken mycelium or conidiophores. For example, release of ^{14}C-labelled exudate was correlated with germination of H. sativum conidia (Knight, 1970) and Puccinia graminis urediospores (Maheshwari and Sussman, 1971). Chet et al. (1977) correlated maximal release of carbohydrates and amino acids with germination of Sclerotium rolfsii sclerotia. Dead conidia of Alternaria brassicae (Tsuneda and Skoropad, 1978) and H. victoriae (Filonow et al., 1981) leaked profusely and rapidly.

Increased flow rates in the leaching system were correlated with increased exudation and decreased germination of H. victoriae conidia (Sneh and Lockwood, 1976; Filonow and Lockwood, 1979; Filonow, 1981). While this is strong evidence supporting the nutrient deprivation hypothesis, it should be noted that it is the only direct correlation between exudation and inhibition of germination. The correlation between increased flow rates and increased exudation would also be expected if hydration

of spores in liquid water causes the initial nonspecific loss of compounds from leaky membranes, and the repair of the membranes is a time-dependent process (Simon, 1974). The nonspecific exudation from spores upon hydration will be discussed later in this review. Furthermore, the correlation between increased exudation and decreased germination was dependent upon the initial, and not the secondary exudate. It was not determined whether there were any quantitative differences in exudation between varying flow rates of the leaching apparatus after the initial ^{14}C exudate was removed. Filonow and Lockwood (1979) and Filonow (1981) compared loss of ^{14}C from spores incubated on the leaching system with spores incubated on 13 soils; ^{14}C loss from spores incubated on soil was always greater. In contrast, in a more limited study, Jackson and Knight (1973) reported that ^{14}C-labelled conidia of H. sativum lost more ^{14}C from spores incubated on dialysis tubing containing water than on tubing containing a soil suspension, even though there was greater inhibition of spores incubated on the soil suspension than on water.

It is generally assumed that spore exudate represents endogenous spore reserves, rather than compounds bound to the external surface of the spore wall or from nonviable spores or fragments of nonspore material. Indeed, some of the exudate certainly originates from within the spores. Spores exuded after a washing treatment prior to leaching

(Filonow and Lockwood, 1979). Furthermore, H. victoriae spores that were leached, then incubated at 4 C without leaching, and leached again, released exudate at the beginning of the second leaching period (Filonow, 1981). However, some of the exudate may be adsorbed onto the spore wall. Numerous spores exude aqueous droplets during propagule formation (Madelin, 1969; Colotelo, 1978); these droplets would presumably be released upon wetting. Fisher and Richmond (1970) found that Penicillium expansum grown on media with a high phosphate content produced conidia which were covered with a surface layer of polyphosphate. The phosphate layer, as well as sugars, were removed by washing with water. In a transmission electron microscope study, Murray and Maxwell (1974) observed that H. carbonum germ tubes were surrounded by a loose fibrillar layer. The layer was reduced or absent in shake cultures and therefore, the authors suggested the material was water soluble and easily removed.

1.1.4. Exudation and sensitivity to fungistasis. Both fungistasis-sensitive and fungistasis-insensitive propagules apparently exude upon hydration. In a single washing in water, non-dried urediospores of Puccinia rubigo-vera and conidia of Neurospora sp. exuded about 10% of the spore dry weight (Lingappa and Lockwood, 1964). Neurospora tetrasperma conidia are sensitive to soil fungistasis and urediospores of Puccinia coronata and P. graminis are insensitive (Ko and Lockwood, 1967);

urediospores of P. rubigo-vera would presumably be the same. It is not known if fungistasis-insensitive spores have comparatively larger internal reserves of nutrients than fungistasis-sensitive propagules, lose less exudate, or can better utilize their own exudates.

Heat activated ascospores of Neurospora tetrasperma were insensitive to soil fungistasis as well as to the inhibition imposed by the leaching system (Ko and Lockwood, 1967). However, such spores may not be a suitable test organism. Even fungistasis-sensitive spores can become insensitive to fungistasis after pretreatments which induce germination. When H. victoriae (Bristow and Lockwood, 1975a) and H. sativum (Knight, 1970) conidia were incubated in germination conducive environments for periods less than required for germ tube emergence, then incubated on soil, germination occurred.

Exudation may be a phenomenon associated with young spores. Drechslera rostrata spores were collected dry, and then aged in bottles at ambient temperatures for up to 3 weeks, (Dix and Christie, 1974). Exudation decreased with increased age of the spores; 3-week-old conidia barely exuded at all. Both 3-month and 2-year-old viable sclerotia of Sclerotium cepivorum did not exude unless the sclerotia were surface-sterilized with calcium hypochlorite (Coley-Smith and Dickinson, 1971).

1.1.5. Exudation and the conversion from nutrient independence to nutrient dependence. After relatively long-term incubation on leached sand or soil, nutrient-independent propagules can become nutrient-dependent (Lockwood, 1977). For example, conidia of H. victoriae and H. sativum incubated on soil or on the leaching system for 7 days lost the ability to germinate without exogenous nutrients (Bristow and Lockwood, 1975a). If nutrient depletion is involved in the loss of nutrient independence (Lockwood, 1977) then the period of nutrient independence should be less in lighter textured soils where exudation loss is greater; this was demonstrated by Filonow (1981). However, the conversion from nutrient independence to nutrient dependence could be attributed to a loss of endogenous reserves via exudation or respiration. It is also possible that leaching or incubation in soil caused a physiological alteration independent of nutrient deprivation. For example, loss of germinability of aged urediospores of Puccinia graminis was attributed to a loss of ability to mobilize and utilize food reserves (Wynn et al., 1966). The spores' ability to assimilate exogenous glucose and acetate decreased during storage. During storage, only glutamic acid, malonic acid and pyrrolidone carboxylic acid decreased in pool size. There was no change in the much larger pool sizes of lipids and carbohydrates. When conidia of H. victoriae were leached until they became nutrient dependent, subsequent incubation

in a constant volume at 4 C for 3.5 days rendered some of the spores able to germinate without exogenous nutrients (Filonow, 1981). Unless nutrient dependent spores took up nutrients at 4 C from nonviable spores, the gross depletion of ^{14}C-labelled exudates did not cause the conversion from nutrient independence to dependence. After Botrytis cinerea conidia were leached with deionized water for 24 hr, the spores appeared to have lost their nutrient independence (Sztejnberg and Blakeman, 1973). However, a high degree of germination occurred when the spores were incubated in Pfeffer's mineral salts solution. It is important to reiterate that even in cases where nutrient independent spores became nutrient-dependent, the conversion occurred well after the majority of the ^{14}C-labelled exudate was released; spores are immediately sensitive to soil fungistasis.

1.1.6. Uptake of spore exudate. It remains undetermined whether propagules can utilize their own exudate in germination-conducive environments. H. victoriae conidia rendered nutrient dependent by leaching were incubated on concentrated ^{14}C-labelled spore exudate (Bristow and Lockwood, 1975a). After a 12 hr incubation, half to three- quarters of the exudate was taken up. It is unknown whether unconcentrated exudate would be taken up in the same manner as concentrated exudate. Moreover, since the spores had germinated long before the end of the incubation period, it is unknown

whether the conidia, germ tubes, or young hyphae absorbed the exudate. Urediospores of Puccinia graminis f. sp. tritici initially exuded 4-9% of the total spore carbon into the medium and then reabsorbed the ^{14}C between the 7th and 24th hour of incubation (Daly et al., 1967). There was no evidence of uptake by the spores themselves, since germ tube production commenced in a fairly synchronous manner after 0.5-1 hr. Similarly, when Drechslera sorokiniana and D. teres conidia were incubated in droplets of water, sugar content of the spore exudate declined by 46% and 38%, respectively, after 16-20 hrs and amino acids declined by 27 and 31%, respectively, after 12-16 hrs (Yadav and Mandahar, 1981). Again, the results indicated that reabsorption of exudate occurred in the post-germination phase.

Assuming that spore exudate is required for germination, soil microbiota must be capable of removing the exudate from the vicinity of the spore so that 1) a diffusion gradient is maintained and 2) the exudate is unavailable to the spore for reutilization. Soil microbes are apparently capable of rapidly utilizing exudate; $^{14}CO_2$ was detected from soil after the addition of ^{14}C-glucose or ^{14}C-concentrated exudate after 3 and 15 minutes, respectively (Bristow and Lockwood, 1975b). The question arises whether spore exudate is removed by a specific group of microflora. Sixty seven isolates of bacteria, actinomycetes, or fungi inoculated into sterile

soil restored soil fungistasis, at least to some extent (Lockwood and Lingappa, 1963). If almost all organisms are capable of inducing fungistasis, then almost all organisms should be more capable of utilizing the exudate than the exudate producing fungus. Although this supposition seems improbable, there is limited evidence to support it. When ^{14}C-labelled Botrytis cinerea conidia were incubated with Pseudomonas sp., conidial germination was inhibited and the bacteria took up and utilized a portion of the ^{14}C exudate (Brodie and Blakeman, 1975). (The bacteria apparantly did not increase the loss of ^{14}C from the spores.) When B. cinerea conidia or Pseudomonas sp. were incubated for 24 hr in 10^{-4} M glutamine, the bacteria and the conidia removed 85 and 10% of the amino acids, respectively. The ability of several leaf surface bacteria and yeasts to remove amino acids from an amino acid/glucose solution was correlated with their ability to inhibit conidial germination (Brodie and Blakeman, 1976).

1.1.7. Autostimulatory exudate. If nutrient deprivation via diffusion is the mechanism of fungistasis, then germination in a small volume of an aqueous solution should increase as cell density increases, since the diffusion gradient is reduced. In addition, supernatant from spores that were germinating should stimulate germination of new spores, since the exuded compounds theoretically promote germination. Actually, germination of most spores appears either unaffected or inhibited by

high cell density. Indeed, there are numerous reports in which washing stimulated rather than inhibited germination. Sussman and Douthit (1973), Allen (1976), Macko et al. (1976) and Gottlieb (1978) reviewed the rather large body of literature on self-inhibitors of fungal spore germination.

In contrast, there are relatively few reports of apparent self stimulation; these will be reviewed here. Myxospores of the prokaryote Myxococcus xanthus required either inorganic ions or a high cell density for germination in distilled water (Dworkin, 1973). It was suggested that the "germination factor" was inorganic phosphate released from myxospores after the induction of alkaline phosphatase and subsequent mobilization of polyphosphate deposits. Ascospores of Sclerotinia curreyana germinated to a greater extent when associated in a group with other ascospores (Aggab and Cooke, 1981). When spores of Rhizopus stolonifera, Mucor plumbeus and Geotrichum candidum were dispersed on the surface of an agar medium, pairs of spores germinated faster than isolated spores. However, spores in pairs were generally negatively autotropic (Robinson, 1973; Robinson, 1980). Dahlberg and Franke (1977) presented evidence which suggested that spores of the myxomycete Fuligo septica contained a soluble autocatalytic factor which stimulated germination. Spores germinated better in filtrates from spore suspensions than in controls. The stimulatory

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activity in the filtrate was dialyzable and stable at 60 C for 5 min. Activity in the filtrate was lost after incubation at room temperature for 24 hrs. With most spores, a plot of the percent germination vs. time gives a sigmoidal curve; a probit transformation straightens the line. Dahlberg and Franke (1977) contended that a germination pattern with a sigmoidal curve indicated that germination was randomly distributed around a mean time interval. However, germination of the myxomycete spores had a better fit to a logarithmic curve; this indicated germination was a controlled, and not a random, phenomenon of a population of spores. Wild type Dictyostelium discoideum spores required an activation treatment (such as heat shock, gamma irradiation, or incubation with amino acids or dimethylsulphoxide) to induce swelling (Dahlberg and Cotter, 1978). However, a mutant spore type germinated as soon as the autoinhibitor was diluted or removed. Autoactivation was associated with the release of a compound. Another compound was released after the spores initiated germ tube emergence. No autoactivating substances were released from heat activated spores.

There are several examples in which identified, endogenous compounds apparently stimulate germination when released into the germination medium. These will be discussed more fully in Part II of this literature review, and only mentioned here. Addition of supernatant of Polysphondylium pallidum spores accelerated germination of

ungerminated microcysts (O'Day, 1974). The stimulatory compounds were identified as spore wall lytic enzymes (O'Day, 1976). Extracellular enzyme activity was also implicated in germination of spores of Dictyostelium discoideum (Jones et al., 1979) and conidia of Microsporum gypseum (Page and Stock, 1974). Rust urediospores contained nonanal, a self-stimulatory volatile compound (Rines et al., 1974).

1.1.8. Inhibition of germination on the leaching system. It is possible the leaching system inhibits germination of spores by a mechanism other than depletion of endogenous carbon compounds. If the leaching medium contains a factor(s) which inhibits germination, then with increasing flow rates, the spore is exposed to increasing quantities of the inhibitory factor(s). Botrytis cinerea conidia were leached for 24 hr with varying concentrations of Pfeffer's mixed salts solution at a flow rate sufficient to inhibit germination (Sztejnberg and Blakeman, 1973). Germinability was assessed later in a static solution with the same or varying concentrations of Pfeffer's solution. Their results strongly suggested that certain concentrations (or total quantities) of ions affected the germinability of spores incubated on the leaching system. Spores leached with 0.5% or 1% Pfeffer's solution germinated as well or better than unleached spores. However, spores leached with deionized water, 0.1%, 10% or 100% Pfeffer's solution germinated far less than the

unleached controls. The concentration of Pfeffer's salts in the static solution used to assess germination generally had no effect on germination. However, spores leached with deionized water germinated as well as unleached controls when the leached spores were later incubated in 100% Pfeffer's solution. If the leaching system was only a model of a diffusion gradient of endogenous nutrients, then the concentration of Pfeffer's solution should not have affected subsequent germination. An analysis of the ionic content of spores before and after leaching might be useful. Other workers have used a leaching system with 100% Pfeffer's solution (Bristow, and Lockwood, 1975a), and 1% Pfeffer's (Filonow and Lockwood, 1979). In addition, spores were leached with water (Bristow and Lockwood 1975b; Sneh and Lockwood, 1976) or phosphate buffer (Bristow and Lockwood, 1975b). Phosphate buffer can be deleterious to biological systems (Good et al., 1966), and water without any solutes can cause osmotic stress. Some spores of Drechslera sorokiniana and D. teres ruptured during leaching with distilled water, but not with a nutrient solution (Yadav and Mandahar, 1981). However, this report of rupturing is an unusual one. There is an unlikely possibility that the leaching medium could become contaminated with materials that are not easily removed. For example, an intermittent decline of soybean protoplasts in the Plant Biology Building at Michigan State University was attributed to the presence of amines in the water

supply (Klein, 1981). The volatile amine morpholine is added to the steam supply in order to prevent scale build up. A deteriorated ion exchange cartridge would no longer remove the morpholine and in addition, deteriorated ion exchange cartridges discharge quaternary amines.

It is also possible the leaching system inhibits germination simply by continuously altering the environment. Spore germination may be dependent upon, say, a certain gas concentration that the spores establish. Constant leaching would disrupt the spores' alteration of their own environment.

1.1.9. Further research. Whether the ^{14}C- and ^{32}P-labelled compounds lost from fungal spores incubated in soil are involved in the immediate inhibition of germination remains unanswered. The following questions may assist in a resolution of the issue. 1) What compounds, qualitatively and quantitatively, are exuded after the initial rapid loss of ^{14}C-labelled compounds? 2) Is there a direct correlation between ^{14}C-exudation and the inhibition of germination in soil? 3) If spores can be hydrated in such a way that initial exudation is reduced, is there any correlation between reduced exudation and decreased sensitivity to soil fungistasis? 4) Is there a correlation between exudation loss from fungal isolates with varying sensitivities to fungistasis? Conversely, is there a correlation between exudation and suppression of germination of spores incubated with bacteria with varying

capabilities of inducing fungistasis? In general, mutants have been underutilized in fungistasis research. 5) Since fungistasis occurs in soils with a range of matric potentials (ψ_m), does exudation from ^{14}C-labelled propagules in soil similarly occur in a range of ψ_m? Historically, experiments on exudation in soil have been maintained at ψ_m approaching 0 bar. 6) With propagules that germinate differently on the leaching system than on soil, are there corresponding differences in exudation? Hsu and Lockwood (1973) reported that the nutrient independent sclerotia of Sclerotium rolfsii and macroconidia of Fusarium solani f. sp. phaseoli germinated poorly in the leaching system but were relatively insensitive to soil fungistasis. Conidia of Alternaria tenuis germinated to a greater degree on the leaching system than on the soil. 6) Are fungistasis-insensitive propagules (other than activated Neurospora tetrasperma ascospores) insensitive to the leaching system inhibition? Propagules that germinated on nonsterile soil included Puccinia coronata and P. graminis f. sp. tritici and conidia of Erysiphe graminis f. sp. hordei and f. sp. tritici (Ko and Lockwood, 1967). (These propagules do not survive in the soil.) Hwang and Ko (1974) reported that conidia of Calonectria crotalariae were insensitive to fungistasis, but these results were disputed by Roth and Griffin (1980). Spores of the vesicular-arbuscular mycorrhizal fungi, Glomus epigaeus, G. mosseae, and

Gigaspora gigantea (Daniels and Trappe, 1980) and Glomus caledonius (Hepper, 1979) germinated in nonsterile soil.

It may also be instructive to design bioassays which could detect volatile stimulatory compounds (such as nonanal) in exudate (Rines et al., 1974; French et al., 1978).

In fungistasis experiments, germination is synonymous with germ tube emergence. With some bacterial (Hashimoto et al., 1969; Gould, 1971), actinomycete (Suarez et al., 1980) and fungal spores (Ekundayo and Carlile, 1964; Farach et al., 1979) germination can be divided into different stages, each having different requirements. However, for nutrient independent propagules, there are no widely used histochemical or spectrographic methods to identify early stages of germination. Such methods may enable the identification of the stage in the germination process that is inhibited by soil fungistasis.

The nutrient deprivation hypothesis essentially suggests that a percentage of the spores' endogenous nutrients act as a sensor of the environment (Lockwood, 1981). If there is relatively little competition, the microbial nutrient sink will impose a relatively small diffusion gradient and exudation loss will be sufficiently low to allow spore germination. On the other hand, if there is high competition, the microbial nutrient sink will impose a steep diffusion gradient and exudation loss will be sufficiently great so that the spore cannot germinate.

The bulk of the results from the leaching system do indeed suggest that germination of nutrient independent spores in soil is an autocatalytic process which is regulated by the loss of a compound(s). However, the leaching system experiments do not differentiate between the loss of nutritional and regulatory compounds. Moreover, the experimental evidence for the hypothesis is based largely on a correlation between increased exudation and decreased germination of conidia incubated on the leaching system. This correlation needs to be established for spores incubated in soil, and a cause and effect relationship between exudation and fungistasis needs to be demonstrated for spores incubated in the model system.

1.2. EXUDATION AND GERMINATION OF SPORES IN AXENIC CONDITIONS

In this section, the loss of compounds from spores suspended in sterile solutions will be reviewed. Particular attention will be focused on 1) the identification and quantification of the exuded compounds, 2) factors affecting exudation, and 3) the effect of exudate on subsequent germination.

Exudation is a common phenomenon amongst germinating and nongerminating conidia (Simon, 1974; Lockwood, 1977). There appear to be two categories of exudation: 1) "nonspecific," in which spores release an amalgam of compounds as soon as the propagules contact liquid water, and 2) "specific" in which spores release a limited number of compounds during the germination process.

1.2.1. Non-specific exudation. The studies cited in the discussion on exudation and fungistasis in the first part of this review appear to be examples of non-specific exudation, and will generally not be repeated here. However, several conclusions will be reiterated. 1) Exudate contained a large number of organic and inorganic compounds. For example, air dried urediospores of Puccinia graminis suspended in water leaked 16 amino acids, 3 organic acids, and mannitol, arabitol and trehalose (Wynn et al., 1966). Conidia of Aspergillus niger released ^{32}P-labelled compounds (Nishi, 1961), and polyphosphate

(Krishnan and Damle, 1956) into solution; the kinetics of the ^{32}P release appeared similar to those of ^{14}C release from other spores. 2) The exudate was nonspecific, i.e., the acidic and basic metabolites accumulating in the germination medium of P. graminis urediospores were representative of the compounds found within the spores (Daly et al., 1967). 3) Exudate comprised a sizeable proportion (10%) of the spore dry weight (Lingappa and Lockwood, 1964). 4) Exudation commenced as soon as the propagules contacted liquid water, maintained at either cold or ambient laboratory temperatures (Sneh and Lockwood, 1976). 5) The logarithm of the loss of ^{14}C-labelled exudate decreased linearly with time, characteristic of passive diffusion (Bristow and Lockwood, 1975a). 6) After the initial loss, little or no further exudation occured (Miller and McCallan, 1957). 7) The initial exudate loss did not reduce subsequent germination (Tsuneda and Skoropad, 1978).

Relatively little is known about factors that affect exudation. Sclerotia of Sclerotium rolfsii washed for 1-5 hr under running water, treated for 3 min with 0.5% NaOCl, or punctured with a needle exuded greater amounts of amino compounds and carbohydrates than nontreated sclerotia (Punja and Grogan, 1981). Air drying conidia may increase exudation when the spores are subsequently hydrated. Conidia of Alternaria brassicae immersed in sterile distilled water at 3 C exuded glutamine, aspartic acid

glucose and fructose. As the period of drying prior to germination increased, leakage of amino acids and sugars also increased (Tsuneda and Skoropad, 1978). If previously dried propagules exude excessively upon hydration, such propagules may have reduced viability. No reports are available on whether air-dried spores which are hydrated by exposure to a water-saturated atmosphere leak less than spores which are hydrated by suspension in solution, or whether leakier spores are more sensitive to fungistasis. Nonetheless, fully dehydrated urediospores of P. graminis f. sp. tritici failed to germinate when rehydrated (Knights and Lucas, 1980). Dried sporangia of Phytophthora infestans had greater viability when rehydrated slowly (Minogue and Fry, 1981). Air-dried urediospores generally germinated to a greater extent if the spores were exposed to a water-saturated atmosphere before assessment for germinability (Strobel, 1965; Wiese and Daly, 1967), although exceptions have been reported (McDonald and Strange, 1976). Air-dried urediospores of Puccinia graminis tritici exposed to -196 C became "cold dormant" (Bromfield, 1964). The thawed urediospores did not germinate when suspended in a solution; germination occurred if the spores were briefly heat-schocked at 40 C or exposed to water vapor before immersion in the germinating medium (Maheshwari and Sussman, 1971). Thawed urediospores germinated somewhat when incubated on 1% water agar, rather than in solution. Thawed, cold-dormant

^{14}C-labelled urediospores suspended directly in a solution exuded 10 x more ^{14}C after 5 min than non frozen or cold-dormant urediospores that were heat shocked. Literature on exudation from plant roots (Shay and Hale, 1973; Martin and Kemp, 1980), yeast cells (Herrera et al., 1956; Lee and Lewis, 1968) and fungal hyphae (Jennings, 1976), suggest that exudation may be greater in cold temperatures (Simon, 1974) and in solutions with either low Ca^{++} or a low Ca^{++} to Na^{+} ratio.

Simon (1974) reviewed papers on leakage of compounds from plant and fungal tissues that were dehydrated, chilled or depleted of functional lipids in the membranes. He concluded, "when dry seeds, spores or lichens are placed in water, soluble cell constituents leak from them for a few seconds or minutes until membrane integrity is re-established," and noted "that 'dry' in this context means having less than about 20% water." In fungistasis experiments, the spores are generally not air dried; spores are collected from agar plates and immersed in a solution in order to wash and/or disperse the propagules. It is unclear whether spores on agar plates are sufficiently dehydrated to have the rehydration injury that Simon (1974) discussed (Hawker and Madelin, 1976). However, regardless of the question of water content of propagules, nonspecific exudation appears to be the result of passive loss of water soluble compounds through temporarily leaky membranes.

1.2.2. Specific exudation. Some spores leak specific compounds into a germination medium (Sussman and Halvorson, 1966). Exudation typically occurs after a lag period; the specificity of the exuded compounds enables a more straightforward assessment of the role of these products in the regulation of germination. There are a few examples in which germination is apparently initiated by exudate. Microsporum gypseum macroconidia suspended in a germination medium rapidly released alkaline protease, Ca^{++}, and a pigment (Page and Stock, 1974). Alkaline protease apparently initiated germination by the non-specific and external lysis of the spore wall. Four additional enzymes were sequentially involved in spore wall degradation, but these enzymes remained localized within the spore. During germination, free amino acids were released into the germination medium; these compounds presumably were the products of proteolytic activity of the alkaline protease (Leighton and Stock, 1970). Extracellular alkaline protease apparently only initiated germination of M. gypseum when the conidia were incubated in a relatively low density (Page and Stock, 1974); germination in high densities was inhibited by other exudation products. Phosphodiesterase, one of the intracellular enzymes involved in spore wall lysis, released phosphate from the spore. Phosphate either precipitated the Ca^{++} in the medium or inhibited the alkaline protease. A high phosphate/protease ratio in the medium inhibited

germination. Parenthetically, Microsporum gypseum macroconidia are nutrient independent, and may be a useful model for fungistasis studies.

There was no measurable leakage from nonactivated spores of Dictyostelium discoideum (Hohl et al., 1978). But, ^{14}C-labelled activated spores in phosphate buffer began exudation before any swelling was evident (after 30 minutes), reached a maximum rate after 1.5 hour and dropped to 0 after 2.5 hours. Cycloheximide did not affect swelling or the kinetics of exudation, although germ tube emergence was inhibited. Hohl et al. (1978) did not identify the exuded material, but Jones et al., (1979) reported that during swelling, two cellulases were excreted into the medium. Spores from another cellular slime mold, Polysphondylium pallidum, exuded 6 enzymes into the medium, two of which reached higher levels of specific activity extracellularly than intracellularly (O'Day, 1974). Studies on two acid proteases suggested that external enzymes caused the initial loosening of the wall which in turn allowed swelling of the spore (O'Day, 1976). Ando (1979) detected a spore lytic enzyme in the germination medium of the anaerobic bacterium, Clostridium perfringens. The enzyme was released after the germination process was initiated.

During germination, bacterial spores exude up to 30% of their dry weight. After activation of Bacillus cereus spores by heat treatment for 30 minutes at 70 C,

germination and the accompaning excretion of cellular products occurred quickly. Spores of Bacillus megaterium released greater than 80% of their K^+ and Na^+ into the germination fluid. H^+ was also released, and was accompanied by a rise in the internal pH of the spore from 6.5 to 7.5, after which spores released dipicolinic acid (DPA), divalent cations and metabolic end products (Swerdlow et al., 1981). B. cereus spores released Ca^{++} and DPA almost coincidentally, but with some indication of a lag in the excretion of Ca^{++}. Detectable metabolism of spores of B. megaterium occurred only after DPA release. Loss of Ca^{++} and DPA was followed by the release of peptidoglycan fragments; this release was accompanied by a change in the absorbance of spore suspensions (Dring and Gould, 1971) and development of stainability (Hashimoto et al., 1969). Glutamate and arginine, contained free within Bacillus sp. endospores, were released into the germination medium at rates nearly identical to DPA loss (Nelson and Kornberg, 1970). A wide variety of other amino acids was excreted into the medium during germination, apparently the result of proteolysis.

Exogenous Ca^{++} and DPA may be involved in the initiation of germination, since spores of both aerobic and anaerobic bacteria germinated in an equimolar mixture of calcium chloride and DPA (Riemann and Ordal, 1961). More conclusive evidence indicates that DPA, present in the dormant spore as a Ca^{++} salt, is involved in spore

dormancy (Gould, 1978). Release of DPA is accompanied by a loss of resistance to heat and toxic chemicals (Hashimoto et al., 1969), and a mutant spore which germinated poorly lost only 75% of the DPA lost by the wild type (Moir, 1981). Activated spores of Bacillus coagulans were restored to dormancy by exposure to an alkaline solution of Ca^{++} (Lewis et al., 1965).

Urediospores from numerous rusts released two volatile, self-stimulatory compounds: nonanal and to a lesser extent, 6-methyl-5-hepten-2-one. (Rines et al., 1974). Nonanal stimulated germination of several rust species and two Penicillium sp. (French et al., 1978) and stimulated growth of numerous wood decomposing fungi (Fries, 1960). As little as 40 ppb nonanal stimulated germination of stem rust uredospores still in pustules (French and Gallimore, 1972).

Numerous fungal spores contain endogenous inhibitors, whose release and removal stimulate germination. In contrast to the examples discussed above, these compounds are apparently released from the spores immediately upon hydration. Endogenous inhibitors which have been identified include methyl cis 3,4 dimethoxy cinnamate in Uromyces phaseoli and several Puccinia spp., methyl cis ferulate in Puccinia graminis (Macko et al., 1976), discadenine in Dictyostelium discoideum (Abe et al., 1981) and quiesone in Peronospora tabacina (Leppik et al., 1972). These compounds have inhibited germination of the parent

species in concentrations from 10^{-6} to 4 X 10^{-11} M and Allen and Dunkle (1971) noted the concentration of methyl cis ferulate required to inhibit urediospore germination was in the same order as the active concentration of hormones; inhibitory metabolites such as "staling factors" (Robinson et al., 1968) required a 1000 X greater concentration for germination inhibition. Methyl cis ferulate did not affect urediospore hydration or swelling, but did inhibit the digestion of a preformed pore plug; the inhibitor was effective until the germ tube emerged from the pore (Hess et al., 1975).

Germinating spores of Streptomyces viridochromogenes released a substance into the medium which inhibited the initiation of germination of other spores of the same species (Grund, 1980). This compound was not detected within dormant spores and had a molecular weight of approximately 900 daltons.

Harper et al., (1980) reported that Fe was involved in the inhibition of germination of conidia of Colletotrichum musae. The Fe was not removed upon hydration, but chelating agents in plant exudate removed the Fe and thereby stimulated germination (McCracken and Swinburne, 1980).

There are few reports in which loss of a specific compound from within the spore inhibited germination. When conidia of Neurospora crassa were suspended in a solution with low water activity (by addition of either electrolytes

or nonelectrolytes), the conidia exuded several Fe transport compounds which were essential for germination (Horowitz et al., 1976).

In the following examples, the compounds released appear to be by-products, rather than regulators, of germination. An efflux of ions was associated with germination of Blastocladiella emersonii zoospores induced by 50mM KCl; zoospores accumulated K^+ and excreted Na^+ (Van Brunt and Harold, 1980). Zoospores were reported to bind Ca^{++} on the external surface of the spore (Van Brunt and Harold, 1980) and to release $^{45}Ca^{++}$ during the course of germination (Gomes et al., 1980). No $^{45}Ca^{++}$ was released from nongerminating zoospores. However, since the calcium ionophore A23187 stimulated the efflux of Ca^{++} without the initiation of germination, Gomes et al. (1980) concluded the early efflux of Ca^{++} was not an initial event in germination.

Germination of spores of Streptomyces viridochromogenes, an actinomycete, was also accompanied by the loss of a heterogeneous mixture of anthrone- and ninhydrin-positive substances (Hirsch and Ensign, 1978). Spores that were treated for 10 minutes at 55 C exuded 12% of their total spore carbon, whereas spores that were not heat-shocked exuded 8% of their total spore carbon. The loss of ^{14}C increased from 0 to 60 minutes and then remained fairly constant (Eaton and Ensign, 1980). There

was a positive correlation between loss of spore carbon and germination.

After heat activation of Saccharomyces cerevisiae ascospores, proline and 7 other amino acids were released into the medium; the release began 1 to 1.5 hours after activation (Rousseau and Halvorson, 1973). After 2-3 hours, the major amino acids were reutilized to some extent. Between 3-4 hours, amino acids were again exuded into the medium. Rousseau and Halvorson (1973) suggested that the excretion of the amino acids may have been the result of proteolytic activity during germination.

Members of the Blastocladiaceae have zoospores and gametangia which germinate through pores in the spore wall (Skucas, 1966). The pores are filled with plugs, or discharge papillae. Youatt (1976) proposed that autodigestion of the Allomyces plug occurred via proteolysis; the plug, unlike the wall, contained a high percentage of protein, and peptides and amino acids accumulated in the suspending medium during germination. Other compounds in the germination medium also appeared to have been derived from plug material. Rise of UV absorption of the filtrate was correlated with the disappearance of plugs.

Numerous fruiting structures exude liquid droplets during propagule formation (Madelin, 1969; Colotelo, 1978). Any compounds not reabsorbed would presumably be released upon suspension of the spores in water. Cooke (1969),

Colotelo (1973, 1978) and Christias (1980) reported a wide variety of compounds in sclerotial exudate: K^+, Mg^{++}, Na^+, free amino acids, free ammonia, fatty acids, oxalic acid, trehalose, mannitol, inositol, glucose, a catalase, peroxidase, polyphenol oxidase, glucosidase, acid phosphatase, acid and alkaline protease, RNA-ase, polygalacturonase and cellulase. Nonetheless, it appeared that the exudate produced during formation of sclerotia was, at least in part, the result of specific excretion of compounds and not random leakage. For example, alanine was the second most abundant free amino acid inside sclerotia of Sclerotium rolfsii, but only a minor constituent of sclerotial exudate (Christias, 1980). Also, mannitol was found inside the sclerotia of Sclerotinia trifoliorum but not in the exudate (Cooke, 1969). Exudation droplets were reported on sclerotia produced both in soil and in agar, but the droplets were most common when the agar medium had a high sugar content. Willetts (1978) suggested that exudation 1) maintained the internal osmotic balance within the sclerotia, and 2) maintained a concentration gradient so nutrients continued to move into the sclerotia. Cooke (1971) suggested that exudation caused the rapid dehydration of developing sclerotia. When the sclerotia matured, the droplets of exudate lost dry matter and decreased in size; certain constituents in the exudate, such as K^+, were reabsorbed (Colotelo, 1973).

In conclusion, I have cited examples of specific spore exudation products that are associated with germination. In some cases, release of certain compounds was incidental and a by-product of germination. While the partial dissolution or weakening of the spore wall may be a critical event in germination in some cases (Hemmes et al., 1972; Ohkawa and Aoki, 1980), the actual presence of wall products in the germination medium does not appear to affect germination. In other cases, exudation was a catalytic event in the course of germination; the extracellular activity of these compounds stimulated germination. Since this section of the literature review concerned exudation from spores in axenic conditions, much of the research cited here is in the category of microbial physiology. Unfortunately there is a gap between microbial physiology and microbial ecology, and it remains to be seen whether any of these examples may be helpful in an assessment of whether spore exudation is involved in soil fungistasis.

1.3. LITERATURE CITED

Abe, H., K. Hashimoto and M. Uchiyama. 1981. Discadine distribution in cellular slime molds and its inhibitory activity on spore germination. Agric. Biol. Chem. 45:1295-1296.

Aggab, A.M. and R.C. Cooke. 1981. Self stimulation and self inhibition in germinating ascospores of Sclerotinia curreyana. Trans. Brit. Mycol. Soc. 76:155-157.

Alexander, M. 1977. Introduction to soil microbiology. 2nd ed. John Wiley and Sons. N.Y. 467p.

Allen, P.J. 1976. Spore germination and its regulation, p. 51-85. In R. Heitefuss and P.H. Williams (eds.) Physiological Plant Pathology. Springer-Verlag, N.Y.

Allen, P.J. and L.D. Dunkle. 1971. Natural activators and inhibitors of spore germination, p. 23-58. In S. Akai and S. Ouchi (eds.), Morphological and biochemical events in plant parasite interaction. The Phytopathological Society of Japan. Tokyo.

Ando, Y. 1979. Spore lytic enzyme released from Clostridium perfringens spores during germination. J. Bacteriol. 140:59-64.

Balis, C. and V. Kouyeas. 1978. Contribution of chemical inhibitors to soil mycostasis. p. 97-106. In B. Schippers and W. Gams (eds.), Soil-borne plant pathogens. Academic press. London.

Bristow, P.R. and J.L. Lockwood. 1975a. Soil fungistasis: role of spore exudates in the inhibition of nutrient independent propagules. J. Gen. Microbiol 90:140-146.

Bristow, P.R. and J.L. Lockwood. 1975b. Soil fungistasis: role of the microbial nutrient sink and of fungistatic substances in two soils. J. Gen Microbiol. 90:147-156.

Brodie, I.D.S. and J.R. Blakeman. 1975. Competition for carbon compounds by a leaf surface bacterium and conidia of Botrytis cinerea. Physiol. Plant Pathol. 6:125-135.

Brodie, I.D.S. and J.P. Blakeman. 1976. Competition for exogenous substrates in vitro by leaf surface micro-organisms and germination of conidia of Botrytis cinerea. Physiol. Plant Pathol. 9:227-239.

Bromfield, K.R. 1964. Cold-induced dormancy and its reversal in uredospores of Puccinia graminis var. tritici. Phytopathology 54:68-74.

Chet, I., D. Timar, and Y. Henis. 1977. Physiological and ultrastructural changes occuring during germination of sclerotia of Sclerotium rolfsii. Can. J. Bot. 55:1137-1142.

Christias, C. 1980. Nature of the sclerotial exudate of Sclerotium rolfsii Sacc. Soil Biol. Biochem. 12:199-201.

Cochrane, V.W. 1966. Respiration and spore germination. p. 201-213. In M.F. Madelin (ed.), The fungus spore. Butterworths, London. 338p.

Coley-Smith, J.R. 1976. Some interactions in soil between plants, sclerotium-forming fungi and other microorganisms. p. 11-23. In J. Friend and D.R. Trelfall (eds.). Biochemical aspects of plant parasite relationships. Academic Press. London. 354p.

Coley-Smith, J.R. and D.J. Dickinson. 1971. Effects of sclerotia of Sclerotium cepivorum Berk. on soil bacteria. The nature of substances exuded by sclerotia. Soil Biol. Biochem. 3:27-32.

Colotelo, N. 1973. Physiological and biochemical properties of the exudate associated with developing sclerotia of Sclerotinia sclerotiorum (Lib.) De Bary Can. J. Microbiol. 19:73-39.

Colotelo, N. 1978. Fungal exudates. Can J. Microbiol. 24:1173-1181.

Cooke, R.C. 1969. Changes in soluble carbohydrates during sclerotium formation by Sclerotinia sclerotiorum and S. trifoliorum. Trans. Brit. Mycol. Soc. 53:77-86.

Cooke, R.C. 1971. Physiology of sclerotia of Sclerotinia sclerotiorum during growth and maturation. Trans. Brit. Mycol. Soc. 56:51-59.

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Dahlberg, K.R. and D.A. Cotter. 1978. Autoactivation of spore germination in mutant and wild type strains of Dictyostelium discoideum. Microbios 23:153-266.

Dahlberg, K.R. and R.G. Franke. 1977. Spore germination in the myxomycete Fuligo septica: evidence for the existence of soluble autocatylytic factor. Mycologia 69:96-108.

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.

Daniels, B.A. and J.M. Trappe. 1980. Factors affecting spore germination of the vesicular-arbuscular mycorrhizal fungus, Glomus epigaeus. Mycologia 72:457-471.

Dix, N.J. and P. Christie. 1974. Changing sensitivity to soil fungistasis with age in Drechslera rostrata spores and associated permeability changes. Trans. Brit. Mycol. Soc. 62:527-535.

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

Dring, G.J. and G.W. Gould. 1971. Sequence of events during rapid germination of spores of Bacillus cereus. J. Gen. Microbiol. 65:101-104.

Dworkin, M. 1973. Cell-cell interactions in the myxobacteria, p. 125-142. In J.M. Ashworth and J.E. Smith (eds.). Microbial differentiation. Cambridge U.P., Cambridge, England.

Eaton, D. and J.C. Ensign. 1980. Streptomyces viridochromogenes spore germination initiated by calcium ions. J. of Bacteriol. 143:377-382.

Ekundayo, J.A. and M.F. Carlile. 1964. The germination of sporangiospores of Rhizopus arrhizus; Spore swelling and germ tube emergence. J. Gen. Microbiol. 35:261-269.

Farach, M.C., H. Farach and P.E. Mirkes. 1979. Control of development in Neurospora crassa: Nutrient requirements for conidial germ tube emergence, elongation, and metabolic activation. Experimental Mycol. 3:240-248.

Filonow, A.B. 1981. Mycostasis and the microbial nutrient sink of soil. Ph.D. dissertation, Michigan State University, E. Lansing, MI. 97p.

Filonow, A.B., D. Chun and J.L. Lockwood. 1981. Enhanced loss of endogenous nutrients from fungal propagules treated with ammonia. Phytopathology 71:1116 (Abstr.).

Filonow, A.B. and J.L. Lockwood. 1979. Conidial exudation by Cochliobolus victoriae on soils in relation to soil mycostasis, p. 107-119. In B. Schippers and W. Gams, (eds.). Soil-borne plant pathogens. Academic Press. London.

Fisher, D.J. and D.V. Richmond. 1970. The electrophoretic properties and some surface components of Penicillium conidia. J. Gen. Microbiol. 64:205-214.

Fraser, A.K. 1971. Growth restriction of pathogenic fungi on the leaf surface. p. 529-535. In T.F. Preece and C.H. Dickinson (eds.), Ecology of leaf surface microorganisms. Academic Press, London.

French, R.C. and M.D. Gallimore. 1972. Stimulation of germination of uredospores of stem rust of wheat in the pustule by n-nonanal and related compounds. J. Agric. Food Chem. 20:421-423.

French, R.C., R.K. Long, F.M. Latterell, C.L. Graham, J.J. Smoot and P.E. Shaw. 1978. Effect of nonanal, citral and citrus oils on germination of conidia of Penicillium digitatum and Penicillium italicum. Phytopathology 68:877-882.

Fries, N. 1960. Nonanal as a growth factor for wood-rotting fungi. Nature 187:166-167.

Gladders, P. and J.R. Coley-Smith. 1980. Interactions between Rhizoctonia tuliparium sclerotia and soil microorganisms. Tran. Brit. Mycol. Soc. 74:579-586.

Gomes, S.L., L. Mennucci and J.C.C. Maia. 1980. Calcium efflux during germination of Blastocladiella emersonii. Devel. Biol. 77:157-166.

Good, N.E., G.D. Winget, W. Winter, T.N. Connolly, S. Izawa and R.M.M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry J. 5:467-477.

Gottlieb, D. 1978. The germination of fungus spores. Meadowfield Press Ltd., Durham, England. 166p.

Gould, G.W. 1971. Methods for studying bacterial spores. p. 326-380. In J.R. Norris and D.W. Ribbons (eds.) Methods in microbiology, Vol. 6A. Academic Press, N.Y.

Gould, G.W. 1978. Practical implications of compartmentalization and osmotic control of water distribution in spores. p. 21-26. In G. Chambliss and J.C. Vary (eds.) Spores VII. Am. Soc. Microbiol., Wash. D.C.

Grund, A.D. 1980. Studies on a germination inhibitor in spores of Streptomyces viridochromogenes. Diss. Abstr. Int. B 41:4338-4339.

Harper, D.B., T.R. Swinburne, S.K. Moore, A.E. Brown and H. Graham. 1980. A role for iron in germination of conidia of Colletotrichum musae. J. Gen. Microbiol. 121:169-174.

Hashimoto, T., W.R. Frieben and S.F. Conti. 1969. Microgermination of Bacillus cereus spores. J. Bacteriol. 100:1385-1392.

Hawker, L.E. and M.F. Madelin. 1976. The dormant spore, p. 1-72. In D.J. Weber and W.M. Hess (eds.) The fungal spore: form and function. John Wiley and Sons, N.Y.

Hemmes, D.E., E.S. Kojima-Buddenhagen and H.R. Hohl. 1972. Structural and enzymatic analysis of the spore wall layers in Dictyostelium discoideum. J. Ultrastructural Res. 41:406-417.

Hepper, C.M. 1979. Germination and growth of Glomus caledonius spores: the effects of inhibitors and nutrients. Soil Biol. Biochem. 11:269-277.

Herrera, T., W.H. Peterson, E.J. Cooper and H.J. Peppler. 1956. Loss of cell constituents on reconstitution of active dry yeasts. Arch. Biochem. Biophys. 65:131-143.

Hess, S.L., P.J. Allen, D. Nelson and H. Lester. 1975. Mode of action of methyl cis-ferulate, the self-inhibitor of stem rust uredospore germination. Physiol. Plant Pathol. 5:107-112.

Hirsch, C.F. and J.C. Ensign. 1978. Some properties of Streptomyces viridochromogenes spores. 134:1056-1063.

Hohl, H.R., M. Buhlmann and E. Wehrli. 1978. Plasma membrane alterations as a result of heat activation in Dictyostelium discoideum spores. Arch. Microbiol. 116:239-244.

Hora, T.S., R. Baker and G.J. Griffin. 1977. Experimental evaluation of hypothesis explaining the nature of soil fungistasis. Phytopathology 67:373-379.

Horowitz, N.H., G. Charlang, G. Horn, and N.P. Williams. 1976. Isolation and identification of the conidial germination factor of Neurospora crassa. J. Bacteriol. 127:135-140.

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.

Hwang, S.C. and W.H. Ko. 1974. Germination of Calonectria crotalariae conidia and ascospores on soil. Mycologia 66:1053-1055.

Imolehin, E.D. and R.G. Grogan. 1980. Factors affecting survival of sclerotia, and effects of inoculum density, relative position, and distance of sclerotia from the host on infection of lettuce by Sclerotinia minor. Phytopathology 70:1162-1167.

Jackson, R.M. and R.A. Knight. 1973. Conidial germination of Cochliobolus sativus on soil and under artificial nutrient stress, p. 201. In G.W. Bruehl (ed.), Biology and control of soil-borne plant pathogens. American Phytopathological Society, St. Paul, Minnesota.

Jennings, D.H. 1976. Transport and translocation in filamentous fungi, p. 32-64. In J.E. Smith and D.R. Berry (eds.), The filamentous fungi, Vol II. Edward Arnold, London.

Jones, T.H.D., M. de Renobales and N. Pon. 1979. Cellulases released during the germination of Dictyostelium discoideum spores. J. Bacteriol. 137:752-757.

Klein, A.S. 1981. Glucan synthesis in soybean cells: properties of the enzymes involved in deposition of glucans during cell wall regeneration. Ph.D. dissertation, Michigan State University. 137 p.

Knight, R.A. 1970. Investigations into the germination of conidia of Cochliobolus sativus with particular reference to soil fungistasis. Ph.D. dissertation, U. of Surrey, England.

Knights, I.K. and J.A. Lucas. 1980. Photo-sensitivity of Puccinia graminis f. sp. tritici urediniospores in vitro and on the leaf surface. Trans. Brit. Mycol. Soc. 74:543-550.

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

Krishnan, P.S. and Damle, S.P. 1956. Phosphate compounds occurring in spore washings of Aspergillus niger. Appl. Microbiol. 4:179-182.

Lee, T.C. and M.J. Lewis. 1968. Mechanism of release of nucleotidic material by fermenting brewer's yeast. J. Food Sci. 33:124-128.

Leighton, T.J. and J.J. Stock. 1970. Biochemical changes during fungal sporulation and spore germination. I. Phenyl methyl sulfonyl fluoride inhibition of macroconidial germination in Microsporum gypseum. J. Bacteriol. 101:931-940.

Leppik, R.A., D.W. Hollomon and W. Bottomley. 1972. Quiesone: an inhibitor of the germination of Peronospora tabacina conidia. Phytochemistry 11:2055-2063.

Lewis, J.C., N.S. Snell, and G. Alderton. 1965. Dormancy and activation of bacterial spores. p. 47-54. In L.L. Campbell and H.O. Halvorson (eds.) Spores III. Amer. Soc. Microbiol., Ann Arbor, MI.

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.

Lockwood, J.L. 1964. Soil fungistasis. Annu. Rev. Phytopathol. 2:341-362.

Lockwood, J.L. 1975. Quantitative evaluation of a leaching model system for soil fungistasis. Phytopathology 65:460-464.

Lockwood, J.L. 1977. Fungistasis in soils. Biol. Rev. 52:1-43.

Lockwood, J.L. 1981. Exploitation competition, p. 319-349. In D.T. Wicklow and G.C. Carroll (eds.). The fungal community. Marcel Dekker, Inc., N.Y.

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

McCracken, A.R. and T.R. Swinburne. 1979. Siderophores produced by saprophytic bacteria as stimulants of germination of conidia of Colletotrichum musae. Physiol. Plant Pathol. 15:331-340.

McDonald, E.A. and R.N. Stange. 1976. Effects of temperature shocks, hydration and leaching on the subsequent germination of uredospores of Puccinia striiformis. Trans. Brit. Mycol. Soc. 66:555-557.

Macko, V., R.C. Staples, Z. Yaniv and R.R. Granados. 1976. Self inhibitors of fungal spore germination, p. 73-100. In D.J. Weber and W.H. Hess (eds.). The fungal spore: form and function. John Wiley and Sons, N.Y.

Madelin, M.F. 1969. Conidium production by higher fungi within thin layers of liquid paraffin: a slide-culture technique. J. Gen. Microbiol. 55:319-324.

Maheshwari, R. and A.S. Sussman. 1971. The nature of cold induced dormancy in urediospores of Puccinia graminis tritici. Plant Physiol. 47:289-295.

Martin, J.K. and J.R. Kemp. 1980. Carbon loss from roots of wheat cultivars. Soil Biol. Biochem. 12:551-554.

Miller, L.P. and S.E.A. McCallan. 1957. Movement of ions and compounds into and out of fungus spores. Science 126:1233. (Abstr.).

Minogue, K.P. and W.E. Fry. 1981. Effect of temperature, relative humidity and rehydration rate on germination of dried sporangia of Phytophthora infestans. Phytopathology 71:1181-1184.

Moir, A. 1981. Germination properties of a spore coat-defective mutant in Bacillus subtilis. J. Bacteriol. 146:1106-1116.

Murray, G.M. and D.P. Maxwell. 1974. Ultrastructure of conidium germination of Cochliobolus carbonus. Can. J. Bot. 52:2335-2340.

Nelson, D.L. and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination XVIII. Free amino acids in spores. J. Biol. Chem. 245:1128-1136.

Nishi, A. 1961. Role of polyphosphate and phospholipid in germinating spores of Aspergillus niger. J. Bacteriol. 81:10-19.

O'Day, D.H. 1974. Intracellular and extracellular enzyme patterns during microcyst germination in the cellular slime mold Polysphondylium pallidum. Develop. Biol. 36:400-410.

O'Day, D.H. 1976. Acid protease activity during germination of microcysts of the cellular slime mold Polysphondylium pallidum. J. Bacteriol. 125:8-13.

Ohkawa, A. and J. Aoki. 1980. Fine structure of resting spore formation and germination in Entomophthora virulenta. J. Invertebrate Pathol. 35:279-289.

Oku, H. 1960. Biochemical studies on Cochliobolus miyabeanus VII. Cellular carbohydrate as an endogenous substrate for conidia germination in Cochliobolus miyabeanus. Plant Cell Physiol. 1:231-239.

Page, W.J. and J.J. Stock. 1974. Sequential action of cell wall hydrolases in the germination and outgrowth of Microsporum gypseum macroconidia. Can. J. Microbiol. 20:483-489.

Papavizas, G.C. 1977. Survivial of sclerotia of Macrophomina phaseolina and Sclerotium cepivorum after drying and wetting treatments. Soil Biol. Biochem. 9:343-348.

Pavlica, D.A., T.S. Hora, J.J. Bradshaw, R.K. Skogerboe and R. Baker. 1978. Volatiles from soil influencing activities of soil fungi. Phytopathology 68:758-765.

Punja, Z.K. and R.G. Grogan. 1981. Eruptive germination of sclerotia of Sclerotium rolfsii. Phytopathology 71:1092-1099.

Riemann, J., Z.J. Ordal. 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science 133:1703-1704.

Rines, H.W., R.C. French and L.W. Daasch. 1974. Nonanal and 6-methyl-5-hepten-2-one: endogenous germination stimulators of uredospores of Puccinia graminis var tritici and other rusts. J. Agric. Food Chem. 22:96-100.

Robinson, P.M. 1973. Autotropism in fungal spores and hyphae. Bot. Rev. 39:367-384.

Robinson, P.M. 1980b. Autotropism in germinating arthrospores of Geotrichum candidum. Trans. Brit. Mycol. Soc. 75:151-169.

Robinson, P.M., D. Park and M.K. Garrett. 1968. Sporostatic products of fungi. Trans. Brit. Mycol. Soc. 51:113-124.

Roth, D.A. and G.J. Griffin. 1980. The response of Cylindrocladium conidia to soil fungistasis. Soil Biol. Biochem. 12:531-536.

Rousseau, P. and H.O. Halvorson. 1973. Physiological changes following the breaking of dormancy of Saccharomyces cerevisiae ascospores. Can. J. Microbiol. 19:547-555.

Shay, F.J. and M.G. Hale. 1973. Effect of low level of calcium on exudation of sugars and sugar derivatives from intact peanut roots under axenic conditions. Plant Physiol. 51:1061-1063.

Simon, E.W. 1974. Phospholipids and plant membrane permeability. New Phytol. 73:377-420.

Skucas, G. 1966. Structure and composition of zoosporangial discharge papillae in the fungus Allomyces. Am. J. Bot. 53:1006-1011.

Smith, A.M. 1972a. Drying and wetting sclerotia promotes biological control of Sclerotium rolfsii Sacc. Soil Biol. Biochem. 4:119-123.

Smith, A.M. 1972b. Nutrient leakage promotes biological control of dried sclerotia of Sclerotium rolfsii Sacc. Soil Biol. Biochem. 4:125-129.

Sneh, B. and J.L. Lockwood. 1976. Quantitative evaluation of the microbial nutrient sink in relation to a model system for soil fungistasis. Soil Biol. Biochem. 8:65-69.

Strobel, G.A. 1965. Biochemical and cytological processes associated with hydration of uredospores of Puccinia striiformis. Phytopathology 55:1219-1222.

Suarez, J.E., C. Barbes and C. Hardisson. 1980. Germination of spores of Micromonospora chalcea: Physiological and biochemical changes. J. Gen. Microbiol. 121:159-167.

Sussman, A.S. and H.A. Douthit. 1973. Dormancy in microbiol spores. Annu. Rev. Plant Physiol. 24:311-352.

Swerdlow, B.M., B. Setlow and P. Setlow. 1981. Levels of H^+ and other monovalent cations in dormant and germinating spores of Bacillus megaterium. J. Bacteriol. 148:20-29.

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.

Tsuneda, A. and W.P. Skoropad. 1978. Nutrient leakage from dried and rewetted conidia of Alternaria brassica and its effect on the mycoparasite Nectria inventa. Can. J. Bot. 56:1341-1345.

Van Brunt, J. and R.M. Harold. 1980. Ionic control of germination of Blastocladiella emersonii zoospores. J. Bacteriol. 141:735-744.

Watson, A.G. and E.J. Ford. 1972. Soil fungastasis - a reappraisal. Annu. Rev. Phytopathol. 10:327-348.

Wiese, M. and J. Daly. 1967. Some effects of pre- and post-germination treatments on germination and differentiation of uredospores of Puccinia graminis f. sp. tritici. Phytopathology 57:1211-1215.

Willetts, H.J. 1978. Scerotium formation, p. 197-213. In J.E. Smith and D.R. Berry (eds.). The filamentous fungi, Vol. 3: Developmental mycology. John Wiley and Sons, N.Y. 464p.

Wynn, W.K., R.C. Staples, B. Strousse and C. Gajdusek. 1966. Physiology of uredospores during storage. Contr. Boyce Thompson Inst. Pl. Res. 23:229-242.

Yadav, B.S. and C.L. Mandahar. 1981. Spore germination of Drechslera sorokiniana and D. teres in relation to leaching. Trans. Brit. Mycol. Soc. 77:219-222.

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

Youatt, J. 1976. Chemical nature of discharge papillae of Allomyces. Trans. Brit. Mycol. Soc. 66:113-122.

CHAPTER 2

EFFECT OF SOIL MICROBIOTA ON GERMINATION OF *HELMINTHOSPORIUM VICTORIAE* CONIDIA

2.1. INTRODUCTION

Helminthosporium victoriae Meehan and Murphy conidia germinated readily when incubated in a sterile environment, but did not germinate when incubated either on top of or buried within soil (Lockwood, 1977). "Fungistasis," or the inhibition of germination of propagules in nature is a widespread phenomenon in soil and generally of microbial origin. Direct contact between the fungus and the surrounding microbiota, or actual parasitism of the fungus, are not required in order for fungistasis to occur; propagules will not germinate when on soil even if the spores are separated from the soil by a membrane that excludes bacteria. In soil, fungistasis is annulled by the addition of energy-yielding nutrients.

Relatively little is known about the organisms which induce fungistasis. Lockwood and Lingappa (1963) inoculated 67 isolates of actinomycetes, bacteria, and fungi individually into autoclaved soil; all of the isolates restored a degree of fungistasis to the soil, regardless of the ability of the isolates to inhibit *Glomerella cingulata* in culture.

The purpose of this research was 1) to develop a soil system with reduced fungistasis and then 2) to use the

system to evaluate the ability of selected isolates to induce soil fungistasis and 3) to determine if fungistatic compounds are involved in fungistasis in this system.

2.2 MATERIALS AND METHODS

A sandy clay loam soil was air-dried, sifted through a 2 mm mesh screen and stored at 21 ± 1 C. One hundred g soil was wetted with water to -0.1 bar and incubated 16-24 hr. One hundred ml water was added and the mixture was shaken for 1 hr. After the suspension was allowed to settle for 5 min, the supernatant contained 14% soil. A fungistatic environment was generated by saturating 10 g sterile clean sand (contained within 5 cm i.d. glass petri dish) with the soil suspension. The final concentration of soil:sand was 4%. In some experiments, the 14% soil suspension was centrifuged for 5 min at 500 g. After filtration through Whatman #1 paper, the soil suspension contained 0.3% soil and when used to saturate sand, the final concentration of soil:sand was 0.1%. Unless indicated otherwise, sand was incubated with the appropriate soil suspension for 16-24 hr at 21 ± 1 C before inoculation with conidia. Routinely used sterile controls included sand saturated with sterile soil extract or sand saturated with 10% White's solution (/L: 8 mg KNO_3, 20 mg $Ca(NO_3)_2 \cdot 4H_2O$, 1.7 mg NaH_2PO_4, 20 mg Na_2SO_4, 36 mg $MgSO_4 \cdot 7H_2O$ and 6.5 mg KCl).

H. victoriae conidia were collected dry with suction from 3 to 5-week-old cultures grown on V-8 juice agar [(200 ml 'V-8 juice' (Campbell Soup Co.), 2g $CaCO_3$, 800 ml H_2O]. The conidia were suspended in cold (2 C) 0.1% Tween 20 and applied to a 0.4 μm Nuclepore membrane filter (Nuclepore Corp., Pleasanton, CA 94566) using mild suction. Approximately 5 x 10^3 conidia, borne on 1 cm^2 pieces of membrane filters, were placed on top of the sand substratum and incubated at 21 C in the dark for either 4 or 6 hr. There were 2 or 3 replicates/treatment. After incubation, the spores were stained with 1.5% Rose Bengal (w/v) in 25% glycerol and 75% ethanol, and stored at 5 C. After the membranes were destained with water, percentage germination was determined microscopically at 150 X. One hundred spores were assessed/membrane; a spore was considered germinated if it contained a germ tube at least 1/2 the width of its conidium.

When indicated, the number of colony forming units/g of the sand-soil mixtures was determined. Duplicate 1 g (wet weight) samples were removed from each replicate and mixed with 10 ml 0.1% water agar. A 10 fold dilution series, most commonly from 10^{-1} to 10^{-8}, was prepared with 0.1% water agar. Individual 1 ml aliquots from the appropriate dilutions were used to cover the surface of two nutrient agar plates. After 4 days, colony forming units were counted.

To determine if a particular size fraction of soil was associated with soil fungistasis, the 0.3% soil suspension was passed through membrane filters with a range of pore sizes from 0.2 to 8 µm. The 8 µm, 0.4 µm and 0.2 µm membranes were produced by Nuclepore and the 5 µm by Gelman (Gelman Sciences, Inc. Ann Arbor, MI 48106).

2.2.1. Sensitivity of fungistasis-inducing organisms to antimicrobial compounds. Before determining which compounds annulled fungistasis, antimicrobial compounds were selected which did not affect the germination of H. victoriae conidia incubated in a sterile environment. The following compounds were individually suspended in sterile 10% White's salts solution and used to saturate a sand substratum for a final concentration of 1,000 ppm active ingredient: streptomycin sulfate, tetracycline, methicillin, novobiocin, rifampin, penicillin g, vancomycin, nitrofurantoin, nalidixic acid, chloramphenicol, mycostatin, 2,3-dihydro-5-carboxanilido-6-methyl-1,4-oxathiin-4,4-dioxide (Oxycarboxin), 2,3-dihydro-5-carboxanilido-6-methyl-1,4-oxathiin (carboxin) 1,4-dichloro-2,5-dimethoxybenzene (chloroneb), sodium (4-(dimethylamino) phenyl) diazenesulfonate (fenaminosulf), 2,6 dichloro-4-nitroaniline (dicloran), or pentachloronitrobenze (PCNB). Technical grades of the fungicidal compounds were generously donated by their manufacturers. H. victoriae conidia were incubated on the treated sand for 6 hr. Any treatments which reduced

germination of H. victoriae to less than 90% of that of a non-treated control were retested successively at 500, 100 and 50 ppm.

The maximum concentration of each compound which did not inhibit H. victoriae germination was added to the suspension with 0.3% soil, mixed, used to saturate sand, and incubated for 16-24 hr. Then, H. victoriae conidia were incubated on the sand substratum for 6 hr to determine which compounds affected fungistasis. Any antimicrobial compounds which annulled fungistasis were successively reduced in concentration to 500, 100, and 50 ppm to determine the lowest of these concentrations in which fungistasis was affected. Fungistasis was considered annulled if germination of the conidia incubated on the sand amended with soil and an antimicrobial compound was 90% or more of the germination of conidia on sand saturated with sterile 10% White's solution. In Tables 2-4 and 2-5, germination is expressed as a percentage of germination in the sterile control.

2.2.2. Evaluation of isolates capable of inducing fungistasis. Seven culture media in three nutritional catagories were used to isolate organisms for testing fungistatic capacity. 1) Low nutrient. a) Soil extract agar (a suspension with 0.3% soil was prepared from 500 g soil as described previously in this section, 1 g glucose, 0.5 g K_2HPO_4, 15 g agar and water for a final volume of 1 L.), b) Water agar (15 g agar in 1 L water) and c) Low

nutrient agar (1% "Bacto" nutrient broth, 15 g agar in 1 L water). 2) High nutrient. a) Modified potato-dextrose agar (10 g dextrose, 2 g yeast extract, 17 g agar, 200 ml potato broth and 800 ml water), b) "Bacto" nutrient agar and c) "V-8 juice" agar. 3) Nutrient agar with reduced O_2. Nutrient agar plates were placed in a desiccator fitted with an inlet and an outlet valve. The desiccator was evacuated and flushed with N_2 three times.

Duplicate 1 g samples of the fungistatic 0.1% soil-sand mixture or the nonfungistatic 10% White's solution-sand mixture were added to 10 ml 0.1% water agar, mixed, and diluted in a 10-fold dilution series with 0.1% water agar. One ml aliquots were used to cover the surface of three plates of each of the seven types of agar medium. Plates were examined under a dissecting microscope after 2,4, and 6 days. Single colonies with differing colonial features were streaked on nutrient agar.

Isolates were removed from 48 hr nutrient agar cultures and individually inoculated into duplicate tubes of sterile 0.3% soil suspension, mixed, used to saturate sterile sand and then incubated 16-24 hr in the dark at 21 $\pm$ 1 C. H. victoriae conidia were inoculated onto the surface as usual. The microbial population was determined as described above, and percentage germination was determined after 6 hr.

Isolates which reduced germination to 10% or less of the non-soil control were added to a sterile suspension

with 0.3% soil mixed, and used to saturate sand. H. victoriae conidia were inoculated within 1 hr. The microbial population and percentage germination were determined as before.

2.2.3. Soil inhibitors. One hundred g aliquots of soil were wetted to -0.1 bar and incubated 16-24 hr. Each aliquot of soil was shaken with either 100 ml chloroform-methanol (2:1, v:v), ethanol, n-butanol, or water for 1 hr. The supernatant was removed, evaporated to dryness in a rotary evaporator operated at 21, 30, or 32 C for chloroform-methanol, alcohol, and aqueous extractions, respectively. The extracts were reconstituted with 0.01 M MES (pH 5) in 10% White's solution so that the chloroform-methanol and water extracts were 12 X more concentrated than the initial soil extract and the alcohols were 2 X more concentrated than the initial extract. The reconstituted alcohol solutions were sterilized by passage through a 0.2 µm Nuclepore membrane filter. (The reconstituted extract was sterilized because in previous experiments conidia incubated on unfiltered solutions germinated less than those in cell-free solutions.) Nuclepore membranes bearing conidia were floated on 1 ml of solutions contained within 20 ml glass scintillation vials for 4 hr at 30 C.

The following experiment was designed to separate inhibitory from stimulatory compounds. A graded series of water-ethanol mixtures (v:v) by 10% increments was

sequentially added and then removed, by vacuum, from the same aliquot of soil. Ethanol is one of the less toxic organic solvents, and it was hoped that ethanol would not cause extensive leakage from soil microflora. Five g soil was wetted to -0.1 bar with water. After 24 hr, 30 ml of water was mixed with the soil, and centrifuged for 5 min at 500 g. The supernatant was decanted and the same soil was mixed with 30 ml of 10% ethanol, centrifuged and decanted as before. Each time this procedure was repeated, the ethanol concentration was increased by 10%, until 100% ethanol was mixed with the soil. The supernatant from each extraction was filtered through Whatman #1 paper, evaporated to dryness in a rotary evaporator at 40 C, and then reconstituted with 4 ml of 0.01 MES (pH5) in 10% White's solution. The solutions were assayed as the previous organic solvent extractions.

To detect volatile inhibitors, petri dishes and air-tight plastic containers, (9.5 cm i.d. X 5.5 cm in height) were used with similar results. Each dish contained conidia borne on two membranes: one placed on top of soil wetted to -0.05 bar or a sterile sand control, and the other on a shallow plastic well which was placed on top of the soil or sand, thus separating the membrane from the soil. The well contained 0.25 ml 0.01 M MES (pH 5) in White's solution. Previous results indicated that the buffer did not affect soil fungistasis.

To trap potential volatile inhibitors, glass petri dishes (5 cm i.d.) containing 5 ml of either water, 2% boric acid, 0.3 or 3 N NaOH or KOH, 2.5 ml 0.3 M $NaHCO_3$ with 2.5 ml 0.3 M Na_2CO_3, or a blank control were placed inside the air-tight containers. The boric acid was intended to remove basic compounds, the NaOH and KOH to remove acidic compounds and the carbonate ions to specifically remove CO_2. The solutions were incubated for 16-24 hr in the containers with a 0.25 cm deep layer of soil, the 0.1% soil-sand mixture, or sand, before membrane filters bearing conidia were incubated on the substratum surface.

In all experiments designed to detect inhibitors, conidia were incubated for 4 hr at 21 C in the dark.

2.3. RESULTS

In order to establish the conditions in which fungistasis could be transmitted to a nonfungistatic environment, autoclaved or nonsterile soil extract containing 0.3% soil, or autoclaved or non-sterile distilled water, was added to autoclaved or nonsterile sand or soil (Table 2-1). H. victoriae conidia incubated on non-sterile soil, sterile sand and autoclaved soil germinated 0, 82, or 98% respectively. When a non-sterile soil suspension was added to sand, the resulting combination inhibited germination of H. victoriae. Non-sterile water did not affect germination of conidia

TABLE 2-1. Germination of Helminthosporium victoriae conidia on sterile or non-sterile sand or soil amended with sterile or non-sterile distilled water or soil suspension.[x]

	% Germination[y]			
	Sterile		Nonsterile	
Substratum	Water	Soil suspension	Water	Soil suspension
Sterile sand	82 ab	82 ab	82 ab	9 c
Nonsterile sand	69 b	82 ab	71 b	3 c
Sterile soil	98 a	98 a	21 c	20 c
Nonsterile soil	0 c	1 c	0 c	0 c

[x] The soil suspension contained 0.3% sandy clay loam and was prepared as described in the text. The sand substratum was saturated with the appropriate solution and incubated for 16-24 hr prior to the inoculation of the conidia.

[y] Spores were incubated for 6 hr. Means which are indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$) are followed by the same letter.

incubated on sterile or nonsterile sand, but did restore fungistasis to autoclaved soil.

Passage of soil suspension through an 8 μm membrane filter did not affect its fungistatic ability, whereas filtration through a 5 μm membrane partially annulled fungistasis (Table 2-2). Fungistasis was fully annulled by passage through 0.8, 0.45, and 0.2 μm filters.

Fungistasis of a soil suspension was also reduced by centrifugation. A suspension containing 14% soil was centrifuged at 0 to 12,000 g for 10 min at 5 C (Table 2-3). After centrifugation, any surface material was removed with a paper towel. The supernatant was pipetted onto sand and the bioassay was performed within 2 hr. Germination on the soil suspension increased as the the centrifugal force increased. The supernatant from a soil suspension centrifuged for 5 min at 6,000 g had no fungistatic capability.

Dilution of the soil suspension with water was the simplest method to partially or fully annul fungistasis. Spores incubated on sand saturated with the undiluted soil suspension germinated 1% whereas spores incubated on a suspension diluted 1:10 germinated 54%. Spores incubated on sand saturated with water or with a soil suspension diluted 1:100 germinated 82%.

2.3.1. Sensitivity of fungistasis-inducing organisms to antimicrobial compounds. Four of the 10 antibacterial compounds and 4 of the 7 antifungal compounds assayed inhibited germination of H. victoriae conidia at all of the

TABLE 2-2. Effect of ultrafiltration of a suspension containing 0.3% soil on its fungistatic capability, as determined by germination of Helminthosporium victoriae conidia.[x]

Pore size, μm[y]	Germination, %[z]
0.2	77 c
0.45	84 c
0.8	76 c
5	31 b
8	0 a
Non-filtered	0 a
Non-soil control	82 c

[x] The soil suspension was prepared as described in the text.

[y] A sand substratum was saturated with the appropriate suspension and incubated for 16-24 hr prior to the inoculation of the conidia.

[z] Germination was determined after 6 hr. Means which are indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$) are followed by the same letter.

TABLE 2-3. Effect of centrifugation of a suspension containing 14% soil on its fungistatic capability, as determined by germination of Helminthosporium victoriae conidia.

Centrifugation, g[x]	Germination, %[y]
0	0 a
500	12 ab
1,000	27 bc
3,000	55 d
6,000	88 e
12,000	86 e
Non-soil control	82 e

[x] The soil suspension was prepared as described in the text and was centrifuged at 5 C for 10 min. A sand substratum was saturated with the appropriate suspension and incubated for less than 2 hr prior to inoculation of the conidia.

[y] Conidia were incubated for 6 hr. Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

concentrations tested (Table 2-4). These compounds were not tested further. Of the 3 antifungal compounds which were not inhibitory to H. victoriae germination, PCNB (500 ppm), carboxin (1,000 ppm) and dicloran (100 ppm) were ineffective in reducing soil fungistasis (Table 2-5). One thousand ppm dicloran significantly reduced fungistasis, but only to a moderate extent. Of the 6 antibacterial compounds, only tetracycline (500 ppm) did not reduce fungistasis. Penicillin G (1,000 ppm) and methicillin (1,000 ppm) annuled fungistasis; penicillin G (100 ppm) moderately reduced fungistasis. Vancomycin (50 ppm), chloramphenicol (50 ppm) and rifampin (50 ppm) eliminated fungistasis since germination was over 90% of the non soil control.

2.3.2. Isolates capable of inducing soil fungistasis. Sand saturated with a soil suspension containing 0.3% soil, after incubation for 24 hr, contained 10^6 colony forming units (CFU)/g of wet soil-sand mixture. 0-3% of the H. victoriae conidia germinated when incubated on the soil extract-sand mixture. Forty-nine isolates obtained from sand saturated either with a fungistatic (soil) or nonfungistatic (sand) substratum were inoculated individually into sterile soil extract and incubated in sand for 24 hr prior to inoculation of H. victoriae conidia. The population of each isolate was determined within 1 hr of the inoculation of the conidia.

TABLE 2-4. Effect of selected anti-microbial compounds on the germination of Helminthosporium victoriae conidia incubated on a sand substratum saturated with 10% White's solution.

Compound	Target population	% Germination at 1000 ppm[V,W]	Germination at concentrations < 1000 ppm	
			ppm[V]	% Germination[W]
Rifampin	Bacteria	98 i	-	-
Methicillin	Bacteria	96 i	-	-
Vancomycin	Bacteria	- [X]	100	100 i
Chloramphenicol	Bacteria	0 a	100	97 i
Penicillin G	Bacteria	99 i	-	-
Tetracycline	Bacteria	38 d	500	94 i
Carboxin	Fungi	92 hi	-	-
Dicloran	Fungi	91 hi	-	-
PCNB	Fungi	42 d	500	94 i
Streptomycin	Bacteria	12 b	50	82 gh
Nalidixic acid	Bacteria	2 a	500	24 c

Table 2-4. (cont'd.).

Compound	Target population	% Germination at 1000 ppm[V,W]	Germination at concentrations < 1000 ppm: ppm[V]	Germination at concentrations < 1000 ppm: % Germination[W]
Novobiocin	Bacteria	0 a	50	87 ghi
Nitrofurantoin	Bacteria	78 g	50	64 f
Oxycarboxin	Fungi	0 a	100	0 a
Fenaminosulf	Fungi	2 a	50	56 e
Mycostatin	Fungi	0 a	500	27 c
Chloroneb	Fungi	0 a	50	50 e

[V] ppm of the antimicrobial compounds were based on sand wet weight.

[W] Germination, expressed as percentage of control, was assessed after 6 hr. The actual percentage germination in the control was 74-100%. Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

[X] - = not determined.

TABLE 2-5. Effect of selected antimicrobial compounds on the fungistatic capability of a suspension containing 0.3% soil, as determined by germination of Helminthosporium victoriae conidia.

	Highest concentration assayed		Lowest concentration assayed	
	ppm	% Germination[x]	ppm	% Germination[x]
Vancomycin	100	92 c	50	91 c
Chloramphenicol	100	96 c	50	97 c
Rifampin	1,000	119 d	50	94 c
Methicillin	1,000	83 c	100	4 a
Tetracycline	500	14 a	-[z]	-
Penicillin G	1,000	96 c	100	53 b
PCNB	500	9 a	-	-
Carboxin	1,000	10 a	-	-
Dicloran	1,000	43 b	100	2 a
Non-soil control	-	100 c		
Soil extract	-	2 a		

[x] Germination, expressed as a percentage of control incubated on a sand substratum saturated with 10% White's solution was assessed after 6 hr; Means followed by the same letter were indistinguishable by the Student-Newman-Keuls Multiple Range test ($P=0.05$).

[y] Actual percentage germination of the nonsoil control was 78-99%.

[z] - Not determined.

Nine isolates had 10^3-10^5, 38 isolates had 10^6-10^7, and 2 isolates had 10^8 CFU/g wet weight. The average percentage germination of the conidia after 6 hr was 50,22, and 10% of the non-soil control, respectively. Thus, germination decreased as the population of microbial cells increased.

In order to examine particular treatment effects, results of the 38 isolates which had 10^6-10^7 CFU/g are presented in Table 2-6; there was no correlation (R=-.24) between inhibition of germination by any of the isolates and concentration of cells in this range. Of these isolates, the 16 organisms isolated from a nonfungistatic environment (sand) induced fungistasis as well as the 22 isolates obtained from a fungistatic soil extract. Nine organisms isolated on low nutrient media were no more effective in inducing fungistasis than 20 organisms isolated on high nutrient media. Nine organisms isolated on high nutrient media with reduced O_2 were no more effective in inducing fungistasis than the 20 from high nutrient agar without provision for reduced O_2.

Nineteen isolates which reduced germination of H. victoriae to less than 10% of the non-soil control value (regardless of the no. CFU/g) were added to soil extract within 1 hr of the time of inoculation of H. victoriae. Since there was no correlation (R=-0.33) between inhibition of germination by any of the isolates and the concentration within the 10^7-10^8 CFU/g range, the 17 isolates in this

TABLE 2-6. Effect of microbes isolated on three types of media from a fungistatic (soil) or a nonfungistatic (non-soil) environment on germination of Helminthosporium victoriae conidia. Each isolate, suspended in sterile soil extract, was used to saturate a sand substratum, and then incubated 24 hr prior to inoculation of the conidia.

	Fungistatic substratum[b]			Nonfungistatic substratum[c]		
Type of medium used to isolate microbes	No. Isolates used	Mean germination[d]	No. of Isolates giving 10% germination[d]	No. Isolates tested	Mean germination[d]	No. of Isolates giving < 10% germination[d]
Low nutrient	4	26	2	5	15	2
High nutrient	12	18	6	8	16	5
High nutrient with low O_2	6	33	1	3	38	1
Total	22	24	9	16	20	8

Table 2-6. (cont'd.).

[a] At the time of inoculation of H. victoriae, there were 10^6-10^7 cells of the microbial isolate/g wet weight of sand.

[b] Microbes were isolated from a sand substratum saturated with a suspension containing 0.3% of a sandy clay loam soil.

[c] Microbes were isolated from a sand substratum saturated with water.

[d] Germination, expressed as a percentage of control incubated on sand saturated with a 10% White's solution, was assessed after 6 hr; s.d. within isolates = 4, with 2 replicates/isolate. The actual percentage germination of the control was 80-85%.

range are included in Table 2-7. These isolates reduced germination to an average of 18% of the non-soil control. These results suggest that prior colonization of the substrate is not required for fungistasis. Results of the previous experiment were confirmed; bacteria induced fungistasis regardless of their isolation from fungistatic (soil) or nonfungistatic (sand) environments or on media with varying nutritional quality.

Eighteen of the 19 isolates which inhibited germination to less than 10% of the control value were bacteria; the other was an actinomycete. Only 1 of the 18 bacteria produced the soluble fluorescent pigment on King's Medium B characteristic of fluorescent pseudomonads. Nine of the bacterial isolates which inhibited germination to 10% or less (when added within an hour of addition of H. victoriae to the sand) were grown in thioglycolate medium to determine O_2 requirements, and nutrient broth to determine shape and motility. Cells from 24-hr-old cultures in nutrient broth were examined under phase contrast microscopy. All nine isolates were obligate aerobes; 8 were motile rods and one was a nonmotile bacillococcus. These isolates and an isolate of Pseudomonas fluorescens, which also inhibited germination to 10% or less, were tested for antibiotic production on nutrient agar. Five x 10^4 conidia in 5 ml of warm nutrient agar were overlaid on 10 ml of this medium in a petri plate. After incubation overnight, 48 hr bacterial cultures (grown on nutrient agar)

TABLE 2-7. Effect of selected microbes isolated on three types of media from a fungistatic (soil) or a nonfungistatic (non-soil) environment on germination of Helminthosporoim victoriae conidia. Each isolate, suspended in sterile soil extract, was used to saturate a sand substratum, which was inoculated with conidia within 1 hr.

	Source of Microbes					
	Fungistatic substratum[b]			Nonfungistatic substratum[c]		
Type of medium used to isolate microbes	No. Isolates tested	Mean germination[d]	No. of Isolates with 10% germination[d]	No. Isolates tested	Mean germination[d]	No. of Isolates causing < 10% germination[d]
Low nutrient	3	33	2	2	6	1
High nutrient	6	15	3	4	3	4
High nutrient with low O_2	1	42	0	1	9	1
Total	10	24	5	7	5	6

Table 2-7. (cont'd.).

[a] At the time of inoculation of H. victoriae, there were 10^7-10^8 cells of the microbial isolate/g wet weight of sand

[b] Microbes were isolated from a sand substratum saturated with a suspension containing 0.3% of a sandy clay loam soil.

[c] Microbes were isolated from a sand substratum saturated with water.

[d] Germination, expressed as a percentage of control incubated on sand saturated with a 10% White's solution, was assessed after 6 hr; s.d. within isolates = 4 with 2 replicates/isolate. The actual percentage germination of the control was 99%.

were streaked onto the agar surface. Plates were observed after 2,4,7, and 11 days. All bacterial isolates reduced the density of growth of the fungus. Similar results were obtained when the bacteria were streaked onto a nutrient agar plate 48 hr before a spore suspension with 10^4 conidia/ml was streaked near the bacterial colonies. Although all the bacterial isolates again reduced the density of growth of H. victoriae, there were no zones of inhibition or hyphal lysis surrounding any bacterial colonies. The cause of the inhibition of growth was not identified since zones characteristic of antibiotics were not observed.

2.3.3. Inhibitors. Extracts of soil made with water, ethanol, butanol or chloroform-methanol did not inhibit H. victoriae germination (Table 2-8). Since the soil extracts increased germination of H. victoriae, it appeared that the soil contained stimulatory compounds. In order to separate stimulatory compounds from possible inhibitors, soil was extracted with a dilution series of ethanol in water from 0-100%. In this experiment, I presumed the theoretical inhibitor was 1) soluble in water, 2) differentially soluble from the stimulatory compounds, and 3) non-volatile. Since germination of the spores in filter-sterilized ethanol extracts of soil was 98-103% of the non-soil control, no ethanol-soluble inhibitors were detected. In order to detect volatile substances in soil, conidia were incubated on soil or on soil extract in sand, with the same results. Spores separated from soil by a plastic barrier germinated

TABLE 2-8. Effect of aqueous and organic solvent extracts of a sandy clay loam soil on germination of *Helminthosporium victoriae* conidia.[x]

	Germination, %	
Treatment	Non-soil control[y]	Treatment[y]
Chloroform-methanol (2:1)	70 b	86 c
Ethanol	66 b	97 c
Butanol	66 b	84 c
Water	70 b	95 c
Non extracted soil	-	0 a

[x] Extracts were prepared as described in the text.

[y] Control conidia were incubated for 4 hr on 1 ml 0.01 M MES (pH 5) in 10% White's solution; "treatment" conidia were incubated on extracts that were evaporated and reconstituted in the control solution; Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

similarly (89%) to spores incubated without any soil, while spores incubated on the soil surface germinated 12%. Thus, there was no evidence that volatile compounds in soil imposed fungistasis. Furthermore, soil or a soil suspension, incubated with trapping solutions for CO_2 or volatile acids or bases, suppressed germination of H. victoriae conidia to the same extent as soil without trapping solutions.

2.4. DISCUSSION

In this paper, a fungistatic substratum was generated by adding a soil suspension to sand. If the soil suspension was poured over sand and incubated 16-24 hr before inoculation of H. victoriae conidia, the degree of fungistasis was greater (0-3% germination) than if the conidia were inoculated within one hour of preparation of the soil suspension (12%). Moreover, there was generally less variability in experiments where suspensions were incubated prior to conidial inoculation.

Historically, researchers have reduced soil fungistasis by: a) the dilution of nonsterile with sterile soil (Chacko and Lockwood, 1966), b) the placement of nutrient agar disks between the spores and the soil (Dix, 1967), c) the addition of a nutrient solution to nonsterile soil (Filonow and Lockwood, 1979), and d) the dilution of soil with silica (Chinn, 1967). All of these assays except that of Chinn (1967) increased concentrations of nutrients in the soil. This could lead to a confusion between the

effects of the inhibition of germination by microbes and the stimulation of germination by nutrients. In this paper, fungistasis was reduced by either the addition of certain antibiotics or by the reduction by dilution, centrifugation or ultrafiltration in the number of soil particles, and hence of microbes, in a soil suspension.

The use of a soil suspension to induce fungistasis has several advantages. 1) As stated previously, this avoids the complications involved when nutrients are added to reduce fungistasis; 2) specific compounds such as antibiotics or nutrients, or microorganisms can be uniformly added to the soil suspension before the bioassay; 3) the suspension contains microsites for microbial attachment (Balkwill and Casida, 1978); 4) since fungistasis is proportional to the concentration of the soil in the suspension, the assay can be used to quantify effects of stimulatory or inhibitory treatments on either germination or growth; and 5) since reduced germination should correlate with the increased concentration of the causal agents of fungistasis, the assay can be used to study the mechanism of soil fungistasis.

When Chinn (1967) diluted 11 soils with silica, only 0.25% of soil was generally required to suppress germination of H. sativum. Here, germination of H. victoriae was suppressed with a concentration of 0.1% soil in sand. The sandy clay loam soil I used apparently contains considerably more fungistatic capacity than is required to suppress H. victoriae germination.

The experiments presented here confirm that soil fungistasis is of microbial origin; fungistasis can be annulled by 50 ppm of either vancomycin, chloramphenicol, or rifampin. Fungistasis can be transferred to sterile soil by inoculation with microbial isolates or with a non-sterile soil suspension. Since non-fungistatic environments contain fungistasis inducing organisms and fungistatic environments become non-fungistatic simply by the dilution of the soil suspension, expression of fungistasis appears dependent upon a sufficient concentration of microbiota. This is supported further by the correlation between suppression of germination of H. victoriae conidia and the number of microbial cells in a soil suspension.

The present experiments also indicated the nonspecific nature of the organisms which induce fungistasis. Previously, vancomycin and chloramphenicol were used to suppress the fungistasis of Glomerella cingulata conidia (Lingappa and Lockwood, 1961) and Phytophthora cinnamomi chlamydospores (Mircetich and Zentmyer, 1969) incubated in soil. Therefore, the organisms responsible for fungistasis of G. cingulata and P. cinnamomi may also suppress germination of H. victoriae. Moreover, most bacterial isolates isolated from either fungistatic (soil extract) or non-fungistatic (non-soil) environments inhibited germination of H. victoriae conidia. Griffin (1962) and Lockwood and Lingappa (1963) previously demonstrated that most organisms (including bacteria, actinomycetes, and

fungi) restored fungistasis when inoculated into autoclaved soil. The present experiments demonstrated that the fungistasis-inducing organisms do not require the relatively large quantities of free nutrients in autoclaved soil in order to induce fungistasis. Since neither nutrients nor prior colonization of the substrate was required for suppresion of germination, it seems unlikely that inhibitory "staling products" (Robinson and Park, 1966) cause fungistasis.

Hattori and Hattori (1980) suggested that growth of many soil bacteria was supported by 1% nutrient broth and suppressed by 100% nutrient broth. I found no evidence that organisms isolated on low nutrient media suppressed germination of H. victoriae conidia significantly better than bacteria isolated on full strength nutrient media. Similarly, organisms isolated on media with low oxygen content were no more effective in suppressing germination than media with atmospheric oxygen.

Balkwill and Casida (1978) demonstrated that soil bacteria were attached to soil particles. Fungistasis-inducing organisms are apparently similarly attached. Ultrafiltration of an aqueous soil suspension with a 5 µm membrane significantly reduced fungistasis, whereas many organisms capable of inducing fungistasis could pass through this pore size. In addition, only soil extracts which contained visible soil particles were fungistatic.

Fungistatic compounds, especially of a volatile nature, were found in some soils (Griffin et al., 1975; Pavlica et al., 1978; Balis and Kouyeas, 1978), although such compounds were not detected in three fungistatic soils (Lingappa and Lockwood, 1961; Lockwood, 1977). In the present work, there was no evidence that antibiotics or other inhibitory compounds, either volatile or non-volatile, caused fungistasis in a sandy clay loam soil.

Watson and Ford (1972) and Hora et al. (1977) argued that soil fungistasis is dependent upon a complex interaction between inhibitory and stimulatory compounds in soil. A complex system was postulated partly because the inhibitory compounds identified thus far have variable effects on different species of fungi or appear limited to specific habitats. The results here confirmed that fungistasis is caused non-specifically by micro-organisms (Lockwood, 1977). Thus, it seems that if fungistasis is caused by inhibiting compounds, one must postulate a fairly simple situation where inhibition is caused by a limited number of widely occuring microbial metabolites. If a complex scenario of inhibitors and stimulators is postulated, then it seems more likely the complexity is in the fungal spore's ability to sense the environment. Ko and Lockwood (1967) and Lockwood (1977; 1981) postulated a fairly simple mechanism by which a spore could determine the suitability for germination in a nonsterile environment. That hypothesis will be addressed in the following two chapters.

2.5. SUMMARY

A fungistatic environment was generated by the saturation of sand substratum with an aqueous suspension containing 0.3 or 14% of a sandy clay loam soil. Fungistasis was reduced by 1) the addition of 50 ppm of either vancomycin, chloramphenicol or rifampin or 2) by the reduction of the number of soil particles, and hence microbes, in a soil suspension by dilution, centrifugation or ultrafiltration. Forty-nine microbial isolates with differing colony morphology were obtained on several culture media from either fungistatic (soil extract) or non-fungistatic (non-soil) environments. The isolates were inoculated individually into sterile soil extract, which was used to saturate sand. Most of the bacterial isolates inhibited germination of Helminthosporium victoriae conidia, regardless of the medium used for the initial isolation. Prior colonization of the sand substratum was not required for the isolates to suppress conidial germination. There was no evidence that inhibitory substances, (antibiotics or other inhibitory compounds, either volatile or non-volatile) caused fungistasis. The results confirmed that fungistasis is caused nonspecifically by microorganisms.

2.6. LITERATURE CITED

Balis, C. and V. Kouyeas. 1978. Contribution of chemical inhibitors to soil mycostasis. p. 97-106. In B. Schippers and W. Gams (eds.). Soil-borne plant pathogens. Academic Press. London.

Balkwill, D.L. and L.E. Casida, Jr. 1978. Attachment to autoclaved soil of bacterial cells from pure cultures of soil isolates. Appl. Environ. Microbiol. 37:1031-1037.

Chacko, C.I. and J.L. Lockwood. 1966. A quantitative method for assaying soil fungistasis. Phytopathology 56:576-577.

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

Dix, N.J. 1967. Mycostasis and root exudation: factors influencing the colonization of bean roots by fungi. Trans. Brit. Mycol. Soc. 58:59-66.

Filonow, A.B. and J.L. Lockwood. 1979. Conidial exudation by Cochliobolus victoriae on soils in relation to soil mycostasis. p. 107-119. In B. Schippers and W. Gams (eds.). Soil-borne plant pathogens. Academic Press. London.

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

Griffin, G.J., T.S. Hora and R. Baker. 1975. Soil fungistasis: elevation of the exogenous carbon and nitrogen requirements for spore germination by fungistatic volatiles in soils. Can. J. Microbiol. 21:1468-1475.

Hattori, R. and T. Hattori. 1980. Sensitivity to salts and organic compounds of soil bacteria isolated on diluted media. J. Gen. Appl. Microbiol. 26:1-14.

Hora, T.S. R. Baker and G.J. Griffin. 1977. Experimental evaluation of hypothesis explaining the nature of soil fungistasis. Phytopathology 67:373-379.

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

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

Lockwood, J.L. 1977. Fungistasis in soils. Biol. Rev. 52:1-43.

Lockwood, J.L. 1981. Exploitation competition, p. 319-349. In D.T. Wicklow and G.C. Carroll (eds.). The fungal community. Marcel Dekker, Inc., N.Y.

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

Mircetich, S.M. and G.A. Zentmyer. 1969. Effect of carbon and nitrogen compounds on germination of chlamydospores of Phytophthora cinnamomi in soil. Phytopathology 59:1732-1735.

Pavlica, D.A., T.S. Hora, J.J. Bradshaw, R.K. Skogerboe and R. Baker. 1978. Volatiles from soil influencing activities of soil fungi. Phytopathology 68:758-765.

Robinson, P.M. and D. Park. 1966. Volatile inhibitors of spore germination produced by fungi. Trans. Brit. Mycol. Soc. 49:639-649.

Watson, A.G. and E.J. Ford. 1972. Soil fungistasis - a reappraisal. Ann. Rev. Phytopathol. 10:327-348.

CHAPTER 3

EFFECT OF TEMPERATURE, PH AND WATER POTENTIAL ON GERMINATION OF HELMINTHOSPORIUM VICTORIAE CONIDIA IN STERILE AND NON STERILE CONDITIONS.

3.1. INTRODUCTION

Conidia of Helminthosporium victoriae Meehan and Murphy (=Cochliobolus victoriae Nelson, =Drechlera victoriae (Meehan and Murphy) Subram. and Jain) have been used as model propagules in experiments on soil fungistasis (Lockwood, 1977). These conidia will germinate, without any special stimulus, in the absence of any organic or inorganic compounds, as long as the environment is free of other microbes. Yet, these spores will not germinate in nonsterile conditions, unless a carbonaceous energy source becomes available. This inhibition of germination in soil, termed "fungistasis," can be simulated in two axenic model systems. In one, conidia are incubated on a sand substratum which is continuously percolated with water, phosphate buffer or a dilute salt solution (Ko and Lockwood, 1967; Lockwood, 1981); in the other, conidia are floated on relatively large (40 or 80 ml) volumes of a dilute salt solution containing 0.05 M MES buffer (pH 6.1) (Epstein and Lockwood, 1981).

Since fungistasis occurs in soil where, in a sterile environment, the pH, temperature and moisture favor germination, the question arises whether the model systems similarly suppress germination in conditions which are

optimum for spore germination. Thus, the purpose of this research was 1) to identify the optimal pH, temperature, and water potential for germination of H. victoriae conidia in axenic conditions and 2) to determine if optimizing environmental conditions would affect fungistasis in soil, or the two model, axenic systems.

3.2. MATERIALS AND METHODS

Spores were produced on either V-8 juice agar [200 ml 'V-8 juice' (Campbell Soup Co.), 2 g $CaCO_3$, 800 ml H_2O] or a defined medium (10g glucose, 10g xylose, 2g asparagine·H_2O, 1.5g KH_2PO_4, 0.75g $MgSO_4 \cdot 7H_2O$, 20g Difco Noble agar, 0.1 mg each: $CuSO_4$, $Fe(SO_4)_3$, $ZnSO_4$, and $Na_2 MoO_4 \cdot 2H_2O$, and 1L H_2O). Sugars and asparagine were autoclaved separately.

Conidia were produced, collected, dispersed, stained and assessed for germination as described in Chapter 2.

3.2.1. Environmental optima in axenic conditions. Conidia borne on Nuclepore membrane filters (0.4 μm pores) were floated on either 1 ml of various aqueous solutions in 20 ml glass scintillation vials, or on 40 or 80 ml solutions in 50 or 100 ml glass beakers. To determine the effects of temperature on germination, conidia were incubated on 1 ml of 10% White's salts solution for 6 hr. For temperature studies, incubators were maintained at 5 C increments from 10-35 C.

For pH studies, conidia were incubated at 21 ± 1 C for 6 hr on 1 ml of 6 different buffers ranging in pH from 3.0-8.5. The buffers, concentrations of stock solutions used, and specific pH range tested (in 0.5 pH unit increments) were as follows: citrate buffer made with 0.05 M citric acid and 0.05 M sodium citrate for pH 3-6, citrate-phosphate buffer made with 0.1 M dibasic sodium phosphate and 0.05 M citric acid for pH 3-7, acetate buffer with 0.1 M acetic acid and 0.1 M sodium acetate for pH 4-5, (2-(N-morpholino) ethanesulphonic) acid buffer (MES) made with 0.1 M MES and 3 N NaOH or KOH for pH 5-7, phosphate buffer made with 0.1 M mono and dibasic sodium phosphate for pH 6-8 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid buffer (HEPES) made with 0.1 M HEPES and 3 N NaOH for pH 6.5-8.5. All buffers were made in 10% White's solution and were prepared according to procedures described by Gomori (1955) or Perrin and Demsey (1974). Solution pH was verified with a pH meter.

To determine the effects of osmotic potential (Ψ_s) on germination, conidia were incubated at 21 ± 1 C on membrane filters floated on solutions with 5 different osmotic values. The volumes of solution, the concentration ranges of the osmotica tested, and the incubation periods were as follows: 80 ml of 0 to 512 g polyethylene glycol (PEG) 4000/kg H_2O for 8 hr or 40 ml of 0 to 512 g PEG 6000/kg H_2O for 8 hr, 1 ml of 0 to 1.0 molal KCl for 8 hr; 0 to 0.2 M MES (pH 5.6) for 8 hr, 40 ml of 0 to 1 X

Hoagland's salt solution for 8 hr, and 40 ml of 0 to 10 X White's salt solution for 8 hr. Ψ_s of PEG 4000 and 6000 solutions were determined from a standard curve for values <-1 bar generated on a dew point microvoltmeter (HR33T Wescor Inc., Logan, Utah 84321). The Ψ_s for KCl was interpolated from a table published by Slavik (1974), and the Ψ_s of White's and Hoagland's solutions were approximated using the formula Ψ_s = (sum of meq/L of cations or anions) X (-0.036) (Bohn et al., 1979).

To determine the effects of matric potential (Ψ_m), sieved sandy clay loam soil was prepared on a ceramic pressure plate to achieve a range of Ψ_m from 0 to 5 bars. A layer of soil with an estimated Ψ_m, 1 cm in depth, was placed in glass petri dishes 10 cm i.d. For treatments requiring sterile soil, the soil was autoclaved for 1 hr, allowed to cool, and then excess moisture was wiped from the lid of the dish with sterile paper towel. Spores, deposited inside 1 in^2 membrane filters that were folded in half, were buried in sterile or nonsterile soil for 4 or 6 hr, respectively. At the end of each experiment, the soils were weighed, dried and then reweighed in order to determine % moisture. Matric potentials were determined from a curve generated on a ceramic pressure plate (Slavik, 1974).

3.2.2. Effect of environmental optima on soil fungistasis. H. victoriae conidia do not germinate when incubated on the surface of soil (Lockwood, 1977). To

determine the effects of environmental optima for germination on soil fungistasis, 10 g of an air-dried sandy clay loam soil contained in glass petri dishes (5 cm i.d.), were wetted to -0.05 bars with water, 0 to 0.1 M MES (pH 5.1), or 0 to 1 X White's solution, and incubated for 24 hr in the dark at 21±1 C or 30 C. Then conidia on 0.4 µm Nuclepore membranes were incubated on the soil surface for 6 hr at the same temperatures.

In order to detect relatively small environmental effects on soil fungistasis, the degree of fungistasis in soil was reduced by 1) using an aqueous suspension of soil to saturate sand and 2) by amending the soil suspension with a series of concentrations of yeast extract or sucrose-peptone solutions (5:1 w/w). To prepare the soil suspension, 100 g aliquots of the air-dried sandy clay loam soil were wetted to -0.1 bar with water and incubated 16-24 hr. One hundred ml of either water ("sub-optimal" conditions) or 0.1 M MES (pH 5.0) in 10% White's solution ("optimal" conditions) was added and the suspension was shaken for 1 or 24 hr. Four ml of the suspension, containing 43% soil, was added to 0-10 mg yeast extract or sucrose peptone (5:1 w/w) in 0.1 ml H_2O, mixed and used to saturate 12 ml sand. Conidia were incubated in darkness on the surface of the soil-sand mixture at 21 ± 1 C ("sub-optimal conditions") or 30 C ("optimal conditions").

3.2.3. Effect of environmental optima on model systems. The "leaching system" has been described

elsewhere (Lockwood, 1977; 1981). Essentially, when fungal propagules on a membrane filter were incubated on sand through which an aqueous solution was continually percolated, germination was inversely correlated with flow rate. To determine the effects of environmental conditions on the fungistatic effect of the leaching system, the apparatus was either incubated at 30 C ("optimal" conditions) or 20 C ("sub-optimal" conditions). Conidia were leached for 4 hr at variable flow rates with 0.01 MES (pH 5.0) in 10% White's solution (optimal) or 10% White's solution (pH 5.8) (sub-optimal).

In the "static volume system," conidia also borne on Nuclepore membrane filters were floated on variable volumes of an aqueous solution; germination was inversely correlated with volume. Solutions and incubation temperatures used were the same as those used for leaching system.

3.2.4. Effect of environmental optima on conidial exudation. Sneh and Lockwood (1976) established a positive correlation between the loss of spore exudate and the decreased germination of H. victoriae conidia incubated on the leaching system. To determine the effect of environmental optima on exudation, ^{14}C-labelled conidia were produced by spreading 1 ml of an aqueous solution containing 5μCi uniformly labelled glucose onto the surface of a glucose-asparagine agar plate. After 24 hr, the plates were inoculatd with a mycelial plug. Conidia from

3-week-old cultures were removed dry from the agar using suction, and then were incubated on the leaching system in the conditions indicated previously. After 30 min, the volume of solution containing exudate was measured, and the exudate was frozen and lyophilized. Exudate was reconstituted in 5 ml H_2O, refrozen in scintillation vials, lyophilized again and then counted in 10 ml of a scintillation cocktail containing toluene:Triton X-100 (2:1) and 4 Omnifluor/L. ^{14}C in conidia was determined by dissolving the conidia and Nuclepore membrane in 0.3 or 0.6 ml chloroform and then suspending the sample in 10 ml of a cocktail consisting of toluene:methanol (3:1, v:v), 4.2 g Omnifluor and 40 g Cab-o-sil/L. Samples were counted on a Packard Tri-Carb Liquid Scintillation Spectrometer for 5 or 10 min. Background counts were determined for a range of different quantities of salts and buffer; however, there were no significant differences. Counting efficiency was determined using a ^{14}C-toluene standard.

3.2.5. Experimental design and statistical analysis. There were 3 replicates/aqueous treatment and 2 or 3 replicates/soil treatment arranged in a completely randomized design; each experiment was performed at least twice to establish reproducibility of results. Each experiment was analyzed with the F test ($P=0.05$). Bartlett's test for homogenity of variance ($P=0.05$) indicated variance was homogeneous and transformation of data was unnecessary. Significant differences between

means in experiments with significant F tests (P=0.05) were detected by the Student-Newman-Keuls Multiple Range Test (P=0.05). When germination was monitored over time, a least squares linear regression was made on a probit transformation of the percentage germination (Finney, 1964). This procedure transforms a sigmoidal curve into a straight line.

3.3. RESULTS

3.3.1. Environmental optima in axenic conditions.

The optimal temperature for germination of H. victoriae conidia incubated in 10% White's solution was approximately 30 C (Figure 3-1). After 6 hr incubation, significantly fewer conidia had germinated at 20 C than at 25-35 C. No conidia germinated at 10 C.

Six buffers were used to determine the optimal pH for germination of H. victoriae conidia (Figure 3-2). Germination occurred most rapidly at pH 5 in citrate and citrate-phosphate buffers; there was significantly less germination at pH 3 and 7 in citrate buffer and at pH 3,6, and 7 in citrate-phosphate buffer. With phosphate, MES and HEPES buffers, germination decreased as pH increased above 5. There were significant differences between germination in different buffers maintained at the same pH. At pH 5, there was significantly less germination in citrate-phosphate or citrate buffers than in MES buffer. No conidia germinated in acetate buffer at pH 4-5 (data not

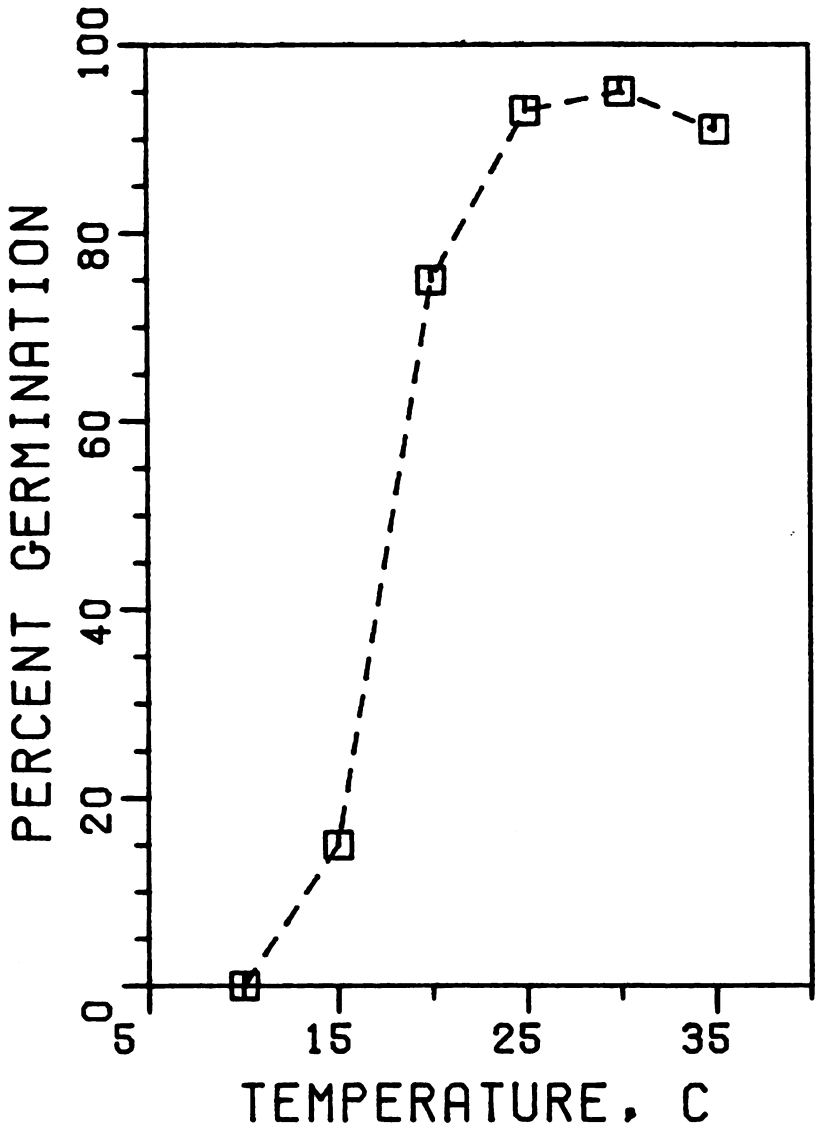

Figure 3-1. Effect of temperature on germination of Helminthosporium victoriae conidia. Spores were incubated for 6 hr on membrane filters floated on 1 ml of 10% White's salts solution; s.d.=4.

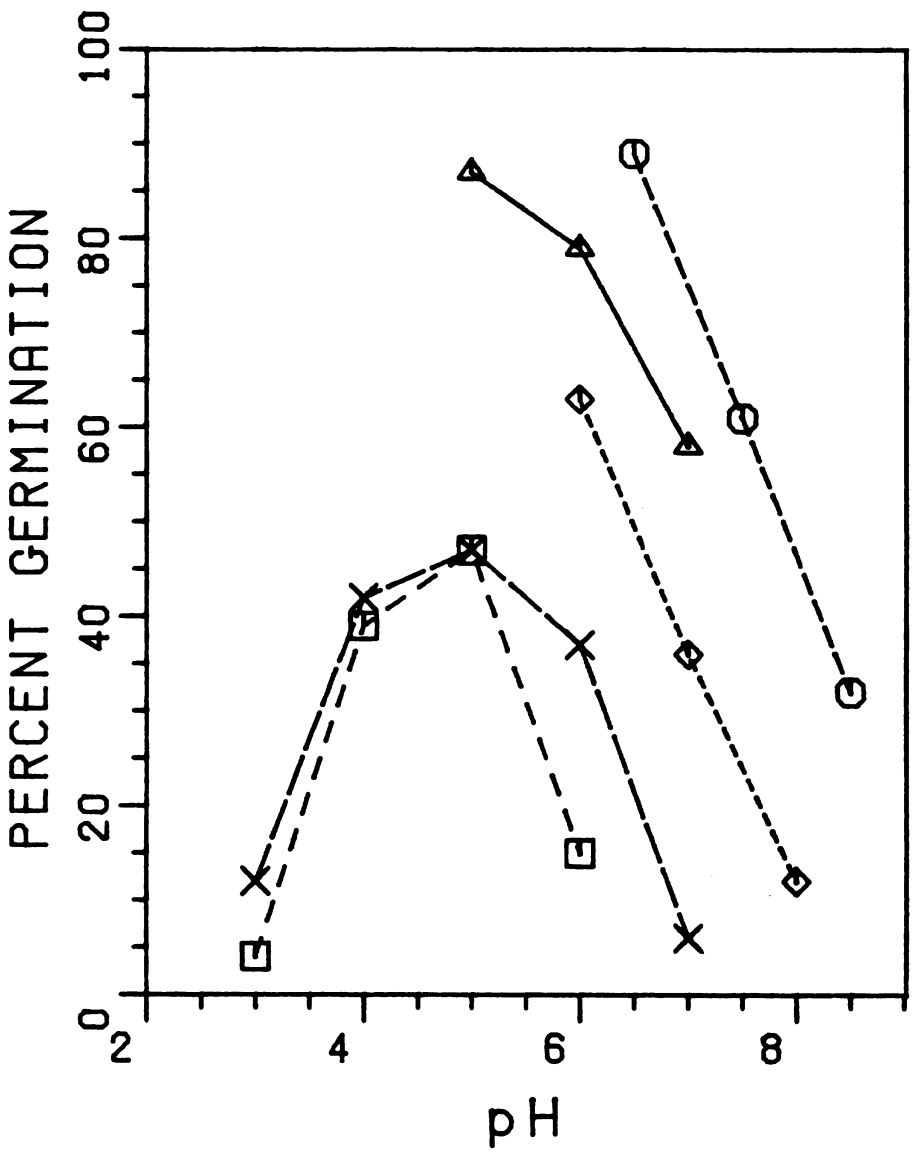

Figure 3-2. Effect of pH with selected buffers on germination of Helminthosporium victoriae conidia. Spores were incubated for 6 hr on membrane filters floated on 1 ml of buffer in 10% White's solution: s.d.=13; X=citrate, □= citrate-phosphate, ◇= phosphate, △= MES, ○=HEPES.

shown). Conidia incubated at pH 7 in MES germinated to a greater extent than those incubated at pH 7 in phosphate buffer. Germination decreased as pH of MES increased, regardless of whether MES was neutralized with NaOH or KOH (data not shown). In subsequent experiments, MES buffer with NaOH (pH 5.0) was used to maintain an optimum pH for germination.

To determine the effect of water potential on germination, spores were incubated on varying concentrations of PEG 4000 and 6000, KCl, MES buffer, and White's and Hoagland's solutions. Osmotic potentials optimal for H. victoriae germination were -0.2 bar in PEG 6000, -0.5 bar in MES (pH 5.6) and -1.0 bar in KCl (Figure 3-3). PEG 4000 and 6000 gave similar results, although in one experiment with PEG 4000, the optimum extended from -0.2 to -3 bars (data not shown). With all osmotica, there was significantly more germination at the optimum Ψ_s than at 0 bar, or at potentials of -5 bars or less. Germination was detected at -9 bars in KCl and at -10 bars in either PEG, but not at -50 bars in KCl or -34 bars in PEG 4000 or 6000. Since 0.01 M MES provided an optimal Ψ_s for germination, this concentration was used in subsequent experiments. These experiments were performed at different times and in different volumes of solution; thus, comparisons of germination in different osmotica should be interpreted conservatively.

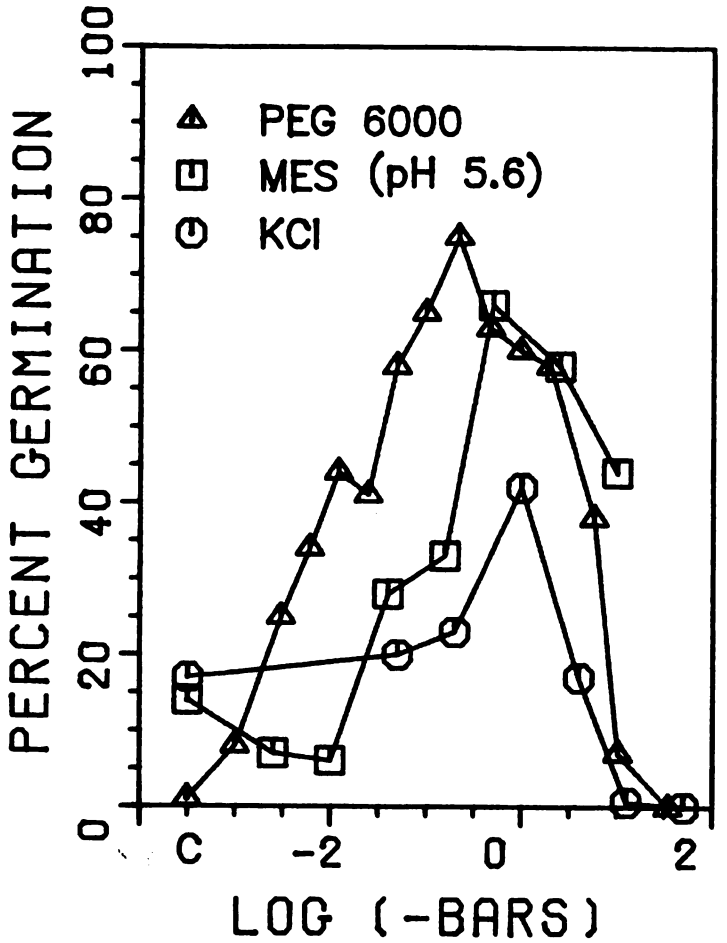

Figure 3-3. Effect of a range osmotic potentials provided by PEG 6000, MES (pH 5.6), and KCl on germination of Helminthosporium victoriae conidia incubated for 8 hr on membrane filters floated on 40, 40, and 1 ml of solution, respectively; C = water control; s.d. = 6, 14 and 4, respectively.

Significantly more conidia germinated in 10 X White's (= -0.9 bar) or 1 X Hoagland's (= -1.2 bar) than in water (Figure 3-4). In order to determine if the increased germination was caused by a specific cation or an osmotic effect, conidia were incubated for 8 hr on 40 ml of either H_2O or 1 mM NaCl, KCl, or $CaCl_2$ (10 X White's solution contains approximately 1 mM Na^+, K^+ and Mg^{++} and 10 mM Ca^{++}). Since none of the treatments significantly stimulated germination, the increased germination in 10 X White's solution was possibly an osmotic effect.

When conidia were incubated in non-sterile soil for 6 hr, there was no germination in Ψ_m ranging from 0 to -5 bars. In sterile soil, germination after 4 hr increased as the matric potential increased from -11 to 0 bars (Table 3-1). Conidia incubated at 0 bar and -0.05 bar germinated significantly more than conidia incubated at -0.3 to 11 bars. None of the conidia germinated at -15 bars. In another experiment, conidia germinated 95% after 2 hrs in sterile soil at 0 bar; conidia incubated at -0.6 bar germinated 42% in 2 hr, and 86% in 4 hr (Table 3-2). There were no significant differences between germination of conidia incubated inside membranes with pore sizes of 0.2, 0.45 or 8.0 μm in sterile soil (data not shown).

Conidia were incubated at either 20 or 30 C on 2 ml of either 10% White's solution (pH 5.8) or 0.01 M MES (pH 5.0) in 10% White's solution (The buffer was used both to reduce the pH and to decrease the water potential). Since there

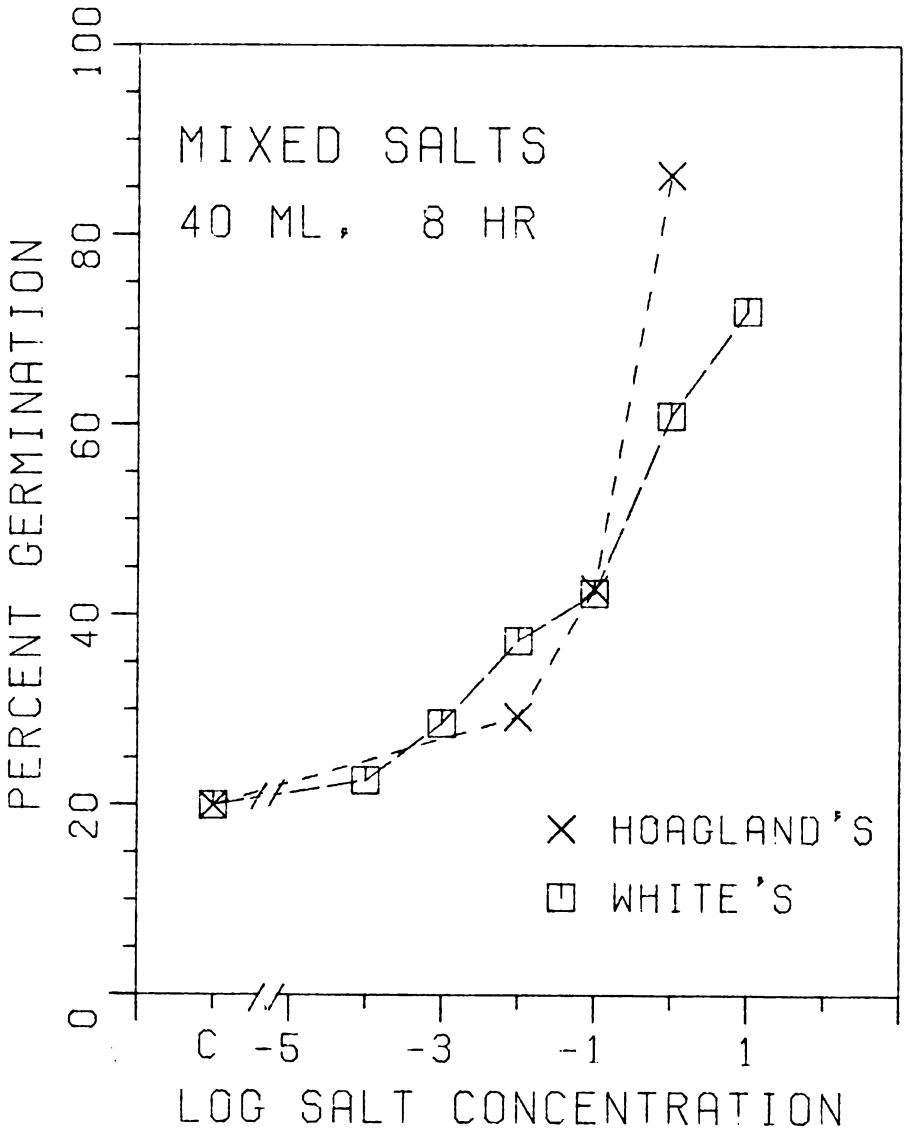

Figure 3-4. Effect of concentration of White's and Hoagland's salts solutions on germination of Helminthosporium victoriae conidia incubated for 8 hr on membrane filters floated on 40 ml solution; C = water control; s.d.=18.

TABLE 3-1. Effect of matric potential on germination of *Helminthosporium victoriae* conidia enclosed within membrane filters and buried for 4 or 6 hr in an autoclaved or nonsterile sandy clay loam soil, respectively.

Soil	Matric potential, - bars	Germination, %
Sterile	0	84 a
	0.05	80 a
	0.3	60 b
	0.6	51 b
	1	52 b
	11	44 b
	15	0 c
Nonsterile	0	2 c
	0.1	0 c
	0.4	1 c
	0.6	1 c
	1.1	0 c
	1.4	0 c
	3	0 c
	5	0 c

[a] Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

TABLE 3-2. Effect of matric potential on germination of Helminthosporium victoriae conidia enclosed in membrane filters and buried in an autoclaved sandy clay loam soil.

	Germination, %[a]	
Time, hr	0 bars	-0.6 bars
0	0 a	0 a
2	95 c	42 b
4	97 c	86 c
6	100 c	100 c

[a] Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

was greater germination of conidia incubated at 30 C on 0.01 M MES in 10% White's solution than in any other solution, this was considered "optimal" and incubation at 20-22 C on 10% White's solution (pH 5.8) was considered "suboptimal" (Table 3-3). It was not determined whether the stimulation by MES was due to a decrease in pH, water potential, or a combination of both. The Ψ_s of 10% White's salts and .01 M MES (pH 5.0) in 10% White's salts are approximately -0.09 and -0.5 bars, respectively.

3.3.2. Effect of environmental optima on soil fungistasis. Germination of H. victoriae conidia incubated on nonsterile soil ranged from 0-3% regardless of matric potentials which ranged from 0 to -5 bars (Table 3-1) or an increase in temperature from 21 to 30 C. Germination of conidia on soil was similarly unaffected by concentrations of MES from 0 to 0.1 M (pH 5.1) or White's solution from 0 to 10 X. Soil fungistasis was not affected when soil was saturated with 0.01 M MES (pH 5.0) in 10% White's solution and incubated at 30 C.

Since optimizing Ψ_m, Ψ_s, temperature or pH did not appear to affect soil fungistasis, the environmental effects on spore germination were assayed on a modified soil with reduced fungistasis. Unfortunately, pH and Ψ_s were not optimized simultaneously; MES at pH 5.0 has weak buffering capacity and 0.1 M MES (pH 5.0) was required to reduce the pH. Thus, the Ψ_s was less than optimal. For these experiments, extraction of soil with 0.1 M MES (pH

TABLE 3-3. Effect of 10% White's salts solution (pH 5.8) and 0.01 MES buffer (pH 5.0) in 10% White's solution at 20 or 30 C on germination of Helminthosporium victoriae conidia incubated for 6 hr on membrane filters floated on 2 ml of solution.

Solution	Temperature, C	Germination, %
White's	20	40 a
MES	20	76 b
White's	30	74 b
MES	30	91 b

[x] Means followed by the same letter were indistinguishable by the Student-Newman-Keuls range test ($P=0.05$).

5.0) and incubation of conidia at 30 C was considered "optimal" and extraction of soil with H_2O and incubation of conidia at 21 C was considered "sub-optimal." The pH's of the soil suspension shaken for 1 hr with 0.1 M MES (pH 5.0) or water were 5.2 and 5.7, respectively. After the soil was shaken for 1 or 24 hr, a nutritional supplement was added to the soil suspension. The soil suspension was used to saturate sand and conidia were inoculated within 1 hr. When a soil suspension was prepared by shaking for 1 hr prior to inoculation of spores, there was significantly greater germination of spores incubated with 0-10 µg yeast extract/sample at optimal than sub-optimal conditions (Figure 3-5A). However, similar quantities of yeast extract were required to completely annul fungistasis of conidia incubated in both optimal and sub-optimal conditions. When the soil suspension was prepared by shaking for 24 hr with buffer or water, there was no germination of conidia incubated at optimal or sub-optimal conditions with 0-10 µg yeast extract (Figure 3-5B) or sucrose-peptone (5:1 w/w) (Figure 3-6). More nutrients were required to annul fungistasis of conidia incubated in optimal than in sub-optimal conditions.

3.3.3. Effect of environmental optima on fungistasis in the leaching system. When conidia were leached in "sub-optimal" conditions for 4 hr with 10% White's solution (pH 5.8) at 20 C, germination decreased from 71 to 2% as flow rate increased from 0 to 176 ml/hr

Figure 3-5. Effect of environmental conditions on the annulment of soil fungistasis by yeast extract. The soil suspension was prepared by shaking 100 g soil, wetted to -0.1 bar with water, with either 100 ml 0.1 M MES (pH 5.0) in 10% White's solution ("optimal conditions") or 100 ml H_2O ("suboptimal conditions") for either 1 (Figure 3-5A) or 24 (Figure 3-5B) hr. Four ml of the soil suspension was mixed with 0-10 mg yeast extract and used to saturate sand, on which conidia were incubated for 6 hr at 30 C (optimal conditions) or 20 C (suboptimal conditions); s.d.=3; C = unamended control.

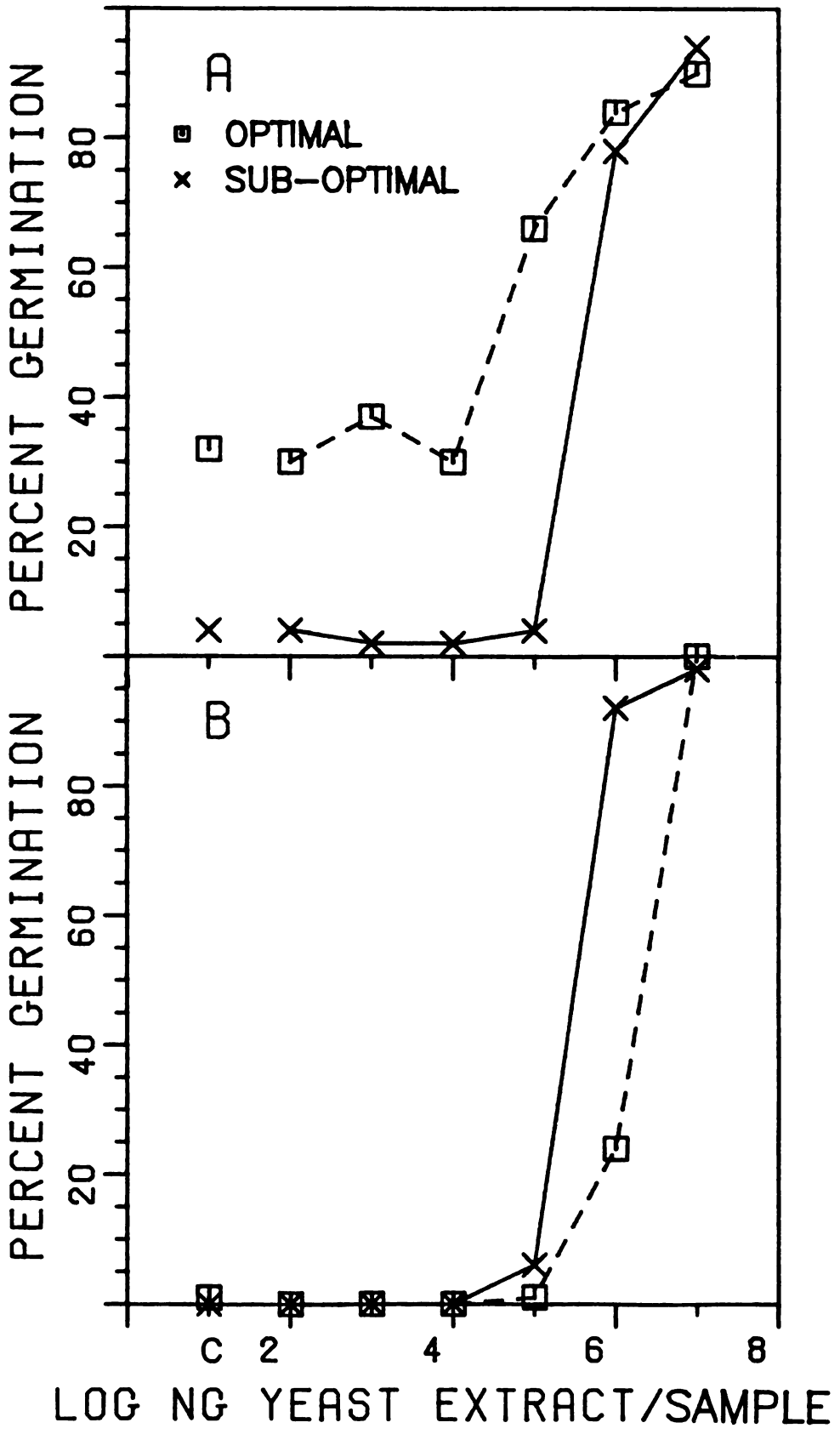
A
OPTIMAL
SUB-OPTIMAL
PERCENT GERMINATION
20
40
60
80
B
PERCENT GERMINATION
20
40
60
80
C
2
4
6
8
LOG NG YEAST EXTRACT/SAMPLE

Figure 3-5

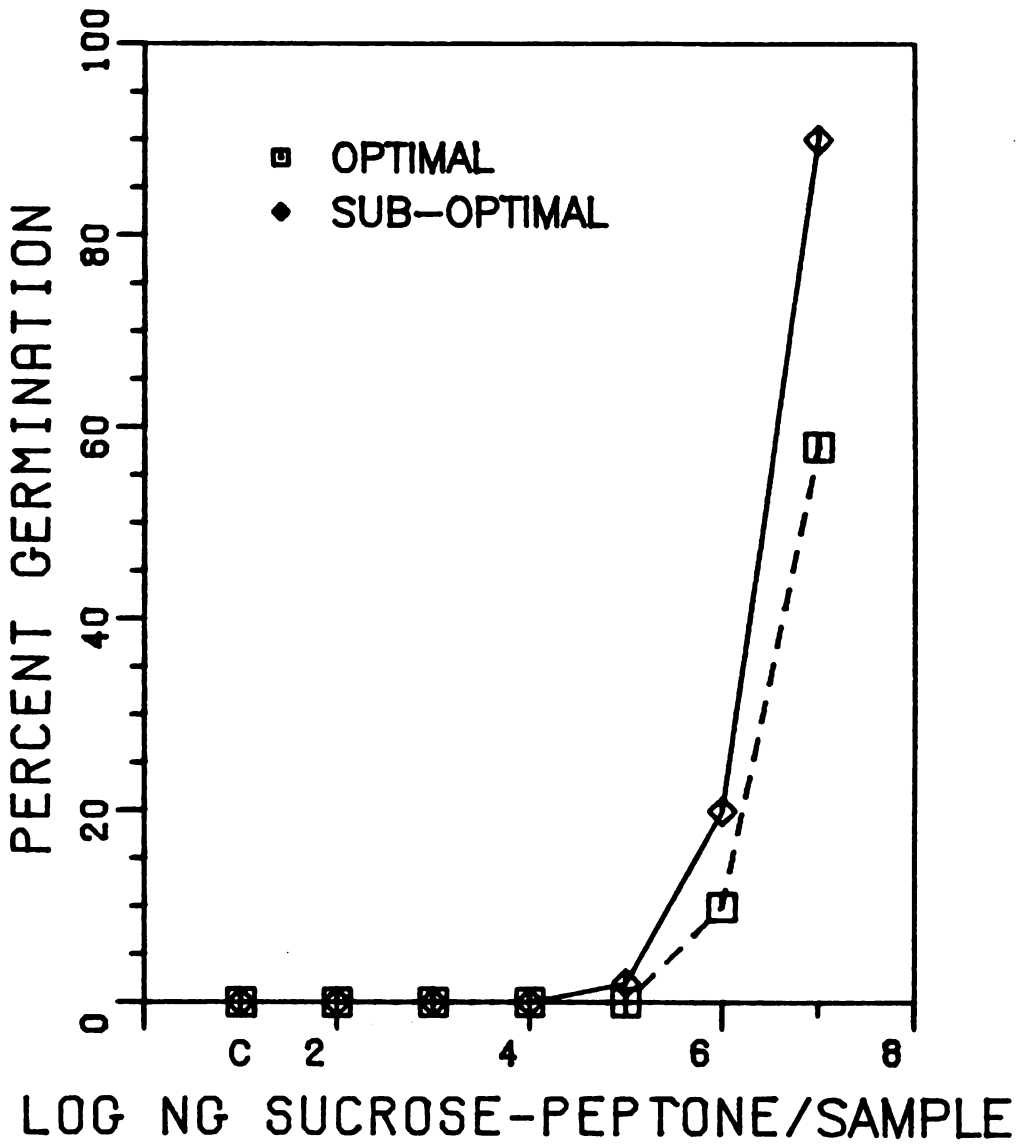

Figure 3-6. Effect of environmental conditions on the annulment of soil fungistasis by sucrose-peptone (5:1, w:w). A suspension was prepared by shaking 100 g soil, wetted to -0.1 bar with water, with either 100 ml 0.1 M MES (pH 5.0) in 10% White's solution ("optimal conditions") or 100 ml H_2O ("suboptimal conditions") for 24 hr. Four ml of the soil suspension was mixed with 0-10 mg sucrose-peptone and used to saturate sand. Conidia were incubated for 6 hr at 30 C (optimal conditions) or 20 C (suboptimal conditions); s.d.=3; C = unamended control.

(Figure 3-7). However, when conidia were leached in optimal conditions for germination with 0.01 M MES, (pH 5.0) in 10% White's solution at 30 C, germination was greater than 77%, regardless of flow rates from 0-136 ml/hr, or in another experiment, at a flow rate of 208 ml/hr. Thus, the leaching system's fungistatic capacity occurred only when the system was operated in conditions which were less than optimal for germination. Exudate in the first 30 min ranged from 0.01-0.3% and from 0.01-0.5% of the total ^{14}C in the spores in two experiments. As flow rate increased, exudation increased regardless of the conditions or the extent of subsequent germination. Consequently, exudation was correlated directly with flow rate, but was correlated inversely with germination only under conditions sub-optimal for germination.

3.3.4. Effect of environmental optima on suppression of germination on the static volume system. Conidia were incubated in "optimal" [0.01 M MES (pH 5.0) in 10% White's solution at 30 C] or "sub-optimal" conditions (10% White's solution at 20 C) on 1, 40 or 80 ml of solution. The rate of conidial germination decreased as the volume increased. Optimizing environmental conditions in a given volume increased the rate of germination (Figure 3-8). The environmental conditions did not appear to affect the time of appearance of the first germ tubes. Statistics from a linear regression of the probit transformation of the percent germination are shown in Table 3-5. In 1 ml, germ

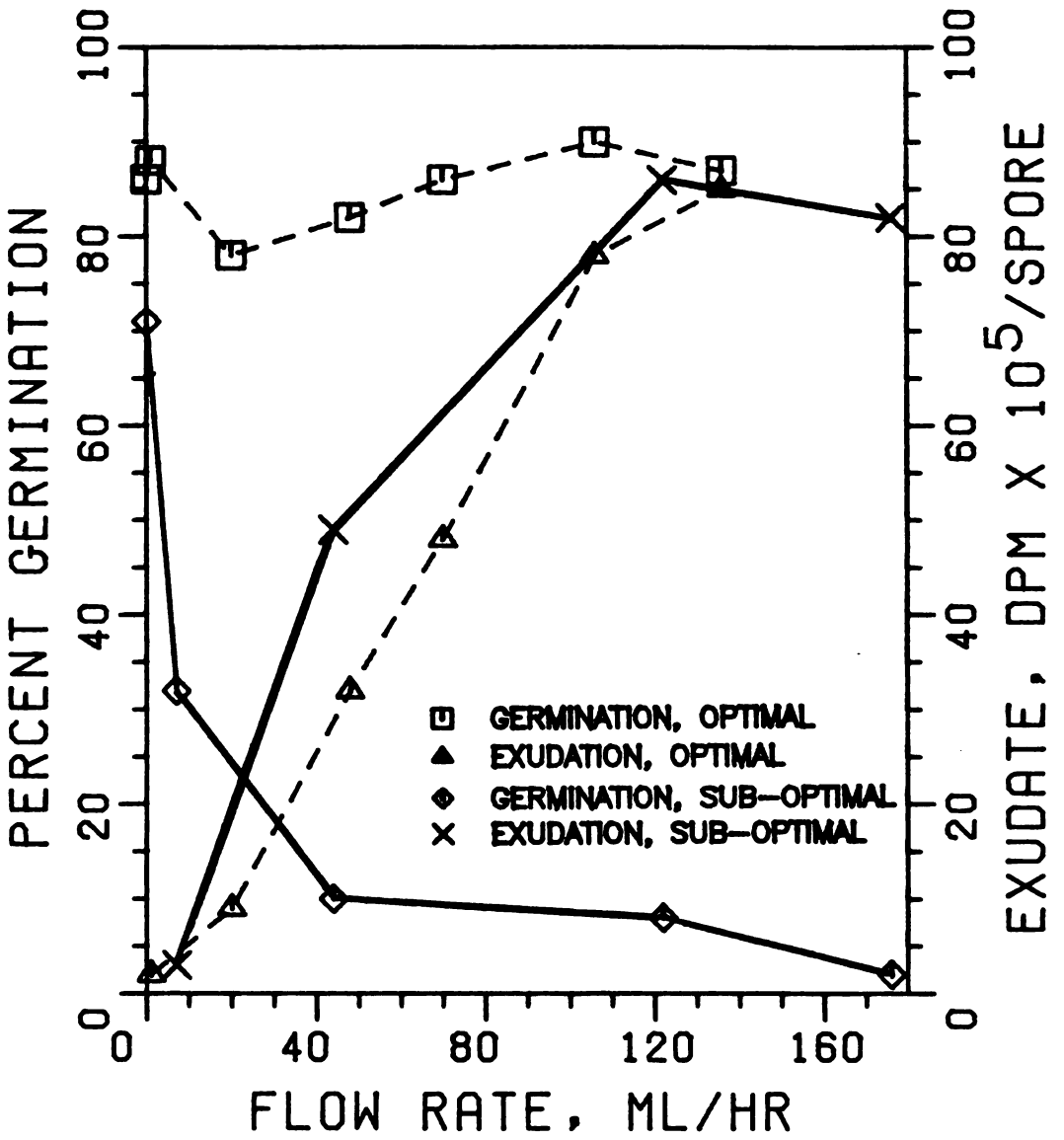

Figure 3-7. Effect of environmental conditions on exudation and germination of Helminthosporium victoriae conidia incubated in the leaching system. Conidia in "optimal" conditions were leached with 0.01 M MES (pH 5.0) in 10% White's solution at 30 C; conidia in "sub-optimal" conditions were leached with 10% White's solution (pH 5.8) at 20 C; Germination was determined after incubation for 4 hr; s.d.=3. Exudate was collected after the leaching system was operated for 30 min.

Figure 3-8. Effect of environmental conditions on germination of Helminthosporium victoriae conidia incubated on membrane filters floated on 1, 40 or 80 ml 0.01 M MES (pH 5.0) in 10% White's solution at 30 C ("optimal conditions") or 10% White's solution (pH 5.8) at 20 C ("suboptimal conditions"); s.d. = 10, 11, and 13 for 1, 40, and 80 ml respectively.

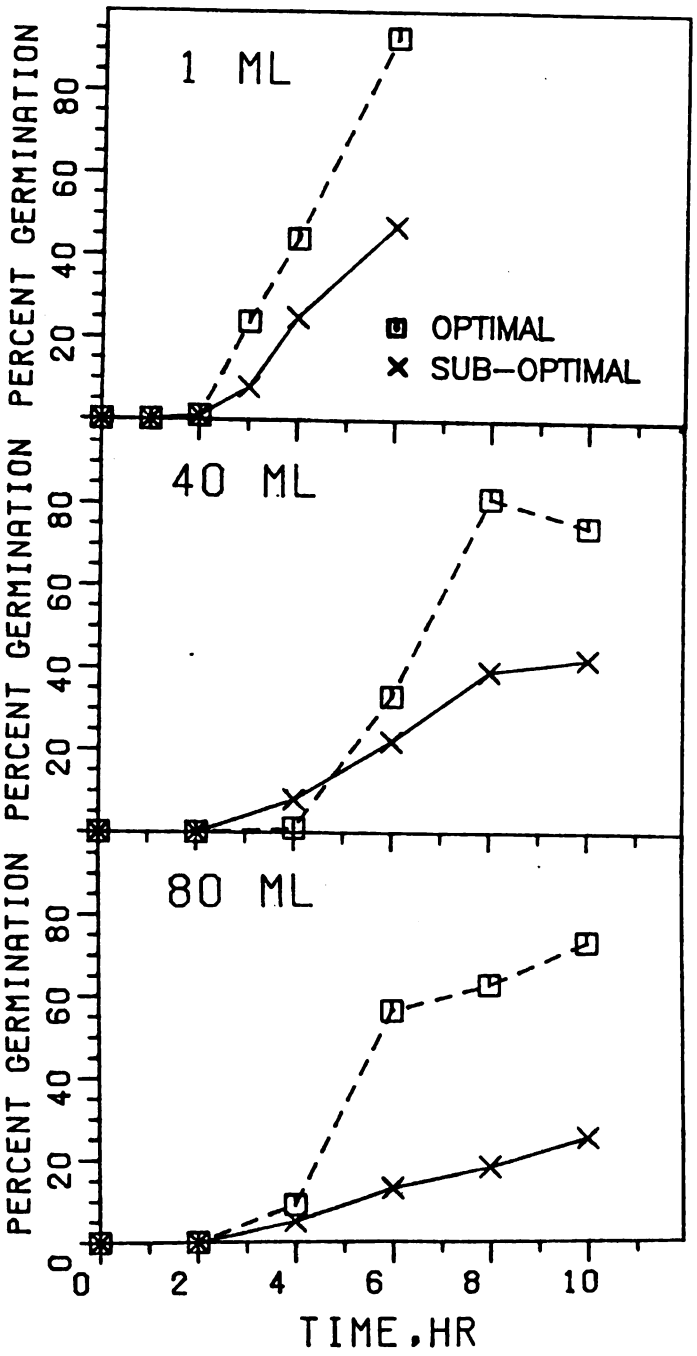
1 ML
40 ML
80 ML
OPTIMAL
SUB-OPTIMAL
PERCENT GERMINATION
PERCENT GERMINATION
0
20
40
60
80
0
2
4
6
8
10
TIME,HR

Figure 3-8

tube production followed a sigmoidal pattern (R=.98), while in 40 and 80 ml, the sigmoidal pattern was somewhat less pronounced (R=0.80 to 0.96). This may be because the standard deviation of the germination means increased slightly as the volume increased (Figure 3-8). Since the slope of the regression line is a measure of the rate of germination of a population of conidia, comparisons of the slopes indicate the extent of treatment effects (Table 3-4). As volume increased, the magnitude of the difference between rate of germination at optimal and suboptimal conditions also increased. Thus, suboptimal conditions had the greatest adverse effects on germination when conidia were incubated in the largest (80 ml) volume. In contrast to the leaching system, optimizing environmental conditions reduced, rather than eliminated, the suppression of germination in the static volume system.

In order to determine whether or not the supression of germination by inhibitory environmental conditions could be affected by nutrients, conidia were incubated for 8 hr on either 1 ml of 10% White's solution (pH 5.8), 0.1 M citrate-phosphate buffer (pH 3.0) or 0.1 M phosphate buffer (pH 8.0) amended with 1 mM glucose, 100 ppm yeast extract, or with no amendment. One hundred mM glucose significantly stimulated germination of conidia incubated in pH 3.0 and 5.8, and 100 ppm yeast extract significantly stimulated germination of conidia incubated at all three pH values (Table 3-5).

TABLE 3-4. Effect of optimizing the environmental conditions on the rate of germination of Helminthosporium victoriae conidia incubated on membrane filters floated on 1,40 or 80 ml of solution.

Conditions[b]	Volume, ml	Linear regression on the probit transformation of the percent germination[a]		% Increase in the slope by optimizing environmental conditions	% Increase in the slope by decreasing volume	
		R	slope		from 40 ml	from 80 ml
Optimal	1	.98	.90	43	76	246
Sub-optimal	1	.98	.63	--	215	600
Optimal	40	.90	.51	155	--	96
Sub-optimal	40	.96	.20	--	--	122
Optimal	80	.91	.26	188	--	--
Sub-optimal	80	.80	.09	--	--	--

[a] Nontransformed germination data are presented in Figure 12.

[b] Optimal=0.01 M MES (pH 5.0) in 10% White's solution at 30 C; Sub-optimal-10% White's solution (pH 5.8) at 21 C.

TABLE 3-5. Effect of glucose and yeast extract on germination of Helminthosporium victoriae conidia incubated for 8 hr on membrane filters floated on 1 ml of solutions with inhibitory pH values.

	Germination %[x]		
Treatment	Control	1 mM glucose	100 ppm yeast extract
10 % White's solution (pH 5.8)	67 d	87 ef	94 f
Citrate-phosphate buffer (pH 3.0)	35 b	49 c	78 c
Phosphate buffer (pH 8.0)	1 a	8 a	53 c

[x] Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test (P=0.05).

3.4. DISCUSSION

Germination of H. victoriae conidia was optimum in a sterile aqueous environment at 30 C, pH 5 and Ψ_s from 0.2 to -1.2 bar. Of the buffers tested, MES was the most favorable for germination. In a sterile sandy clay loam soil, the greatest germination occurred at a Ψ_m of 0 bar. These results are fairly typical of soil-borne fungal propagules (Waggoner and Parlange, 1976; Morrall, 1977). When conidia were incubated in environmental conditions that favored germination, the rate of germination increased and thus, the conidia germinated more synchronously (Cotter and Rapper, 1968). When the pH was unfavorable, the germination rate was increased after the addition of nutrients.

In experiments on effects of environmental conditions on germination in two axenic model systems, 10 mM MES buffer, (pH 5.0) in 10% White's solution was used to maintain both optimal pH and Ψ_s. In soil studies either 10 or 100 mM MES (pH 5.0) in 10% White's solution was used, because 100 mM MES was required to reduce the pH of the soil suspension. Since soil has a Ψ_s of its own, it was not possible to adequately optimize the pH and the osmotic potential in soil at the same time. Nonetheless, it seems that fungistasis in the nonsterile sandy clay loam is relatively unaffected by environmental conditions which favor H. victoriae germination; this confirms published results (Lockwood, 1977; 1981). In the sandy clay loam,

for example, there was more fungistatic capacity than was required to suppress germination of C. victoriae conidia (Chapter 2). Environmental effects on soil fungistasis were only detected in assays under conditions which reduced soil fungistasis. This confirms previous claims (Lockwood, 1977). When conidia are incubated on top of a water agar disc that is placed on soil, soil fungistasis is reduced. In this type of experiment, pH and Ψ_m have been found to affect the expression of fungistasis. Schuepp and Frei (1969) found that fungistasis was reduced as soil acidity was increased. It is unknown whether this is an effect of decreased bacterial activity at low pH or a more favorable pH for spore germination (Lockwood, 1977). An acidic pH stimulated germination of H. victoriae conidia under axenic conditions. There are also reports that fungistasis is reduced at high matric potentials, although Lockwood (1977) has suggested that this may be an effect of increased diffusion of nutrients. H. victoriae conidia germinated in autoclaved soil most readily at Ψ_m of 0 bar; this too could be due either to nutrient stimulation or to enhanced availability of water. In my experiments, soil fungistasis was reduced when soil extract was diluted with sand. Optimizing environmental factors increased germination (reduced fungistasis) only if the soil suspension was prepared by shaking soil with buffer for 1 hr before conidia were inoculated. When H. victoriae conidia were incubated in the absence of nutrients, favorable

environmental conditions stimulated an increase in germination from 4%-32%. But, in order for conidia to germinate fully (i.e, completely annul fungistasis), conidia in both optimal and suboptimal conditions required similar quantities of nutrients. When the soil suspension was prepared by shaking soil with buffer for 24 hr, the amount of nutrients required to annul fungistasis was greater in "optimum" than in "sub-optimum" environmental conditions. It seems that conditions which favor germination of H. victoriae conidia similarly favor the activity of fungistasis-inducing organisms.

Changes in the environment had greater effects on germination of conidia incubated on the two model systems than on conidia incubated on soil. When conidia were leached at 20 C with 10% White's solution, germination was inversely correlated with flow rate, confirming earlier reports (Lockwood, 1977). But, when conidia were leached at 30 C with 0.01 M MES (pH 5.0) in 10% White's solution, germination commenced, regardless of the flow rate. Thus, unlike soil fungistasis, the stasis imposed by the leaching system appears to be dependent upon environmental conditions which are not optimum for conidial germination. Conidia incubated on the static volume system were somewhat affected by environmental conditions; when conditions in the static volume system were optimized, the rate of germination in a given volume was increased. However, even

in optimized conditions, germination decreased as volume increased.

Lockwood and co-workers have demonstrated a high degree of correlation between the inhibition of germination of spores incubated on soil and on the leaching system (Ko and Lockwood, 1967; Hsu and Lockwood, 1973; Bristow and Lockwood, 1975b). Of 17 species of nutrient independent propagules, 14 germinated similarly in soil and on the leaching system. In addition, the rates of the following processes were similar on soil and the leaching system: 1) the conversion of H. victoriae and H. sativum from nutrient independence to nutrient dependence (Bristow and Lockwood, 1975a; Filonow, 1981); 2) the germination of H. victoriae conidia that were preincubated in a nonfungistatic environment before incubation on either system (Bristow and Lockwood, 1975a); 3) the reversion in the progress towards germination of Penicillium frequentans conidia that were alternately incubated on a germination-conducive and -suppressive environment (Yoder and Lockwood, 1973); and 4) the lysis of mycelia (Ko and Lockwood, 1970). The present study indicates that in certain soil conditions there is a lack of correlation between the suppression of germination in soil and on the leaching system.

MES buffer, used here to both reduce pH and decrease water potential, was developed by Good et al. (1966) because it is not metabolically active. Unfortunately, MES has the lowest pK_a (6.1) of any of the nonmetabolically

active buffers; therefore, no other buffers were used to confirm the leaching system results. While it is conceivable that MES stimulates germination in the model systems, I found no evidence in the literature or in the laboratory to support this. First, conidia incubated in 0.1 M MES (pH 5.0) in 10% White's salts did not increase in dry weight during a 4-day period (data not shown). And second, in the static volume system, temperature and buffer both contributed to the stimulation of germination (Table 3-3).

Exudation from conidia incubated in both environmental conditions was correlated with flow rate, although suppression of germination occurred only in "sub-optimal" conditions. In "optimal" conditions, therefore, exudation was not associated with fungistasis. Ko and Lockwood (1967) originally proposed that fungistasis in the leaching system and in soil was caused by the loss of spore exudate. The present work raises questions about the significance of the loss of this material. Further experiments on the role of exudation in germination will be discussed in the final chapter.

3.5. SUMMARY

Helminthosporium victoriae conidia germinated optimally when incubated in a sterile solution at 30 C, pH 5, with an osmotic potential (Ψ_s) from -0.2 to -1.2 bar. Of the buffers tested, MES was the most favorable for

germination. In a sterile sandy clay loam soil, germination was most rapid at a matric potential of 0 bar.

In experiments on the effects of the environment on germination in soil and in two axenic model systems that simulated soil fungistasis, 10 mM MES buffer (pH 5) in 10% White's solution was used to maintain both optimal pH and Ψ_s. Environmental factors had far greater effects on the stasis imposed by the two model systems than on fungistasis in soil. Germination was inversely correlated with flow rate when conidia incubated on sand were leached at 20 C with 10% White's solution. In contrast, conidia germinated when leached at 30 C with 0.01 M MES (pH 5.0) in 10% White's solution at all flow rates. The evidence suggested that fungistasis in soil occurs even when pH, temperature and Ψ_s favor germination, whereas the stasis in the leaching system apparently occurs only when environmental conditions are not optimum for conidial germination.

Since exudation from ^{14}C-labelled conidia leached for 30 min was positively correlated with the flow rate in either germination-suppressive or germination-conducive environments, the role of this initial exudate in spore germination is questioned.

3.6. LITERATURE CITED

Bohn, H.L., B.L. McNeal and G.A. O'Conner. 1979. Soil chemistry. John Wiley and Sons. N.Y. 329p.

Bristow, P.R. and J.L. Lockwood. 1975a. Soil fungistasis: role of spore exudates in the inhibition of nutrient-independent propagules. J. Gen. Microbiol. 90:140-146.

Bristow, P.R. and J.L. Lockwood. 1975b. Soil fungistasis: role of the microbial nutrient sink and of fungistatic substances in two soils. J. Gen. Microbiol. 90:147-156.

Cotter, D.A. and K.B. Raper. 1968. Factors affecting the rate of heat-induced spore germination in Dictyostelium discoideum. J. Bacteriol. 96:86-92.

Epstein, L. and J.L. Lockwood. 1981. A bioassay for detecting compounds involved in the annulment of fungistasis of nutrient independend spores. Phytopathology 71:215 (Abstr.).

Filonow, A.B. 1981. Mycostasis and the microbial nutrient sink of soil. Ph.D. thesis. Michigan State University, E. Lansing, MI, 97p.

Finney, D.J. 1964. Statistical method in the biological assay. 2nd ed. Hafner Pub. Co., N.Y. 668p.

Gomori, G. 1955. Preparation of buffers for use in enzyme studies, p. 138-146. In S.P. Colowick and N.O. Kaplan (eds.). Methods in enzymology, Vol. 1. Academic Press, Inc., N.Y.

Good, N.E., G.D. Winget, W. Winter, T.N. Connolly, S. Izawa and R.M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5:467-477.

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.

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

Ko, W.H. and J.L. Lockwood. 1970. Mechanism of lysis of fungal mycelia in soil. Phytopathology 60:148-154.

Lockwood, J.L. 1977. Fungistasis in soil. Biol. Rev. 52:1-43.

Lockwood, J.L. 1981. Exploitation competition, p. 319-349. In D.T. Wicklow and G.C. Carroll (eds.). The fungal community. Marcel Dekker, Inc., N.Y. 855p.

Morrall, R.A.A. 1977. A preliminary study of the influence of water potential on sclerotium germination in Sclerotinia sclerotiorum. Can. J. Bot. 55:8-11.

Perrin, D.D. and B. Dempsey. 1974. Buffers for pH and metal ion control. Chapman and Hall, London. 176p.

Schuepp, H. and E. Frei. 1969. Soil fungistasis with respect to pH and profile. Can J. Microbiol. 15:1273-1279.

Slavik, B. 1974. Methods of studying plant water relations. Springer-Verlag, N.Y. 449p.

Waggoner, P.E. and J.Y. Parlange. 1976. Germination of Alternaria solani spores in changing osmotic pressures. Phytopathology 66:786-789.

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

CHAPTER 4

RELATION OF EXUDATION TO GERMINATION OF *HELMINTHOSPORIUM VICTORIAE* CONIDIA

4.1 INTRODUCTION

Fungistasis is generally defined as an inhibition of fungal germination and growth (Lockwood, 1977); it is a widespread phenomenon in soil, generally, but not always, due to the presence of living microbes and is generally annulled by the addition of energy-yielding nutrients. Direct contact between the fungus and the surrounding microbiota, or actual parasitism of the fungus, are not required in order for fungistasis to occur; propagules will not germinate on soil, even if the spores are separated from the soil by a membrane that excludes bacteria (Lockwood, 1977; Watson and Ford, 1972).

"Nutrient independent" propagules will germinate, without any special stimulus, in the absence of any organic or inorganic compounds, as long as the environment is free of other microbes (Ko and Lockwood, 1967). Yet, many of these same spores will not germinate in nonsterile conditions, unless a carbonaceous energy source becomes available. In this chapter, I will report on the germination of the nutrient independent conidia of *Helminthosporium victoriae* Meehan and Murphy (=*Cochliobolus victoriae* Nelson, =*Drechslera victoriae* (Meehan and Murphy) Subram. and Jain) the soil-borne causal agent of Victoria blight of oats.

Lockwood and his co-workers have proposed that fungistasis is associated with the loss of germination-promoting nutrients from the spore to the environment. In this model, soil microflora serve as a "nutrient sink" which constantly drains the spores of metabolic reserves, thereby preventing germination. Losses may eventually occur to such an extent that the spores may lose the ability to germinate without exogenous energy. This hypothesis has been referred to as the "nutrient deprivation hypothesis" (Lockwood, 1977; 1981). Evidence in support of the hypothesis is based primarily on the observation that many nutrient-independent spores will not germinate in a sterile model system in which a sand substratum is continuously leached with either water, phosphate buffer, or a dilute salt solution (Hsu and Lockwood, 1973). There is generally a correlation between germination behavior (i.e. sensitivity to fungistasis) on the sterile system and in the soil. Heat-activated ascospores of Neurospora tetrasperma will germinate on soil as well as on the leaching system (Ko and Lockwood, 1967; Hsu and Lockwood, 1973). In the leaching system, germination is inversely proportional to the flow rate of leaching solution (Lockwood, 1975, Sneh and Lockwood, 1976). Similarly, when H. victoriae conidia were floated on distilled water, germination decreased as the volume of water increased (Sneh and Lockwood, 1976). In the leaching system, inhibition of germination was correlated with the

loss of ^{14}C-label from the spores (Sneh and Lockwood, 1976; Filonow and Lockwood, 1979). Loss of ^{14}C from labelled non-germinating spores on the leaching system was less than ^{14}C loss from spores incubated on soil (Sneh and Lockwood, 1976; Filonow and Lockwood, 1979). Germination was partially restored when the spores were incubated with ^{14}C-labelled compounds leached from the same or other spores (Bristow and Lockwood, 1975). These results suggested that the loss of the ^{14}C-labelled compounds inhibited germination (Lockwood, 1977).

The H. victoriae exudate contained anthrone- and ninhydrin-positive compounds; more than 90% of the carbohydrate was glucose (Bristow and Lockwood, 1975). Fungistasis of H. victoriae conidia incubated on the leaching system was partially annulled by a nutrient solution containing glucose and casein hydrolysate, in concentrations which approximated the carbohydrate and amino compound composition of H. victoriae exudate. Neither exudate nor the defined nutrient solution affected germination of unleached spores, but both increased germination of leached spores to a similar extent (Bristow and Lockwood, 1975).

The purpose of this research was to further investigate the role of exudation in H. victoriae germination. More specifically, I 1) identified nutrients that stimulated germination, 2) identified nutrients in exudate and determined conditions in which exudate

stimulated germination, 3) investigated the consequences of the loss of exudate on germination, and 4) attempted to determine whether the leaching system inhibited germination by imposing a diffusion gradient.

4.2. MATERIAL AND METHODS

4.2.1. Model systems. Germination of Helminthosporium victoriae conidia was suppressed in two aseptic model systems. In the "static volume system," conidia, borne on Nuclepore membranes, were floated on 1, 40 or 80 ml 10% White's mineral solution (Chapter 2) for 4 or 8 hr. As the volume of solution increased the rate of germination decreased (Chapter 3). Since detection of stimulatory treatments was facilitated at larger volumes, which were more inhibitory, 80 ml was generally used. In the "leaching system," conidia borne on a Nuclepore membrane were incubated on a bed of sterile sand. Germination was inversely proportional to the flow rate of a dilute salt solution percolated through the sand, and was strongly suppressed at a flow rate of 70-80 ml/hr.

4.2.2. Growth medium. Conidia were produced on a defined medium (10 g glucose, 10 g xylose, 2 g asparagine·H_2O, 1.5 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 20 g Noble Agar, 0.1 mg each: $CuSO_4$, $MnSO_4$, $Fe(SO_4)_3$, $ZnSO_4$, $Na_2MoO_4 \cdot 2H_2O$, and 1L H_2O), modified from that of Garraway and Evans (1977). Sugars and asparagine were autoclaved separately. Medium with 2 g

asparagine·H_2O/L produced significantly more spores than 1 or 4 g/L, or with the equivalent moles of N as KNO_3 (Table 4-1).

When specified, conidia were produced on either V-8 juice agar [200 ml 'V-8' juice, (Campbell Soup Co.), 2 g $CaCO_3$, 20 g agar and 800 ml H_2O] or carrot agar (decoction from 30 g carrots in 250 ml H_2O, 20 g agar, and 750 ml H_2O).

4.2.3. Germination bioassay. Conidia were collected, inoculated, incubated, stained and counted as described previously (Chapters 2,3). The experimental design and statistical analysis were also the same as in Chapter 3.

In all experiments, conidia were maintained in axenic conditions. All solutions were made with reagent grade chemicals and distilled-deionized water. Solutions were sterilized by autoclaving, except glucose, which was sterilized by ultrafiltration.

The concentration of conidia in a solution was determined by removing 4-1 µl aliquots with a "Ziptrol" microsyringe pipette (Drummond Scientific Company, Broomall, PA 19008) and then microscopically counting all conidia in each droplet. To determine conidial dry weight, after conidial density was determined, conidia from 7 collections of exudate were deposited onto 0.45 µm Gelman membrane filters, dried at 75 C, and weighed.

4.2.4. Exudate. Conidia from 3-5-week-old cultures on glucose-asparagine agar (or V-8 juice agar when

TABLE 4-1. Effect of N source and concentration on growth and reproduction of *Helminthosporium victoriae* in culture[w,x].

Nitrogen source	Concentration, g/L	Growth parameter[y]			
		Colony diameter, mm	No. of spores/mm^2	Spore length, m	Germination, %[z]
KNO_3	1.4	50 a	1310 a	93 a,b	99 a
	2.7	50 a	884 a	92 a,b	96 a
	5.4	51 a	1110 a	88 a	99 a
L-asparagine·H_2O	1.0	66 b	1220 a	115 b	97 a
	2.0	65 b	1790 b	109 a,b	99 a
	4.0	61 b	1190 a	94 a,b	96 a

[w] See text for list of other ingredients in medium.

[x] Means in each row followed by the same letter were indistinguishable by the Student-Newman-Keuls rang test ($P=0.05$).

[y] Colony diameter and the other growth parameters were determined 7 and 14 days after inoculation, respectively.

[z] Spores were incubated for 21 hr on filter paper saturated with 10% White's solution before germination was assessed.

indicated) were collected dry using suction and suspended at the rate of approximately 10^5 conidia/ml in 10% White's solution and stirred gently for 0.5-1 hr. (Exudate obtained from conidia soaked for 0.1, 0.5, 1 or 2 hr, showed no significant differences in subsequent stimulatory activity). When specifically indicated, 10^5 conidia/ml 10% White's solution were placed in an ultrasonic cleaner for 30 min. Conidia were removed from the exudate by deposition on a 0.22 μm Gelman or Nuclepore membrane filter. Unless a further treatment was indicated, exudate was assayed for stimulatory activity on freshly harvested conidia within 1-2 hr.

For the carbohydrate analysis of exudate, 3×10^6 conidia were collected by suction, suspended in 80 ml water at 4 C and washed by centrifugation at 10,000 g for 20 min. After the supernatant was decanted, the conidia were suspended in 0.7 L water at 21 C and stirred gently for 4 hr. Conidia were removed from the exudate by passage through a 0.22 μm Millipore filter. Exudate was frozen in an ethanol bath chilled with dry ice and then lyophilized. The lyophilized material was reconstituted in a small volume, refrozen and dried again. Sugars were derivatized as the trimethylchlorosilane-o-methyl glycosides and analyzed in a Perkin Elmer 910 gas chromatograph using a SP 2100 column. The procedure and materials are described in D.T.A. Lamport's Protocol #P06178 (Plant Research Laboratory, Michigan State University) as adapted from

Bhatti et al., (1970). Standards included arabitol, rhamnose, fucose, xylose, mannose, galactose, glucose, mannitol and inositol. For ion analysis, conidia were removed from agar plates with vacuum and 4 x 10^4 conidia/ml were suspended in 1 mM MES (pH 6.1) for 1 hr. Conidia were deposited by suction onto a 0.22 µm Gelman filter and the filtrate (exudate) was evaporated under reduced pressure. The evaporated sample was rehydrated in a small volume of water, frozed, lyophilized and rehydrated. Ionic analysis of the exudate and a buffer control were performed with a Spectrametrics SMI III Emission Spectrophotometer (204 Andover St., Andover, MA 01810), courtesy of Ray Harris, USDA Forestry Service, Michigan State University.

4.2.5. <u>^{14}C-labelled conidia, exudate, or substrate</u>. ^{14}C-labelled conidia were produced by spreading 1 ml containing 1-10 µCi of uniformly labelled glucose (Chapter 3) onto the surface of glucose-asparagine, or when specified, carrot agar. After 24 hr, the agar surfaces were inoculated with a mycelial plug. When the cultures were 3 weeks old, the conidia were removed dry from the agar using suction. For the experiment on effects of temperature on exudation, conidia, produced on carrot agar containing 1 µCi ^{14}C-glucose/10 cm i.d. plate, were collected dry and suspended in 20 ml of 0.1% Tween 20 maintained at either 2 or 22 C. After 5, 15, 30, or 45 min, 4 ml aliquots of spore suspension were removed and filtered through a 0.2 µm Nuclepore membrane filter. Ten

ml aqueous scintillation fluid (Chapter 3) was added to 1 ml of exudate.

For experiments on the uptake of exudate by conidia, unlabelled conidia were incubated on 1 ml 10% White's solution containing 186 or 421 dpm of ^{14}C-labelled exudate. At 0, 1, 2, 4, and 8 hr, membranes were removed from the solution and wiped gently on a paper towel. ^{14}C in conidia and the solution was determined as described in Chapter 3.

For experiments on the uptake of 10^{-2}, 10^{-4}, or 10^{-7} M ^{14}C-labelled glucose or a mixture of ^{14}C-labelled amino acids containing 10^{-9} moles/ml, unlabelled conidia were incubated on 1 ml of substrate in 10% White's solution for 0.5, 1, 2, 3, 4.5, 6, or 8 hr. Conidia incubated for approximately 30 sec were used as a zero time uptake control. After the alloted time period, conidia were removed and stained to determine % germination and the radioactivity in the solution was counted to determine uptake.

4.3. RESULTS

4.3.1. Effect of selected nutrients on germination. Conidia were incubated on solutions containing 1 μM - 1 mM of selected nutrients (Table 4-2). Germination was stimulated by 1 μM fructose or 10 μM glucose, or higher concentrations. Glucose in combination with an equimolar concentration of asparagine was no more stimulatory than

TABLE 4-2. Effect of 1 μM-1 mM of selected nutrients on germination of Helminthosporium victoriae conidia incubated for 8 hr on 80 ml 0.05 M MES (pH 6.1) in 10% White's solution.

	% Germination[x]					
		Log μM of compound				Standard
Compound	Control	0	1	2	3	deviation
D-Glucose	25 a	23 a	53 b	75 c	88 c	9
β-D-Fructose	18 a	58 b	86 b	76 b	90 b	16
D+Galactose	4 a	4 a	3 a	2 a	7 a	3
D+Xylose	2 a	6 a	1 a	2 a	0 a	3
D-Ribose	2 a	10 a	5 a	8 a	5 a	3
L-Proline	10 a	9 a	28 ab	35 ab	46 b	10
DL-α-Alanine	10 a	16 ab	14 ab	15 ab	30 b	7
D-Glucose + L-Asparagine	10 a	9 a	28 b	80 c	90 c	7
α-D Galacturonic Acid	31 a	26 a	19 a	11 a	5 a	11
Picolinic Acid	31 a	27 ab	16 ab	2 b	2 b	11
Succinic Acid	39 a	19 a	34 a	31 a	27 a	11

Table 4-2 (cont'd.).

[x] Means within each row followed by the same letter are indistinguishable by the Student-Newman-Keuls, (P=0.05).

glucose alone. One mM, but not lower concentrations, of proline or alanine also stimulated germination; no stimulation was detected with 1 μM -1 mM galactose, xylose, ribose, galacturonic acid, picolinic acid or succinic acid. One hundred μg/ml casein hydrolysate and 5 μg/ml yeast extract also stimulated germination, but lower concentrations did not.

4.3.2. Nutrients in exudate. To identify the sugars in exudate, 3×10^6 conidia were submerged in 0.7 L distilled-deionized water for 4 hr; germination was 0%. Conidia released glucose, xylose, galactose, mannose and rhamnose (Table 4-3); glucose accounted for 52% of the exuded sugars. With 5×10^{-3} conidia/ml, exudate contained from 3 to 40×10^{-7} M of each of the 5 sugars. This is equivalent to 8 to 70×10^{-14} moles/spore or 1 to 12 pg/spore of each of the detected sugars. Since each conidium released 23 pg of sugar, and each conidium weighed approximately 3 ng (s.d.=2 ng), conidia exuded 0.08% of their dry weight as sugars.

The ionic composition of exudate was partially determined by emission spectrophotometry (Table 4-4). Of the 13 metals analyzed, exudate contained significantly more Mg (7.2 X), P (6.3 X), K (2.5 X) and Cu (1.3 X) than was present in the buffer control. Per spore, conidia released 2, 3, and 4×10^{-18} moles Mg, P, and K, respectively, and 3×10^{-21} moles Cu. However, since 10% White's solution contained 7×10^{-6} M Mg, 1 X

TABLE 4-3. Sugars in Helminthosporium victoriae exudate.[a]

Sugar[b]	Amount released/ spore (X 10^{14} moles)	pg/spore	Concentration in exudate (X 10^{7} M)
Glucose	6.6	12	3
Xylose	2.8	4	1
Galactose	2.3	4	1
Mannose	1.2	2	0.6
Rhamnose	0.8	1	0.4

[a] 5 x 10^{3} spores/ml were soaked in water for 4 hr.

[b] TMS-O-methyl glycoside derivatives of the sugars were identified by gas chromatography.

TABLE 4-4. Metals in concentrated exudate from Helminthosporium victoriae conidia.[a]

Metal	Metal, ppm, in: exudate	control	Standard deviation	Exudate, ppm/ control, ppm	Moles X 10^{18} released/spore
Mg	5.56	0.78	.04	7.2	4
P	4.30	0.68	.11	6.3	2
K	13.7	5.45	.33	2.5	3
Cu	.11	.08	.002	1.3	0.003
Pb	0.10	.06	.02	NS	-
Fe	.06	.04	.02	NS	-
Mn	.03	.02	.01	NS	-
Ca	5.52	4.94	.17	NS	-
Al	.13	.13	.01	NS	-
Zn	.37	.35	.08	NS	-
Na	28.6	29.1	.30	NS	-
B	1.98	2.06	.02	NS	-
Ni	.014	.015	.004	NS	-

Table 4-4 (cont'd.).

[a] Exudate was collected by mixing 4 x 10^4 spores/ml in 1 mM MES with NaOH (pH 6.1) for 1 hr. Samples were concentrated to 2% of their original volume and analyzed by emission spectrophotometry.

[b] The ratios presented were significantly different from the control by the Least Significant Difference (LSD) test (P=0.05); NS - not significant by the LSD.

10^{-5} M P, and 2 X 10^{-4} M K, and 5 X 10^{3} conidia incubated on 40 ml exuded 5 X 10^{-13} M Mg, 2 X 10^{-13} M K, and 4 X 10^{-16} M Cu, it seems unlikely that the inorganic component of exudate influenced its activity. Exudate did not contain more Fe, Mn, Ca, Zn, Pb, Al, Na, B, or Ni than controls.

4.3.3. Effect of exogenous exudate on germination. When freshly harvested H. victoriae conidia were incubated on freshly collected spore exudate, germination was significantly stimulated in 35% of the experiments (Table 4-5). Unsuccessful attempts were made to remove enzymes or other agents from the exudate which might inactivate stimulatory compounds. Methods used included: dilution of the exudate, collection of the exudate and bioassay in darkness, removal of volatiles with He_2, and separation of the exudate by ultrafiltration with a 30,000 molecular weight exclusion size. With the last method, the filtrate and any material retained by the membrane were assayed. Conidia produced on V-8 juice agar were no more sensitive to exudate stimulation nor produced more stimulatory exudate that conidia produced on the defined agar medium. Conidia soaked in an ultrasonic cleaner produced exudate that significantly stimulated germination in each of four experiments. In order to determine if conidia could take up exudate, conidia were incubated for up to 8 hr on 1 ml of ^{14}C-labelled exudate with 186 or 421 dpm. No uptake by the spores or decrease of ^{14}C in the exudate

TABLE 4-5. Effect of exudate from Helminthosporium victoriae conidia on germination of other H. victoriae conidia.

Growth medium[a]	Collection of exudate[b]	Treatment of exudate after collection	No. experiments with stimulatory exudate/ no. experiments[c]	No. experiments in which the fresh exudate was stimulatory
Glu-asp	Diffusion	None	8/23	-
V-8	Diffusion	None	0/2	-
Glu-asp	Ultrasound	None	4/4	-
Glu-asp	Diffusion	Autoclaved	1/6	1
Glu-asp	Diffusion	Freeze-dried	0/4	1
Glu-asp	Diffusion	Evaporated	0/2	1
Glu-asp	Diffusion	Ultrafiltration for separation at 30,000 daltons	0/4	0
Glu-asp	Diffusion	Volatiles removed with He and collected with cold trap	0/2	1
Glu-asp	Diffusion	Diluted	0/2	1

Table 4-5 (cont'd.).

[a] Conidia were collected from 3-5-week-old cultures grown on "glu-asp" (glucose asparagine-xylose agar) or "V-8" (V-8 juice agar). See the text for the composition of the media.

[b] Conidia were collected dry and then approximately 10^5 conidia/ml were stirred gently in 0.15-0.75 L 10% White's solution for 0.5 or 1 hr (Diffusion) or were placed in an ultrasonic cleaner for 30 min (Ultrasound).

[c] Conidia were incubated on 80 ml exudate or 10% White's solution for 8 hr, or on 40 ml for 4 hr.

was detected, even after the conidia commenced germination.

Since Bristow and Lockwood (1975) reported that H. victoriae conidia incubated on the leaching system were stimulated by spore exudate, the leaching system and the static volume system were compared (Figure 4-1). Exudate was obtained by soaking 8 x 10^4 conidia/ml 10% White's solution in an ultrasonic cleaner for 30 min. After 5 hr of incubation on the model systems, conidia were exposed to similar quantities of exudate. But at 20 hr, conidia on the leaching system were exposed to 4 X more exudate than conidia on the static system. Conidia incubated on the leaching system without exudate were suppressed throughout the 20 hr experimental period, whereas similar control conidia incubated on the static system were suppressed only during the first 4 hr. Significant stimulation from exudate was detected after 4 hr on the static system and after 8 and 20 hr sampling periods on the leaching system. There was greater germination of conidia on the static system exudation treatments than with static system controls after 8 and 20 hr. These differences were not significant (s.d.=12) by the Student-Newman-Keuls multiple range test, but were significant by the Least Significant Difference procedure.

In order to determine if stimulatory activity of exudate was lost upon processing, conidia borne on membranes were arranged inside leaching dishes so that any

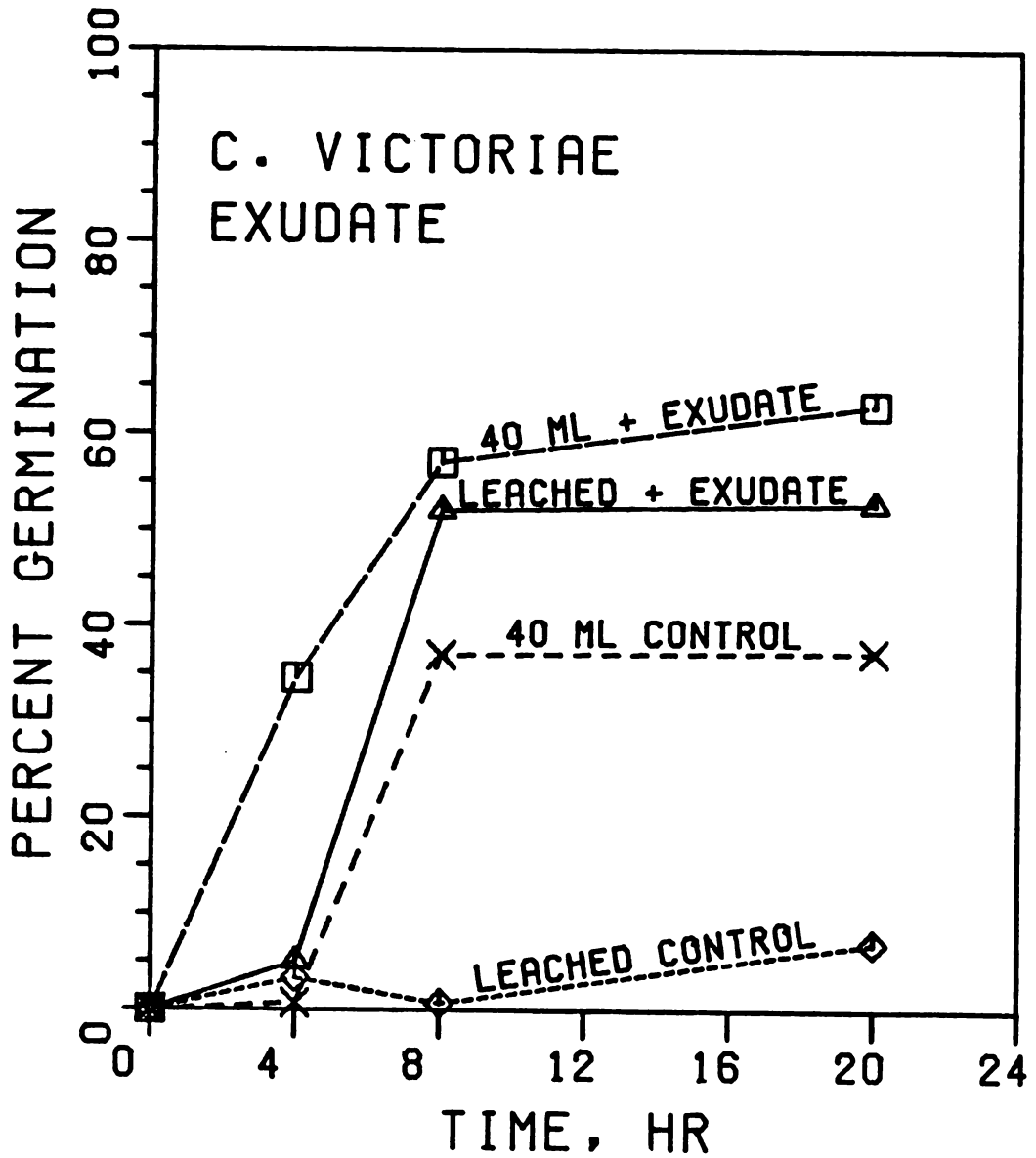

Figure 4-1. Effects of exudate collected from conidia of Helminthosporium victoriae on germination of H. victoriae conidia incubated in the leaching and static volume assay systems. In the static system, conidia were incubated on 40 ml of 10% White's solution with or without exudate from 3 X 10^6 conidia. In the leaching system, the leaching medium was 10% White's solution containing exudate from 5 X 10^5 conidia, and was used at a flow rate of 70 ml/hr. Exudate was collected by soaking 7.5 X 10^4 conidia/ml 10% White's solution in an ultrasonic cleaner for 30 min.

stimulatory activity would be immediately bioassayed. Duplicate membranes with either 5 X 10^3, 7 X 10^5 or 3 X 10^6 conidia were placed across the middle of each dish. Additional membranes, each bearing 5 X 10^3 conidia were placed "upstream" and "downstream" of the membranes in the middle. Conidia were either unleached or leached at 75 ml/hr for 8 hr. Since there were no differences between germination directly above or below membranes bearing 5 X 10^3 to 3 X 10^6 conidia, no stimulatory activity was detected (Table 4-6).

In case conidia freshly collected from media were unique in their lack of response to exudate, 2 x 10^3 conidia/ml 10% White's solution were soaked for 1 hr before germination was bioassayed for 8 hr on 80 ml exudate or 10% White's solution. Conidia treated in this manner were not stimulated by exudate to any greater extent than those which were not soaked before the bioassay.

To test whether exudation was affected by the initial suspension of the conidia in a cold (4 C) solution, 10^6 conidia were incubated in 20 ml 0.1% Tween 20 at either 2 or 22 C for up to 45 min (Figure 4-2). From 15 to 45 min, there was significantly greater exudation from conidia at 22 that at 2 C. With both treatments, for the first 30 min, there was a linear increase of the log dpm with time. After 30 min the rate of exudation decreased; the decrease was more pronounced at 22 than at 2 C. Thus, exudation is

TABLE 4-6. Effect of exudate from conidia of *Helminthosporium victoriae* on germination of *H. victoriae* conidia incubated on the leaching system.

Flow rate, ml/hr[a]	No. spores on middle membrane	Placement of spores	Germination, %[b]
0	-	Upstream	78 a
	5×10^3	Middle	69 a
	-	Downstream	76 a
75	-	Upstream	5 b
	5×10^3	Middle	4 b
	-	Downstream	3 b
75	-	Upstream	1 b
	7×10^5	Middle	-
	-	Downstream	2 b
75	-	Upstream	2 b
	3×10^6	Middle	-
	-	Downstream	2 b

[a] Conidia were leached with 10% White's solution for 8 hr.

[b] Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$).

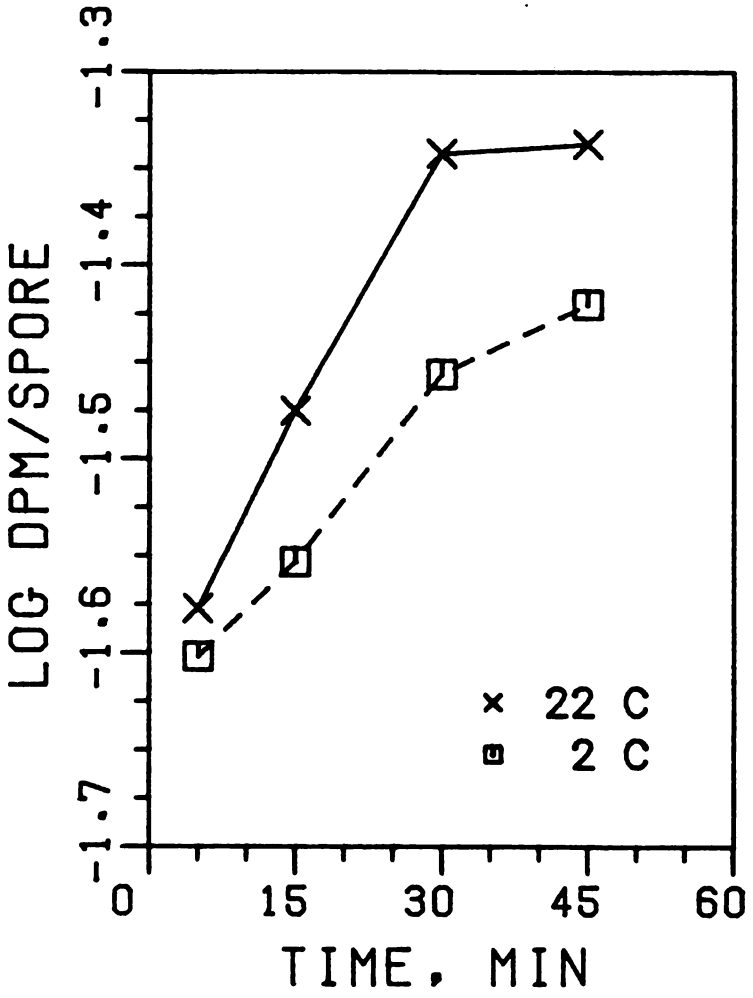

Figure 4-2. Exudation from ^{14}C-labelled Helminthosporium victoriae conidia incubated at either 2 or 22 C. 1 X 10^6 conidia were incubated in 20 ml 0.1% Tween 20. Exudation was determined after removal of conidia by ultrafiltration.

apparently the result of passive diffusion and exudation rates are temperature dependent.

4.3.4. Effect of exogenous glucose on germination. To determine if glucose triggered germination of H. victoriae, conidia were incubated on 10 ml 0.05 M MES (pH 6.1) with or without 1 mM glucose. After 2, 12, or 24 min, the spores were washed by floating the membrane on 2 changes of 40 ml 10% White's solution, each for 1 min. Spores were then incubated on 80 ml of 10% White's solution for 8 hr less the time required for the buffer or glucose treatment. One mM glucose did not affect germination whether it was present during the first 2, 12 or 24 min of incubation.

To determine if glucose in conidial exudate affected germination, conidia were incubated at 30 C for 2,4 or 6 hr on 1 ml of 0.1 M MES (pH 5.1) in 10% White's solution with or without 0.2 units glucose oxidase (Table 4-7). Since 0.2 units enzyme can oxidize 12 μmoles glucose/hr and 5 X 10^3 conidia released 3 x 10^{-4} μmoles glucose (Table 4-3), the quantity of glucose oxidase was more than sufficient to oxidize any glucose in the exudate. Conidia incubated with the glucose oxidase germinated as well as the controls. Thus, there was no indication that glucose had an exogenous role in germination.

To determine if glucose or amino acids were taken up before germ tube emergence, conidia were incubated on 1 ml of 10% White's solution containing 10^{-2}, 10^{-4}, or 10^{-7} M ^{14}C-labelled glucose or a mixture of

TABLE 4-7. Effect of exogenous glucose oxidase on germination of Helminthosporium victoriae conidia.

	% Germination[a]	
Time, hr	Control	Glucose oxidase
2	3 a	2 a
4	78 b	79 b
6	91 c	86 bc

[a] Conidia were incubated at 30 C on 1 ml 0.01 M MES (pH 5.1) in 10% White's solution with or without 0.2 units insoluble glucose oxidase. Means which are indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$) are followed by the same letter.

^{14}C-labelled amino acids containing a total of 10^{-9} moles/ml. Percentage germination and ^{14}C removed from solution were determined periodically. In all treatments, a significant percentage of germ tubes emerged before significant uptake of ^{14}C was detected. Although it is essentially impossible to chose a time that differentiates between pre- and post-germ tube emergence in an asynchronously germinating population, ^{14}C glucose and amino acids appeared to be primarily taken up by germ tubes and young hyphae, rather than ungerminated conidia.

4.3.5. Effect of loss of exudate on germination. In order to determine if the loss of exudate affected subsequent germinability of H. victoriae, conidia were subjected to fungistatic conditions and then incubated in non-fungistatic environments. Germination of these spores was compared to that of conidia not previously exposed to fungistatic treatments. Conidia, borne on 0.4 µm Nuclepore membrane filters, were either incubated for 1-4 hr on sterile sand saturated with 10% White's solution (nonfungistatic) or for 2 hr on saturated soil which was covered with a 0.2 µm Nuclepore filter (fungistatic). (Soil fungistasis was expressed through a double thickness of membrane filter.) Conidia incubated on soil were then transferred to sterile sand for 1-4 hr. Following the 2 hr incubation on soil, conidia commenced germination after 1-2 hr on sand, whereas conidia that were not pre-incubated on soil commenced germination after 2-3 hr (Table 4-8). Once

TABLE 4-8. Effect of incubation of Helminthosporium victoriae conidia on soil for 2 hr on subsequent germination on sand.

	% Germination[z]	
Time on sand, hr	Not pre-incubated	Pre-incubated for 2 hr on soil[b]
1	0 a	0 a
2	0 a	32 ab
3	38 ab	69 bc
4	59 bc	84 c

[a] Means which are indistinguishable by the Student-Newman-Keuls multiple range test ($P=0.05$) are followed by the same letter.

[b] Conidia, borne on a 0.45 µm Nuclepore membrane, were placed on top of a second Nuclepore membrane (0.2 µm) which covered the soil surface. Conidia incubated on soil for 6 hr germinated 0%.

germination began, the rates appeared similar with both treatments. Therefore, the loss of exudate which occurs on soil does not adversely affect subsequent germination on sand.

Conidia were either incubated for 8 hr on 80 ml of 10% White's solution (a fungistatic environment) and then transferred for 8 additional hrs on 1 μM to 1 mM glucose in 0.05 M MES (pH 6.1) in 10% White's solution (a non-fungistatic environment), or were incubated continually on buffered glucose (Table 4-9). Only 8% of the conidia germinated in 80 ml of 10% White's solution. Conidia incubated first on White's solution and then on buffer without glucose germinated significantly better than conidia incubated continually on buffer. Spores pre-incubated on White's solution and then incubated for 8 hr on 1 μM to 1 mM glucose in MES buffer, germinated as well as conidia which were only incubated on buffered glucose. Again, any loss of exudate in a germination suppressive environment did not adversely affect subsequent germination in a conducive environment.

4.3.6. Effect of increasing mass of spores in a given volume. Lockwood and co-workers (1977) proposed that the leaching system inhibits germination by providing a continuous diffusion gradient away from the propagules. In an attempt to alter the diffusion gradient, the mass of spores in 2 or 40 ml of 10% White's solution was increased, and the effect on germination was measured. Each of 4

TABLE 4-9. Effect of pre-incubation of Helminthosporium victoriae conidia on 80 ml of 10% White's solution for 8 hr on subsequent germination of the same conidia on 1 μM - 1 mM glucose in 80 ml 0.05 M MES (pH 6.1) in 10% White's solution for 8 hr.

		% Germination	
Germination medium	Glucose, log μM	Not pre-incubated	Pre-incubated
10% White's solution	-	-	8 a
Buffer	-	46 b	62 c
Buffer + glucose	0	66 c	74 cd
Buffer + glucose	1	81 d	76 cd
Buffer + glucose	2	75 cd	83 d
Buffer + glucose	3	79 d	84 d

[a] Means which are indistinguishable by the Student-Newman-Keuls range test ($P=0.05$) are followed by the same letter.

experiments included 6-8 treatments. The experiments included a range of concentrations from 1 X 10^1 to 1 X 10^6 conidia/ml. No significant changes in percentage germination with increasing numbers of spores were detected.

4.4. DISCUSSION

The experiments in this paper were designed to investigate the role of the glucose-rich exudate in germination of H. victoriae conidia. H. victoriae conidia exuded glucose and four other sugars when incubated in water. ^{14}C-labelled conidia released exudate when incubated at either 2 or 22 C but the rate of loss was greater at 22 C. The log of the loss of ^{14}C was linear with respect to time for the first 30 min. After 30 min at both temperatures, the rate of loss of ^{14}C decreased. These results generally agree with those of Bristow and Lockwood (1975). Simon (1974) suggested that conidia exuded during hydration until membrane integrity is re-established. There are numerous reports of an initial non-specific exudation from propagules; at least some exudation apparently occurs regardless of sensitivity to fungistasis or subsequent germination (Wynn et al., 1966; Yadav and Mandahar, 1981).

In the experiments presented here, there was no indication that the initial loss of glucose-rich conidial exudate was causally involved in fungistasis. The exudate does not represent substrate which is required for

germination. Conidia were pre-incubated in germination-inhibiting conditions where exudation loss occurs, i.e., on soil for 2 hr (Table 4-8) or on 80 ml of 10% White's solution for 8 hr (Table 4-9). After conidia were transferred to germination-conducive environments, germination of the pre-incubated conidia proceeded unimpaired. In fact, the experiment with soil suggests that during the time of greatest exudation, the germination process is initiated; thus, soil fungistasis inhibits germ tube production sometime after the first 30 min of incubation. When H. sativus conidia were incubated for 1 day on non-sterile soil and then transferred to sterile soil, conidia preincubated on non-sterile soil germinated in 38% less time than conidia which were incubated only on sterile soil (Yoder and Lockwood, 1973). Conidia of Penicillium frequentans, which required nutrients to germinate in sterile or non-sterile conditions, initiated germination in non-sterile soil. Initiation only required water, was partially inhibited at 1 C, and accounted for approximately 20% of the total germination time. Thus, germination appears to be a two-stage process: in the first, the propagules are uninhibited by soil funsistasis or the loss of exudate, and in the second, the propagules are inhibited by fungistasis imposed by soil or the leaching system.

There was also no evidence that the glucose rich exudate, once released, was exogenously involved in germination. During the period of greatest exudate release

(2-24 min), germination was not stimulated by 1 mM glucose. When conidia were incubated with glucose oxidase for up to 6 hr, germination was also unaffected. Moreover, exudate obtained by suspending spores in 10% White's solution was not taken up by freshly harvested conidia. The exudate unpredictably stimulated germination of new conidia; significant stimulation of germination by exudate occurred in 35% of the trials. There are two possible explanations for the variable stimulation of exudate. i) The stimulatory compound in exudate was quickly inactivated or highly volatile. Unsuccessful attempts were made to separate possible inhibitors or inactivating agents, especially enzymes, from any stimulatory compounds. ii) Stimulation was dependent upon a quantity of nutrients that was greater than the quantity that was generally exuded. Of the nutrients tested, only glucose at 10 μM and fructose at 1 μM stimulated germination; exudate contained only 0.3 μM glucose and from 40 to 100 nM of four other sugars. Conidia suspended in water or incubated on the leaching system (Chapter 3) exuded less than reported for previous studies (Bristow and Lockwood, 1975; Sneh and Lockwood, 1976; Filonow and Lockwood, 1979; Filonow, 1981). In addition, there are previous reports that germination of H. victoriae made nutrient dependent, was partially restored when spores were incubated with ^{14}C-labelled compounds leached from the same or other spores, or with a solution of 3×10^{-6} M glucose and 0.084 ppm casein

hydrolysate--concentrations which approximated the carbohydrate and amino compound composition of H. victoriae exudate in their study (Bristow and Lockwood, 1975). Whether the difference in reports on stimulation of germination by exudate is due to variation in experimental procedure or in possible isolate differences is unknown. However, regardless of differences in exudation, in all studies the conidia were nutrient independent in axenic conditions and sensitive to fungistasis in soil and the leaching system. Stimulatory exudate was consistently obtained in the present study from conidia that were incubated for 30 min in an ultrasonic cleaner. Whereas conidia unquestionably contain compounds which stimulate germination of other conidia (Mandels, 1981), it is unknown whether there is any ecological significance in the stimulation if it is only observed when high concentrations of stressed spores are extracted.

These results do not disprove the essence of the nutrient deprivation hypothesis. That is, fungistasis may be controlled by the loss of a germination-regulating compound(s) into soil or an aqueous medium. However, based on the present results, it seems more likely that 1) such compounds would be released in the second stage of germination, and 2) that the compounds serve as a means of sensing the suitability of the environment for germination, rather than actually depleting the spores of its own metabolic reserves. The compounds released in the second

stage of germination are unidentified; characterization of the composition and the kinetics of the release of these compounds may be useful in delineating the mechanism of fungistasis.

4.5. SUMMARY

Helminthosporium victoriae conidia exuded glucose and four other sugars when incubated in water. The main flush of exudation occurred during the first 30 min at 2 or 22 C. Glucose, which accounted for 52% of the exuded sugars, stimulated germination in 10 µM or greater concentrations. Glucose in combination with an equimolar concentration of asparagine was no more stimulatory than glucose alone. H. victoriae conidia also exuded Mg, P, K, and Cu.

The exudate did not represent substrate required for germination. When conidia were incubated in germination-inhibiting conditions, then transferred to germination-conducive environments, conidia pre-incubated in the inhibiting conditions germinated as rapidly as conidia incubated only in the conducive environment. The primary glucose-rich exudate was apparently not required exogenously for germination. Exudate inconsistently stimulated freshly harvested conidia and ^{14}C-labelled exudate was not taken up by ungerminated conidia.

LITERATURE CITED

Bhatti, T., R.E. Chambers, and J.R. Champ. 1970. The gas chromatographic properties of biologically important n-acetylglucosamine derivatives, monosaccharides disaccharides, trisaccharides, tetrasaccharides and pentasaccharides. Biochim. Biophys. Acta 222:339-347.

Bristow, P.R. and J.L. Lockwood. 1975. Soil fungistasis: role of spore exudates in the inhibition of nutrient independent propagules. J. Gen. Microbiol. 90:140-146.

Filonow, A.B. 1981. Mycostasis and the microbial nutrient sink of soil. Ph.D. thesis, Michigan State University, E. Lansing, MI. 97p.

Filonow, A.B. and J.L. Lockwood. 1979. Conidial exudation by Cochliobolus victoriae on soils in relation to soil mycostasis, p. 107-119. In B. Schippers and W. Gams (eds.). Soil-borne plant pathogens. Academic Press. London.

Garraway, M.O. and R.C. Evans. 1977. Sporulation in Bipolaris maydis: enhancement by xylose. Phytopathology 67:990-993.

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.

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

Lockwood, J.L. 1975. Quantitative evaluation of a leaching model system for soil fungistasis. Phytopathology 65:460-464.

Lockwood, J.L. 1977. Fungistasis in soils. Biol. Rev. 52:1-43.

Lockwood, J.L. 1981. Exploitation competition, p. 319-349. In D.T. Wicklow and G.C. Carroll (eds.). The fungal community. Marcel Dekker, Inc., N.Y.

Mandels, G.R. 1981. Compartmentation of metabolic systems in the regulation of dormancy in fungus spores. Exp. Mycol. 5:278-291.

Simon, E.W. 1974. Phospholipids and plant membrane permeability. New Phytol. 73:377-420.

Sneh, B. and J.L. Lockwood. 1976. Quantitative evaluation of the microbial nutrient sink in relation to a model system for soil fungistasis. Soil. Biol. Biochem. 8:65-69.

Watson, A.G. and E.J. Ford. 1972. Soil fungistasis - a reappraisal. Annu. Rev. Phytopathol. 10:327-348.

Wynn, W.K., R.C. Staples, B. Strousse and C. Gajdusek. 1966. Physiology of uredospores during storage. Contr. Boyce Thompson Inst. Pl. Res. 23:229-242.

Yadav, B.S. and C.L. Mandahar. 1981. Spore germination of Drechslera sorokiniana and D. teres in relation to leaching. Trans. Brit. Mycol. Soc. 77:219-222.

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