DYNAMICS OF FUNGAL SPORE GERMINATION IN SOIL

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
DAVID L. YODER
1971

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Dynamics of Fungal Spore
Germination in Soil

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Plant Pathology

John L. Lockwood
Major professor

Date June 14, 1971

O-7639

ABSTRACT

DYNAMICS OF FUNGAL SPORE
GERMINATION IN SOIL

By

David L. Yoder

It is widely known that fungus spores fail to germinate in natural soil, but spores will germinate completely in soil amended with energy-yielding metabolic substrates or in sterilized soil which is also an energy-rich medium. Experiments were designed to determine (i) the nutritional requirements for inducing fungal spore germination in soil; (ii) the reversibility or irreversibility of the accumulated progress toward germination completion under competitive conditions in the soil; (iii) the fate of accumulated energy-yielding metabolic substrates in cases of regression in the germination process; and (iv) the role of water in the germination process.

The same total time of exposure to sterilized soil was required for GT_{50} (the time required for germ tube production by 50% of the spores) of Penicillium frequentans conidia whether the incubation was continuous or was

interrupted by alternate 4 hr periods on unamended-natural soil. In addition, conidia initially incubated 3 hr on sterilized soil then transferred to natural soil for 3 or 6 days and subsequently returned to sterilized soil required the same total exposure time to sterilized soil for GT_{50} as did control conidia incubated continuously on sterilized soil.

Conidia of P. frequentans and Aspergillus ustus and chlamydospores of three Fusarium species initially incubated on sterilized soil for periods slightly less than that required to germinate, then transferred to natural soil for 6 days, required significantly longer total exposures to sterilized soil to reach the GT_{50} than did control spores. A. ustus conidia initially incubated on sterilized soil and then transferred before germ tube production to natural soil for increasingly longer periods required progressively longer total exposures to sterilized soil for GT_{50}. These data indicated that a retrogression in the germination process, or a reversal in the time accumulated toward germination, occurred during the natural soil incubation period.

Germinating conidia of P. frequentans, labeled in a ^{14}C-glucose medium, lost radioactivity when subsequently incubated on natural soil. The percent loss of radioactivity from ^{14}C-glucose labeled conidia was directly correlated with the percent reversal in the time accumulated toward

germination when these conidia were incubated on natural soil for increasingly longer times. In addition, about 50% of the radioactivity was lost from ^{14}C-glucose labeled conidia incubated in a closed leaching system designed to imitate the microbial-nutrient sink of natural soil. About 50% of the radioactivity lost was found in the leachings and spore supporting materials and the remainder in $^{14}CO_2$.

Conidia of P. frequentans pre-incubated on natural soil or on glass beads saturated with subsoil extract or water germinated subsequently on sterilized soil more quickly than did control spores not pre-incubated. No significant reduction of the GT_{50} was found when pre-incubation was made at 1°C. With the exception of chlamydospores of two Fusarium oxysporum form species, pre-incubation of spores on natural soil resulted in decreased GT_{50} values for all spores tested when they were then transferred to sterilized soil and compared with untreated control spores.

Conidia of P. frequentans pre-incubated on natural soil and subsequently dried over P_2O_5 required no longer to germinate on sterilized soil than did pre-incubated conidia not dried. These results indicated that, unlike the nutrient-mediated phase of the germination process, the water-mediated phase was irreversible.

Kinetic studies of water uptake indicated that during a twenty-four hour period of incubation of P. frequentans conidia on glass beads saturated with tritiated water, a rapid, early uptake and incorporation of water into metabolites occurred. During the incubation period permeability seemed to increase and permit a freer exchange of the labeled water within the spores with unlabeled water when the spores were subsequently washed. In addition, tritium labeled metabolites were lost from the spores during incubation and collected as a labeled residue after evaporation of the tritiated water on which the spores were incubated. The increased permeability, exudation of labeled metabolic products, and exchange of the tritium on labeled products within the spores with unlabeled water during washing resulted in near total loss of the previously accumulated tritium from the spores.

Conidia pretreated in water had an increased rate of ^{14}C-glucose uptake when compared with control conidia not pretreated.

From these data it is concluded that the germination process of spores in soil was initiated by exposure to water in soil, and that the nutrient-mediated phase of the germination process is a dynamic process dependent on the presence and duration of utilizable carbon nutrients.

DYNAMICS OF FUNGAL SPORE GERMINATION IN SOIL

By

David L. Yoder

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1971

To Marian

ACKNOWLEDGMENTS

Deep appreciation is expressed to Dr. J. L. Lockwood for his guidance and constant encouragement during the course of this investigation and in preparation of the manuscript.

Thanks are also due to Drs. N. E. Good, M. L. Lacy, C. L. Schneider, and A. R. Wolcott for making helpful suggestions and for critical evaluation of the manuscript.

Appreciation is expressed to Dr. H. J. Kende for the use of the tritium oxidizer.

This study would not have been possible without the patience, optimism, and encouragement shown by my wife, Marian. In more subtle ways Sandi and Gretchen provided their own inspiration.

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CHAPTER I

INTRODUCTION

Recent evidence presented by Ko and Lockwood (39) indicated that fungal spores in soil failed to germinate because the soil lacked essential nutrients, or that nutrients were lost from spores due to a sink effect imposed by competing organisms, rather than as a result of microbially produced, diffusible, inhibitory substances (21).

Fungal spore germination in soil does indeed occur as a result of amendment with nutrients (7, 27, 66) or in the rhizospheres of plants (4, 14, 16, 17, 27, 28, 34, 37, 42, 56, 57, 58, 66). Steiner and Lockwood (60) recently reported that the duration of exposure to an energy source nutrient was the determining factor in whether germination occurred or not. Fungal spores exposed to nutrients for periods less than those required for formation of a germ tube, then transferred to non-nutritive conditions and later returned to nutritive conditions, required the same total exposure time (although interrupted) to nutrients for germination as for spores continuously exposed to nutritive conditions. They concluded that the effect of a nutrient stimulus on the germination process was cumulative and irreversible.

My research was initiated as an attempt to reexamine and extend the results of Steiner and Lockwood. Three primary issues were considered: (a) the effect of continuous or interrupted exposure to nutrients on spore germination; (b) the reversibility or irreversibility of the accumulated progress towards GT_{50} under competitive conditions of soil; and (c) the fate of accumulated energy substrates in cases of regression towards dormancy in the germination process.

CHAPTER II

LITERATURE REVIEW

Fungal spore germination in soil has received considerable attention since the discovery of the phenomenon of soil fungistasis by Dobbs and Hinson in 1953 (20). A preponderance of the papers during this interval has either attested to the existence of this characteristic of soils or presented evidence as to the nature of soil fungistasis. Lockwood has recently reviewed the literature related to this subject (47). Relatively few investigations have been made into the sequence of morphological and physiological events occurring in the fungal spore during germination in soil. However, it is well known that propagules of soil or root-inhabiting fungi germinate, grow and develop in microsites of decaying organic matter and in the rhizospheres of plants (4, 14, 16, 17, 27, 28, 34, 37, 42, 56, 57, 58). The physiological aspects of fungal spore germination have recently been reviewed by Allen (1). My review will consider the literature dealing with the aspects of spore germination which relate to specific areas covered in the present research.

The germination phenomenon.--The fungus spore is a nucleated portion of the fungus body which is delimited

from the main thallus. It is characterized by a cessation of cytoplasmic movement, low water content, slow metabolism, a lack of vacuoles, and is specialized for dispersal, reproduction, or survival (33). The process of resumption of active development is termed germination; it may be fixed by the first irreversible stage that is recognizably different from the dormant organism, as judged by physiological or morphological criteria (62). Morphologically, germination has been recognized to include (a) a preliminary stage of swelling, (b) the emergence of the germ tube, and (c) the early development of the germ tube after emergence (15).

Nutrient requirements for germination.--Germination of fungal spores is a growth process which requires prescribed environmental conditions for its initiation. The two extremes found in fungal species are (a) complete dependence on, or (b) complete independence of exogenous nutrients. Some species also exhibit a borderline character, in that within a population of spores a low percentage will germinate in the absence of exogenous substrates (28).

Nutritional requirements for spore germination have been established for many fungi. Basically, it has been found that an energy source such as glucose is essential and may often be the only requirement. Many fungi require, in addition, some form of nitrogen, either organic or inorganic; and for some species, specific carbon or

nitrogen sources or specific treatments to break dormancy may be essential for the initiation of active growth (22, 25, 28, 43, 46, 49, 61, 67).

Initial stages of germination.--Spore swelling has been recognized to occur as a general phenomenon in the germination process of many fungi. With no firm data to support the hypothesis, it was early suggested that swelling resulted from an uptake of water and was not metabolically controlled (35, 44, 63). Perhaps the supposition that spore swelling resulted from water uptake was made because seeds of angiosperms and gymnosperms imbibe water prior to germination. However, imbibition by seeds is not a characteristic of living tissues alone, for heat-killed seeds imbibe water as strongly as viable seeds by virtue of the hydrophilic colloids within the seed (52). More recent evidence indicates the swelling process of fungal spores is metabolically controlled and results in activation and/or synthesis of respiratory enzymes necessary for energy production.

Using spores of Penicillium atrovenetum, Gottlieb and Tripathi (32) demonstrated that swelling occurs only in the presence of a utilizable carbohydrate. Swelling and germ tube production did not occur when spores were incubated in distilled water or in Czapek-Dox solution minus glucose. When the complete medium was used, 96% of the spores swelled and 97% produced germ tubes within

8 hr. Dibasic potassium phosphate also stimulated some swelling and germ tube production. When this salt was absent from the complete medium, 38% of the spores exhibited swelling and 37% produced germ tubes. Calcium phosphate, but not potassium sulfate, could substitute for the potassium form of the salt. Fusarium roseum (18), Penicillium notatum and Trichoderma lignorum (51) also required an exogenous energy source before swelling occurred, and F. culmorum required both glucose and ammonium sulfate for maximum swelling (49, 50). Swelling in Aspergillus niger, as in P. atrovenetum, required an exogenous carbohydrate and the phosphate ion and, in addition, a reduced nitrogen form such as L-proline or L-alanine (68). Yanagita reported that conidia of this fungus underwent two swelling phases: (a) an endogenous phase which occurred in the first 1.5 hr at all temperatures tested, although she mentioned only 30° and 33°C, and (b) an exogenous swelling which occurred after 1.5 hr at these temperatures. However, at 33°C germ tubes were not produced. The exogenous phase was inhibited by CO_2-free air. The first swelling was minimal. Unincubated spores averaged 3.2 μ in diameter and those which had swollen endogenously were 3.2-4.8 μ across. Exogenous swelling resulted in conidial diameters exceeding 4.8 μ.

Some other fungal species reported to swell on germination include Aspergillus niger (6), Cicinnobolus

cesatii, Colletotrichum trifolii, and Sclerotinia fructicola (68), Myrothecium verrucaria (48), Penicillium griseofulvum (25), Rhizopus arrhizus (23), R. stolonifer (11, 12) and Trichoderma sp. (3). In all of these species, germination was investigated under some form of nutrient condition with the implication that swelling occurs only in the presence of some utilizable energy source.

Ekundayo and Carlile (23) have recently made intensive studies of the swelling phase of germination with Rhizopus arrhizus. Spores of this fungus germinated on a complete-nutrient agar medium within 8 hr. Swelling began about 1 hr after plating, and by 3 hr the spore diameters had nearly doubled. Eight hr later the diameters had increased to 3-4 times the original size. This increase in size throughout the incubation period was approximately linear, with no significant lag, indicating very early metabolic and/or physical alterations. Nutrient conditions supporting maximum swelling and germ tube production included the presence of glucose, dibasic potassium phosphate, and ammonium chloride in the agar medium. Incubation on water agar alone resulted in no swelling or germ tube production. Glucose or fructose, but not sucrose or lactose, in agar brought about an increase in diameter from the original size of 4.5 μ to 7.5 μ, and 20-30% of the spores produced germ tubes. The

addition of ammonium chloride resulted in a further increase in diameter to 9.5 μ and all spores produced germ tubes; the further addition of dibasic potassium or sodium phosphate allowed swelling equal to that on the complete medium (14 μ) and all spores germinated.

Conidia incubated on cellophane strips on a complete-nutrient agar medium for 2 hr increased in diameter from 4.5 to 8.0 μ. If then transferred to water agar no further swelling occurred, but all spores produced germ tubes. However, conidia incubated initially on water agar containing glucose and then transferred to the complete-nutrient agar medium lacking glucose germinated no more than spores held on glucose-agar (20-30%). Hence, utilization of nutrients other than glucose required the simultaneous presence of glucose in the medium. Thus, requirements for maximum swelling were more exacting than those for maximum germination.

In further studies, Ekundayo (24) showed that R. arrhizus spores incubated in D_2O freely exchanged the water within the spore for D_2O without swelling. This indicated that even though swelling required the presence of utilizable energy-source nutrients the spores were permeable to water. Fletcher and Morton (26) confirmed free permeability to D_2O with ungerminated conidia of P. griseofulvum using the same technique.

To determine if swelling was metabolically controlled, Ekundayo (24) incorporated inhibitors of metabolic pathways into a complete-nutrient germination medium. 5×10^{-3} M NaN_3, an inhibitor of the cytochrome system, prevented swelling and germ tube formation for up to 24 hr. Spores then transferred to media without the inhibitor all swelled normally and produced germ tubes in 8 hr. If spores were initially incubated on the complete medium for 3 hr then transferred to the same medium containing 10^{-3} M NaN_3, neither swelling nor germination occurred in 5 addtional hr. However, if transferred instead to water agar with or without the inhibitor, again no further swelling resulted, but 90% of the spores produced normal germ tubes. This indicated that swelling ceased when uptake of utilizable energy sources was blocked by inhibition of the electron transport system. The author suggested that NaN_3, like essential nutrients mentioned previously, was not taken into the spores in the absence of exogenous energy sources.

Fletcher and Morton (26) reported that P. griseofulvum conidia failed to swell or germinate in water. Germination occurred in a complete-nutrient liquid medium in 16 hr, but if glucose was deleted from this medium no swelling or germination occurred in 24 hr. If conidia were incubated in the complete medium for 6 hr then transferred to the same medium, but with glucose concentrations

ranging from 5.0 to 0.04%, the total percentage germination was not altered after 16 total hr. They concluded that the glucose concentration was of significance only during the first one-third to one-half of the germination period. Although no swelling determinations were made, it is possible that the swelling phase in this fungus has more exacting nutrient requirements than does the remainder of the germination process as was found with R. arrhizus.

To test the oxygen requirements for swelling and germ tube production, Wood-Baker (67) incubated sporangiospores of Mucor hiemalis and M. rouxianus in 2% malt-extract through which air or nitrogen gas was bubbled for 8 hr. M. hiemalis germinated 70% in the air treatment and 0% in the nitrogen treatment. M. rouxianus germinated in air, but only 11-12% under anaerobic conditions. If M. hiemalis was incubated aerobically for 4 hr and then in anaerobic conditions for a further 4 hr, only 8.6% germination occurred; but when incubated in the reverse conditions 60.4% of the spores germinated. The same pattern was found for M. rouxianus. Although she assumed from her results that the initial swelling stage of germination occurred under anaerobic conditions, she made no observations of swelling, and thus the only valid conclusion from her work is that aerobic conditions were essential for production of germ tubes.

Bussel et al. (11, 12) reported that sporangiospores of Rhizopus stolonifer swelled in a liquid medium under anaerobic conditions, but if held longer than 3 hr viability was rapidly lost. R. arrhizus spores swelled incompletely under anaerobic conditions, increasing from 4.5 μ to 8.0 μ (23). Fletcher and Morton (26) used 0.05 M KCN and anaerobic conditions and showed that conidia of P. griseofulvum under both conditions swelled minimally, but increased in diameter at least 1.6 X under aerobic conditions. They did not rule out traces of O_2 in the medium or cyanide stable respiration.

Conidial swelling in Trichoderma sp. was investigated by Barnes and Parker (3) in aerated malt-extract medium containing chlorocresol (an antimicrobial agent) in concentrations from 0.005 to 0.05%. Viability of spores was tested after the chlorocresol treatments. After 1 hr contact with 0.05% chlorocresol some 60% of the spores failed to germinate in the medium minus the antimicrobial agent. At all concentrations of chlorocresol used, spores swelled during the first hour of incubation; but only at the lower concentrations did further increase in size occur and germ tubes develop. They attributed the failure of spores to swell, treated at the higher concentrations of chlorocresol, to death occurring during the first hour; the swelling observed in this time period was considered due to "inanimate"

water uptake. However, since Trichoderma spores required 6 hr before germ tube formation, death might have occurred at any time prior to 6 hr when a lethal concentration of the antimicrobial agent was obtained at the active site in the spore. Therefore, the evidence does not rule out metabolic swelling during the first hour.

Macroconidial swelling of Fusarium culmorum may comprise both external and internal alterations. Marchant and White (49) demonstrated a 25% increase in macroconidial width in a near linear pattern during 6 hr incubation in a liquid medium containing glucose, ammonium sulfate, and phosphate buffer. In glucose-phosphate buffer medium no swelling occurred. The rapid increase in conidial width was considered to be due to a thin external matrix which developed on conidia incubated in any medium containing glucose, but this interpretation appears to contradict the observation of no conidial swelling during incubation in glucose-phosphate buffer medium. Studies with tritiated water in the complete medium demonstrated a 32% increase in free water in the spores thus treated over those treated in buffer control which also contained tritiated water. Therefore, the swelling of macroconidia of F. culmorum, at least in part, may be attributed to water uptake within the spore.

The investigations of Ekundayo (24), Fletcher and Morton (26), and Marchant and White (49) using deuterated,

deuterated, and tritiated water, respectively, have demonstrated that spore membranes are freely permeable to water, but that water uptake by spores during the swelling process occurs only when utilizable energy substrates and/or nitrogen-containing compounds are present. However, the fact that swelling may occur under anaerobic conditions indicates that a minimum expenditure of energy is required.

Several investigations (5, 29, 69) have demonstrated increases in fresh or dry weights of spores incubated for periods less than that required for germ tube production. Others (5, 13, 30, 31, 32, 36, 48, 53, 59, 64, 68) have presented evidence of increased metabolic activity and changes in protein, lipid, and/or nucleic acid content.

With the exception of the nutrient-independent fungi, few investigations have been made of the influence of water on the germination process of fungal spores. Scheld and Perry (55) reported that basidiospores of Lenzites saepiaria responded immediately to water treatment by increased respiratory activity. Doran (22) early reported germination of 24 species, including a broad spectrum of taxonomic types, in water alone; and Broadfoot (9) reported germination of Fusarium lini in water. More critical studies have demonstrated that most of these fungi, except for the rust and powdery mildew fungi, require exogenous substrates for germination. Possibly

these early investigations were made with unwashed spores or in nutrient-contaminated glassware.

Germination in soil.--Despite a general deficiency of energy-yielding substrates in soil we would expect germination to occur in soil among those fungal species which are independent of exogenous sources of nutrients. However, these fungi, as well as the nutrient-dependent species, fail to germinate in soil in the absence of added nutrients when all other known conditions for the resumption of active growth appear to be met (7, 39).

The nature of this phenomenon has been debated repeatedly. It is agreed that microorganisms are in some way responsible for this inhibition of germination. Two possible explanations of the nature of fungistasis are (i) diffusible inhibitory substances produced by microorganisms which are active in the soil complex (21), and (ii) the general deficiency of essential energy-yielding nutrients or the loss of such nutrients from nutritionally independent spores imposed by the pervasive sink resulting from the complex of hungry organisms in the soil (39).

Ko and Lockwood (39) presented evidence that fungal spore germination in soil is inhibited because of the deficiency of utilizable energy substrates in the environment, and the rapid disappearance of such substances from the general soil mass. To test the hypothesis that this condition is the determinant of the widespread

fungistasis of soil, model systems were developed to place spores under nutrient stress by slowly leaching them with water or phosphate buffer. The response of spores to this condition was then compared to that of spores on unamended-natural soil. Testing seven fungal species, which represented the nutritionally dependent and independent groups, each responded similarly to both soil and the leaching system. For example, activated Neurospora tetrasperma ascospores germinated on the soil and also when leached; Helminthosporium victoriae conidia did not germinate on soil or when leached; and conidia of Penicillium frequentans failed to germinate on soil or on the leaching system. Thus the artificial nutrient sink created by leaching imitated natural soil by inhibiting spore germination to the same extent and for the same spectrum of fungi. They concluded that this evidence, coupled with documentation of rapid loss of utilizable carbohydrates added to natural soil, indicated the significance of nutrient stress in restricting germination of fungal spores in soil.

In studies of the sensitivity of fungal spores to soil fungistasis, Steiner and Lockwood (60) reported that spores having relatively small volumes required long periods for germination while larger spores germinated more quickly. For example, conidia of H. victoriae had a volume of 6333 μ^3 and germinated in 1.5 hr. Fusarium

solani f. sp. pisi macroconidial volume was 427 μ^3 and they germinated in 4 hr. Glomerella cingulata conidial volume was 212 μ^3 and required 6 hr for germination, and Aspergillus fumigatus conidia had a volume of 13 μ^3 and germinated in 13 hr.

Gottlieb (30) has suggested that the time required for germ tube production is a reflection of the enzymatic capabilities of the spore. Uredospores of two rust fungi, Puccinia graminis tritici and Uromyces phaseoli, contained a full complement of the enzymes required for anaerobic respiration, those for the oxidation of the intermediate organic acids in the Krebs cycle, and complete terminal electron-transport systems. These two fungi germinated within 2 hr. Penicillium oxalicum, also shown to possess a full complement of enzymes, required 6-8 hr for germination, but Ustilago maydis only began germination after 12 hr, which Gottlieb reported as the time required for a full set of enzymes to be synthesized. Spores of this fungus lacked seven of the essential enzymes required for glucose catabolism, but each was synthesized during the germination process. Waid (66) suggested that the rapidity of response of a soil-substrate colonist depends upon its potential enzyme complement and the availability of nutrients for the generation of active protoplasm.

Those fungi which require long periods for spore germination have been reported by Steiner and Lockwood to

be the most sensitive to soil fungistasis (60). Thus P. frequentans, which required 7 hr for germination had a sensitivity index of 20 (sensitivity indexes were determined by the ratio of sterile to natural soil which would allow 50% germination), but Helminthosporium sativum, which germinated in 2.5 hr, had a sensitivity index of 2.5. In soil, microbial competition resulted in rapid depletion of available utilizable substrates. Thus it would appear that fungal spores in soil requiring long periods to complete germination would be at an ecological disadvantage without some compensatory mechanism which would allow them to benefit from periodic exposures to nutrients in soil. Such exposures may result from alternate wetting and drying, freezing and thawing, animal activity, lysis of microorganisms, death of roots, etc.

The results of Steiner and Lockwood (60) indicated that fungi are able to maintain their position in the germination process once this phenomenon has begun. By exposing spores of three fungal species, Verticillium albo-atrum, P. frequentans, and Aspergillus ustus, to a nutrient solution and water for alternating 4 hr periods, they showed the total time of exposure to nutrients, whether uninterrupted or temporarily suspended, was the determining factor in germination; i.e., there was no difference in the time required for germination whether the exposure was continuous or discontinuous. This was

confirmed by exposing spores of G. cingulata, P. frequentans, and A. ustus to sterilized soil for a time period approximately 1.5 hr less than the time required for germination, then transferring the spores to natural soil for 0, 2, 4, 6, and 11 days before returning them to sterilized soil. Spores so treated held their relative positions in the time course of the germination process. For example, conidia of P. frequentans required 11 hr to germinate when continuously incubated on sterilized soil. Conidia incubated 2, 4, 6, and 11 days on natural soil following an initial exposure to sterilized soil required a total exposure to sterilized soil of 11.5, 12, 13, and 12.5 hr, respectively, for germination. These results led Steiner and Lockwood to propose that nutrients are not germination-triggering mechanisms for many fungi, as has been reported for ascospores of Neurospora spp. (62), but that germination requires an exposure to energy-supplying nutrients for a given length of time, depending on the species. They concluded that fungal spores in soil may periodically be exposed to nutrients, with each increment shortening, irreversibly, the remaining time required for germination. The result would be a population of spores in different states of readiness for germ tube production.

CHAPTER III

METHODS AND MATERIALS

Maintenance of fungi.--*Aspergillus ustus* (Bainer) Thom and Church was maintained on V-8 juice agar (per liter: 200 ml V-8 juice [Campbell Soup Co.], 2 g $CaCO_3$, 20 g agar). *Helminthosporium sativum* Pam., King, and Bakke was maintained on sterilized wheat straws. The following fungi were maintained on potato dextrose agar: *Penicillium frequentans* Westling, *Fusarium solani* (Mart.) Appel and Wr. f. sp. *pisi* (Jones) Snyd. and Hans., *F. oxysporum* Schlect, f. sp. *lycopersici* (Brushi) Snyd. and Hans., *F. oxysporum* f. sp. *melonis* (Leach and Curr.) Snyd. and Hans., and *Mucor ramannianus* Moeller.

Characteristics and preparation of soil.--Conover loam soil was stored up to four months in 5 gal covered containers at 25 ± 2°C. This soil possessed the following characteristics: pH 6.7, organic matter 3.8%, clay 7.5%, silt 42.8%, sand 49.7%, and water holding capacity 42.7%. For some experiments the soil was air dried, sieved, and stored up to two weeks in covered plastic containers. For making soil plates for spore germination assays (45), soil was remoistened with 12 ml H_2O/50 g air dried soil, added

to plastic petri dishes (90 X 15 mm) and the surface smoothed with a bent spatula. These plates were incubated ca. twelve hours before use. Control plates of sterile soil were prepared in glass petri dishes (95 X 15 mm) and autoclaved at 121°C (15 psi) for 30 min.

Collection and preparation of spores.--Conidial suspensions of the fungal species to be tested were prepared by adding sterile glass-distilled water to slant cultures of the organism and gently agitating the mycelial colony with a transfer loop. Conidial suspensions were transferred to sterile centrifuge tubes and washed 2-3X by refrigerated centrifugation.

Chlamydospores of Fusarium solani f. sp. pisi, F. oxysporum f. sp. lycopersici, and F. oxysporum f. sp. melonis were obtained by a method similar to that of Alexander et al. (2). One liter of distilled water was added to 1 kg Conover loam soil and shaken for one hour. The suspension was centrifuged at 3000 g for 10 min and the clear supernatant solution was made up to 1 liter with distilled water. Two-tenths g sodium polypectate was dissolved in the soil extract and the solution sterilized by membrane filtration (Millipore Corp., Bedord, Mass., pore size 0.22 μ). Fifty ml of the filtrate was dispensed into sterile 250 ml Erlenmeyer flasks and unwashed conidia of the different Fusarium species were introduced. Flasks were incubated on a platform shaker

for up to 30 days until chlamydospores were abundant. Chlamydospores were detached from mycelia by blending in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) for 1 min at approximately 5000 rpm or by grinding in a tissue homogenizer with a Teflon pestle (Tri-R Instr., Inc., Jamaica, N.Y.). The suspension was passed through a 325-mesh sieve to remove mycelial fragments. Chlamydospores, reasonably free of mycelial fragments, were collected and washed 3X by refrigerated centriguation.

Spore germination assays.--Germination assays and spore treatments were performed on Acropor membrane filters (Gelman Instr. Co., Ann Arbor, Mich., pore size 0.20 μ) placed on the smooth surface of soil plates. When natural soil was used, a larger membrane (2 X 2 cm) was placed directly on the soil surface and two smaller (1 X 1 cm) membranes were located on the larger filter. Sufficient moisture was essential to maintain good contact between membranes and soil; therefore, one drop (0.04 ml) water was dispensed on the soil surface at the location of each membrane. On sterilized soil plates only the smaller, single membrane filters were utilized. In some experiments water or a nutrient solution (extract of autoclaved subsoil containing 0.1% glucose or a solution of 0.1% glucose and 0.1% peptone) was added to petri dishes containing 200 μ diameter glass beads to a depth of 5-7 mm. Large membrane filters, used for support, and small membrane filters

were placed on the surface of the saturated beads as described for the soil plates.

For spore germination tests, 0.05 ml of a dilute spore suspension was placed on each small filter with a sterile Pasteur pipette. Periodically, duplicate membranes were removed, stained with phenolic rose bengal, air-dried and mounted on glass slides in mineral oil for clearing. Two hundred spores per membrane were examined microscopically for germination. Thus, for each time period 400 spores were counted, and the time required for 50% germination was determined by plotting germination percentages as probits against the log of time. The time required for 50% of the spores to germinate was called the GT_{50}.

In some experiments, Helminthosporium sativum conidia were thoroughly mixed in 10 g air-dried, remoistened, nonamended- or glucose-amended- (0.3% w/w) natural soil and incubated in screw-cap test tubes for various periods. To recover the conidia, a modification of Ledingham's (43) flotation technique was employed. Ten ml mineral oil was added and mixed with the soil, then 10 ml water was pipetted into the tubes. Each tube was shaken vigorously for 3-4 minutes and the emulsion layer containing the conidia removed with a Pasteur pipette. Soil particles were removed by repeated refrigerated centrifugation (3000 g) which broke the emulsion leaving the spores in the oil layer. Detergent (0.05 ml Tergitol nonionic TMN) and

10 ml water were added to the separated oil layer. Centrifugation resulted in pelleting the conidia in the water layer. Conidia were then washed 3-4X with sterile glass-distilled water by further centrifugation. Germination of these conidia was determined on sterilized soil or in small petri dishes (50 X 15 mm) containing 1.5 ml Czapek-Dox salts solution (per liter: $NaNO_3$, 2g; K_2HPO_4, 1 g; KCL, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $FeSO_4$, 0.01 g) plus 0.01% glucose and 0.01% peptone. Two hundred spores per duplicate sample were examined for germination and the GT_{50} determined. All experiments were run in duplicate and were repeated two or more times.

Radioisotope studies.--Uptake and loss of an energy source and water from spores was studied using ^{14}C-glucose (uniformly labeled) and $^{3}H_2O$. In ^{14}C studies conidia were incubated either directly in 1 ml liquid medium in screw-cap test tubes or on membrane filters placed on 200 μ diameter glass beads saturated with the medium. When membrane filters were used, controls consisted of filters without spores incubated under the same conditions. The medium used was an extract of autoclaved subsoil prepared by shaking 1 kg autoclaved subsoil in 1 liter of water for 1 hr and clarified by centrifugation. The sterile extract was supplemented with glucose (0.1%) and ^{14}C-glucose to give a final concentration of 1 μCi/ml (specific activity 1 μCi/5.55 mM glucose).

After incubation directly in the medium, conidia were washed 3X by refrigerated centrifugation and a standard volume was then pipetted onto membrane filters. Triplicate filters bearing the spores were used for ^{14}C determination. Conidial concentration to be applied to the filters was determined using a hemacytometer.

An artificial nutrient sink designed to imitate the effect on germination of incubation on natural soil was constructed. This was similar in principle to that described previously by Ko and Lockwood (39), except that the modified model provided for a completely closed chamber for incubating the spores and was arranged so that spore exudates and CO_2 evolved could be collected quantitatively (Figure 8). Sterile distilled water was slowly dripped (15-20 ml/hr) from a separatory funnel attached to an inlet in the top of a ground-glass weighing bottle (90 X 15 mm) containing 65-70 g glass beads (200 μ diameter). Water slowly percolated through the glass beads to an outlet at the bottom on the opposite side which was connected to a large flask for collection of the leachings. Air, dried by passage through $CaCl_2$ was directed through a mixture of methyl cellosolve and ethanolamine (7:3 v/v) to remove CO_2. The air from the CO_2 trap was then directed through an inlet and outlet in the top of the leaching chamber to remove CO_2 from spores on membrane filters aseptically placed on the glass beads.

The air was then dried and directed to a second CO_2 trap of methyl cellosolve and ethanolamine. The air flow rate was ca. 10-12 ml/min.

Radioactivity in the spores was determined using either a gas-flow Geiger-Mueller counter with a Micromil window or by scintillation in 15 ml modified Bray's scintillation fluid (8)(toluene, ethyleneglycol-monomethylether 2:1 v/v; 2, 5-diphenyloxazole (PPO), 4 g/l; and 1, 4-di(2-(5-phenyloxazolyl)-benzene)(POPOP), 0.05 g/l; using a Packard Tri-Carb spectrometer.

For experiments with tritiated water, conidia were incubated on Nuclepore membrane filters (General Electric Co., pore size 0.5 μ) on 200 μ diameter glass beads saturated with tritiated water diluted with glass distilled water to 5 μCi/ml. Conidial concentrations were determined by direct counts. Following incubation, conidia and membranes were washed with 5 ml Tergitol nonionic TMN detergent (0.01%) followed by 50 ml water in a Millipore filtration apparatus. Filters and spores were oxidized in a Packard Tritium Oxidizer and the tritium collected in scintillation fluid (naphthalene, 100 g; PPO, 5 g; dimethyl-POPOP, 0.3 g; dioxane, 730 ml; toluene 135 ml; absolute methanol, 35 ml). Radioactivity was determined using a Packard Tri-Carb spectrometer (efficiency 36%). All experiments were repeated at least twice with essentially the same results.

CHAPTER IV

RESULTS

Effect of intermittent exposure to sterilized soil on germination time.--Steiner and Lockwood (60) reported that the time required for 50% germination of spores of 3 fungal genera was not altered by discontinuous incubation in nutrient solution. Spores alternately incubated in a nutrient solution and in water required the same total time in nutrient solution to reach GT_{50} as control spores incubated continuously in nutrient solution. Experiments were designed to determine if alternate exposure to sterilized and natural soil would reproduce the response observed by Steiner and Lockwood.

Washed conidia of Penicillium frequentans were incubated on sterilized soil to determine the GT_{50} of the conidia. After 4 hr on sterilized soil some conidia were transferred to natural soil plates for a further 4 hr period. The same two treatments were repeated and followed by a final incubation on sterilized soil. After each incubation some conidia were retained on the same soil plate and the GT_{50} was determined.

The GT_{50} for conidia continuously incubated on sterilized soil was 15 hr (Figure 1). In the first natural soil incubation subsequent to the initial 4 hr incubation on sterilized soil, no germination occurred after 24 hr. No germination occurred after the second 4 hr incubation on sterilized soil, but conidia left on sterilized soil achieved the GT_{50} in 6 additional hr (total = 14 hr). On the second natural soil incubation no germination occurred in 24 hr. About 10% germination was found after the third 4 hr incubation on sterilized soil and after 2 additional hr the GT_{50} was achieved (total = 14 hr). Thus the total time needed for 50% germination of spores intermittently exposed to sterilized soil was 14 hr as compared to 15 hr for spores continuously exposed to sterilized soil. Germination appeared to be a continuous, cumulative process dependent upon the duration of exposure to the nutrient supply. The fact that conidia treated on natural soil germinated 1 hr more quickly than the control spores suggested that exposure to natural soil may have decreased the GT_{50} values to a limited degree.

In natural environments of soil, fungal propagules may be exposed to nutritionally favorable conditions of insufficient duration to allow germination to be completed, which may be followed by periods of essentially starvation conditions; therefore, experiments were

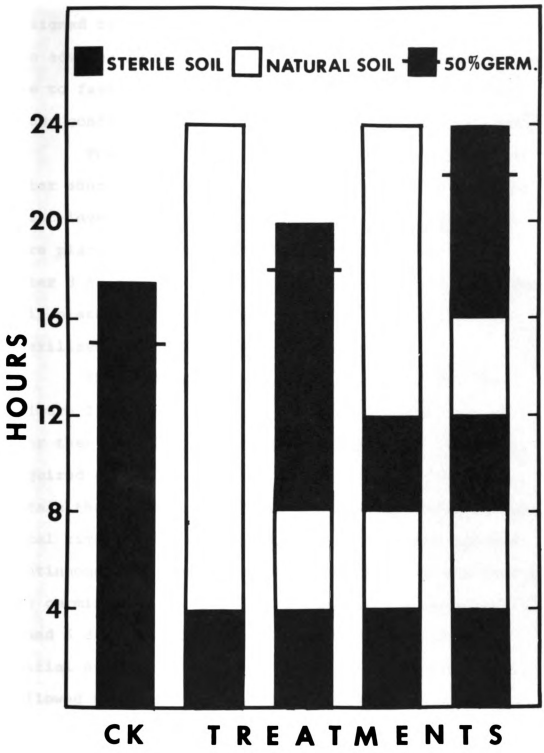

Figure 1. Effect of alternate 4 hr exposures to sterilized and natural soil on the GT_{50} of P. frequentans conidia.

designed to determine whether fungal spores maintained the advance in the germination process acquired by exposure to favorable conditions when subsequently placed under conditions of starvation before germination occurred.

The effect of longer exposures to natural soil after short initial incubations on sterilized soil was first investigated. Washed conidia of P. frequentans were placed on membrane filters on sterilized soil. After 3 hr some of the conidia were transferred to natural soil plates for 3 or 6 days and subsequently returned to sterilized soil to determine the GT_{50}.

The control conidia reached GT_{50} in 10.2 hr (Figure 2). Conidia incubated on sterilized soil for 3 hr then transferred to natural soil for 3 or 6 days required 6.1 and 5.6 additional hr, respectively, to attain the GT_{50} point. There was no difference in the total time required for germination of spores exposed continuously to sterilized soil and those interrupted in the germination process by incubation on natural soil for 3 and 6 days ($P>5\%$). Thus under conditions of short initial exposures to favorable nutrient circumstances, followed by relatively longer incubation periods on nutrient-deprived media, conidia appeared to maintain their progress initially made towards germination, or possibly even to have advanced somewhat farther. It could

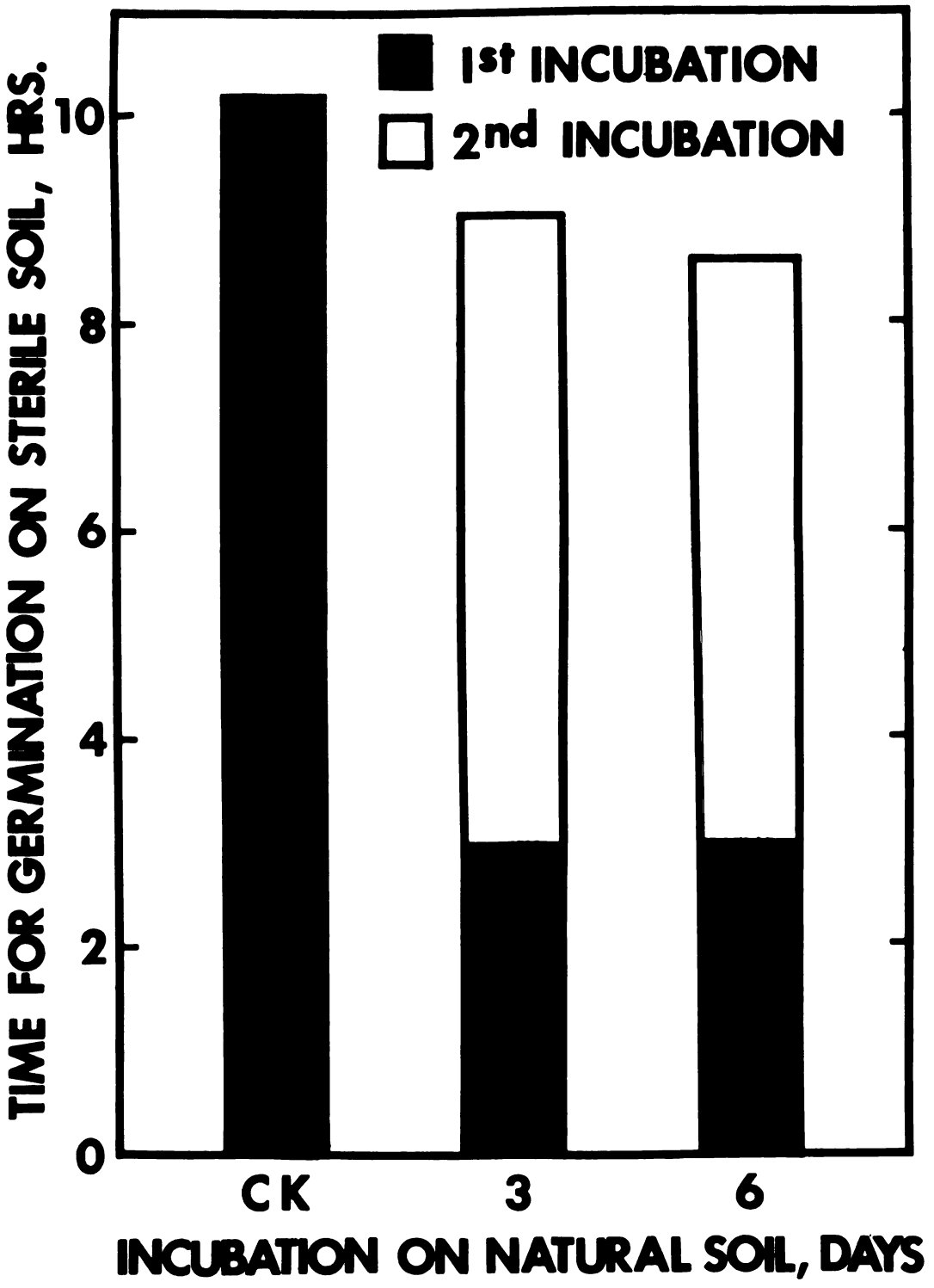

Figure 2. GT_{50} of P. frequentans conidia incubated on sterilized soil for 3 hr, transferred to natural soil for 3 or 6 days, and subsequently returned to sterilized soil.

be concluded from the above data that the germination process was indeed cumulative and irreversible.

Experiments were next designed to determine whether fungal spores nearing completion of the germination process remained at that stage during exposure to starvation conditions. Washed conidia of P. frequentans were incubated on sterilized soil for 9 hr then transferred to natural soil for 3 or 6 days. After 3 or 6 days the conidia were returned from natural soil to sterilized soil for germination.

Conidia incubated on sterilized soil without interruption needed 11.9 hr to reach the GT_{50} (Figure 3). Conidia initially incubated on sterilized soil for 9 hr then transferred to natural soil for 3 days required 3.2 additional hr on sterilized soil to germinate and those on natural soil for 6 days needed 7.2 additional hr. Thus the total time (12.2 hr) needed for germination of conidia incubated on natural soil for 3 days was not different from the control conidia ($P>5\%$). By contrast, conidia incubated 6 days on natural soil then returned to sterilized soil required a total sterilized soil exposure 4 hr longer than that of controls (total = 16.2 hr; $P<5\%$). Thus, it was concluded that conidia nearing germination completion were sensitive to starvation conditions of the environment, which was reflected in a regression in the time accumulated towards germination.

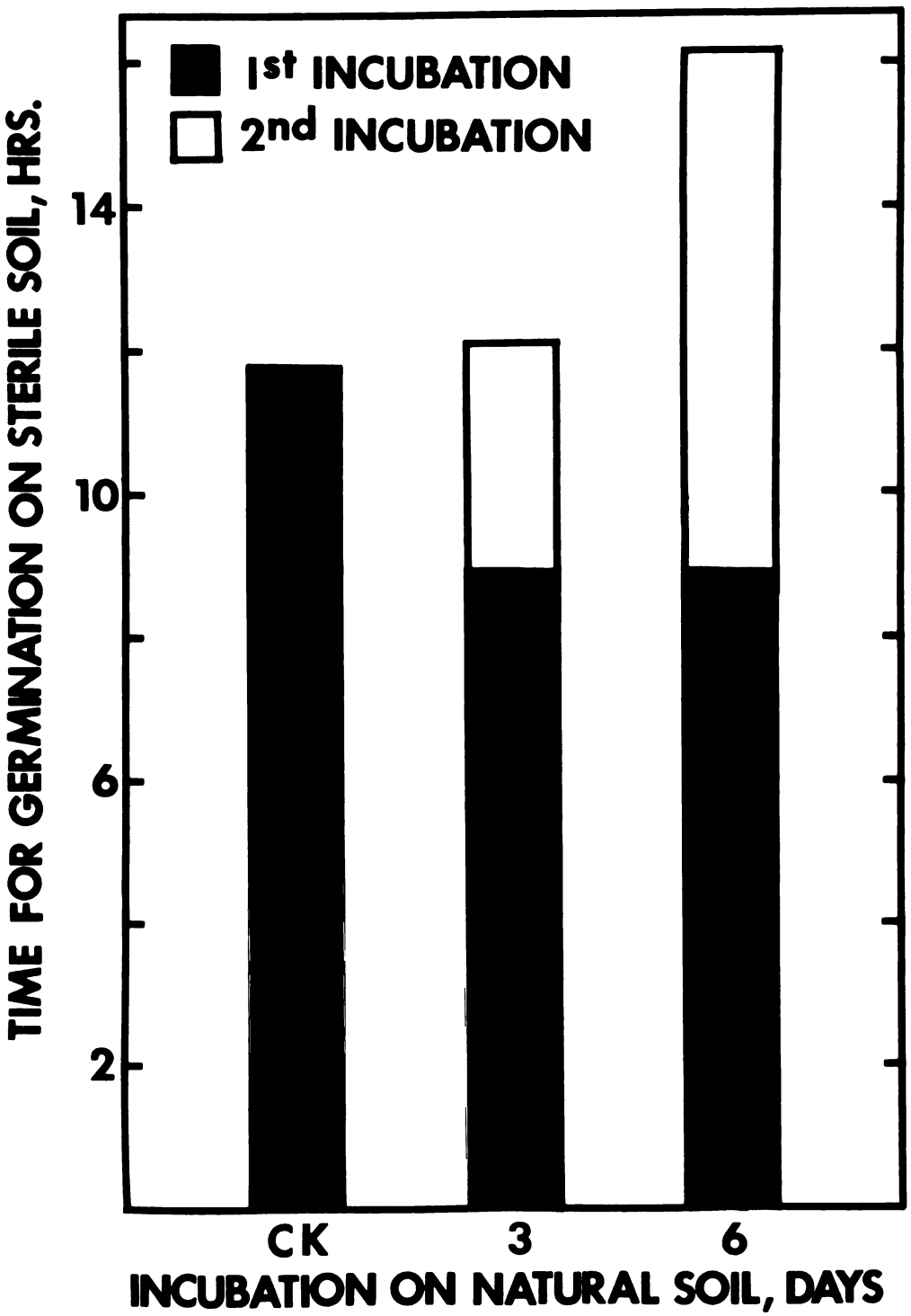

Figure 3. GT_{50} of P. frequentans conidia incubated on sterilized soil for 9 hr, transferred to natural soil for 3 or 6 days, and subsequently returned to sterilized soil.

There was also a suggestion of this reversal in the germination process with the conidia incubated 3 days on natural soil. These spores required a total time equivalent to the control spores for germination, but in the previous experiment (Figure 2) spores initially incubated on sterilized soil for 3 hr had an apparent shorter GT_{50} than control spores.

To determine if this phenomenon was peculiar to P. frequentans or was characteristic of other fungi, including soil fungi, conidia of Aspergillus ustus and chlamydospores of Fusarium solani f. sp. pisi, F. oxysporum f. sp. lycopersici, and F. oxysporum f. sp. melonis were incubated on sterilized soil for periods less than those required for 50% germination. The spores were then transferred to natural soil for 6 days and subsequently returned to sterilized soil for the GT_{50} determination. In all cases a regression in germination time was found (Figure 4; $P<5\%$). For example, A. ustus conidia required 15.3 hr uninterrupted incubation on sterilized soil for GT_{50}. Conidia transferred to natural soil after 11 hr required 9.5 hr subsequent incubation on sterilized soil to achieve the GT_{50} point; i.e., a total of 20.5 hr of sterilized soil incubation was needed. The same pattern was observed with chlamydospores of the Fusarium spp.

Conidia of Helminthosporium sativum presented a special problem due to their ability to germinate on some

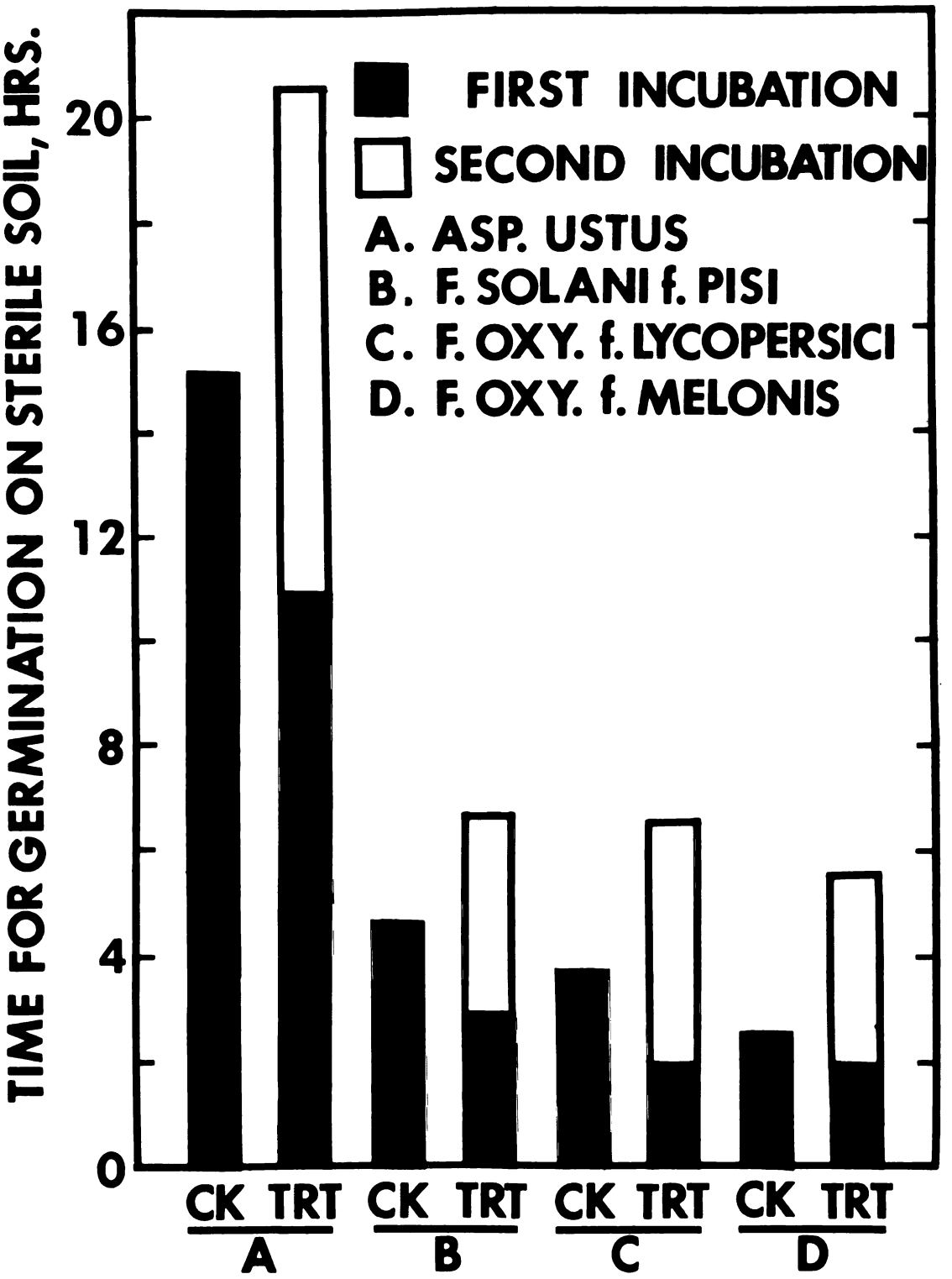

Figure 4. GT_{50} of spores incubated initially on sterilized soil for long periods relative to their GT_{50} on sterilized soil, then transferred to natural soil for 6 days and subsequently returned to sterilized soil.

membrane filters on natural soil, though they did not germinate directly on soil. To circumvent this problem, conidia were incubated in screw-cap tubes containing 10 g air-dried natural soil amended with a glucose solution to give 0.3% w/w glucose concentration and 57% WHC. After incubation and extraction of conidia, GT_{50} was determined in a nutrient solution. Glucose concentration in soil was also assessed after incubation periods corresponding to those of conidial incubation. Two water extractions were made of each soil sample and the extracts combined and sterilized by membrane filtration. Glucose was determined with the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.) used as specified by the manufacturer. Glucose, at concentrations of 20, 40, and 80 µg/ml, was used as a standard. The samples were read in a colorimeter at 400 mµ.

Conidia placed in amended soil and removed immediately reached GT_{50} after 3 hr incubation in the germination medium. Those incubated 4 hr in the amended soil required 3.3 hr and those incubated 24 hr needed 4.6 hr to reach the GT_{50}. No evidence was found for germination directly in the amended soils. The glucose concentration in soil decreased from 2.06 mg/g at zero time to 1.94 mg/g at 4 hr and 1.36 mg/g at 24 hr. Thus, in an environment of decreasing nutrient concentrations the conidia responded by increasingly lengthened

germination times. Since it was not possible to determine the time period in which these conidia were exposed to utilizable nutrients in a concentration sufficient to enable them to complete the germination process, it was not possible to determine the time accumulated towards germination completion which was diminished during incubation in glucose-amended natural soil. Nevertheless, the lengthened GT_{50} values after longer incubation periods is consistent with the results obtained with other fungi using membrane filters.

Effect of increasing time of incubation on natural soil on accumulated germination time.--The above data indicated that a reversal in the germination process could be detected after 6 days' incubation on natural soil. It was of interest to determine if this reversal was a progressive process and could be detected after shorter periods of incubation on natural soil. Washed conidia of A. ustus were incubated on sterilized soil for 10 hr then transferred to natural soil plates for increasing periods of time, and subsequently returned to sterilized soil for germination. The GT_{50} values of conidia incubated on natural soil for 0, 2, 4, and 6 days were 13 hr, 16.1 hr, 17.7 hr and 19.4 hr, respectively. Thus, incubation on natural soil resulted in a progressive reversal of accumulated germination time which was proportional to the time

of exposure to natural soil.

Conidia of P. frequentans did not germinate on natural soil. When air-dried soil was amended with either glucose (0.3% w/w) or ascorbic acid (0.01% w/w) alone, only 10-20% germination was observed. However, when both glucose and ascorbic acid were used as amendments, or when conidia were incubated 12 hr on soil with one amendment and then placed 12 hr on soil amended with the second nutrient, 98-100% germination occurred. Advantage was taken of this property to further test reversal in the germination process. Washed conidia were incubated on glucose-amended soil for 12 hr then transferred to one of the following for 12 hr: (a) glucose-amended soil, (b) ascorbic acid-amended soil, or (c) glucose and ascorbic acid-amended soil. Some conidia were also transferred to natural soil plates for 2, 4, 6, and 8 days, after which they were incubated on the three types of amended soils for 12 hr, when germination percentage was determined.

Spores germinated 17% after 12 hr on glucose-amended soil (Figure 5). Conidia placed on glucose-amended soil and then transferred to a fresh glucose-amended soil plate for a further 12 hr germinated 60% whether the germination process was interrupted by incubation on natural soil for up to 8 days or no exposure to natural soil was made. When transferred to glucose and ascorbic acid-amended

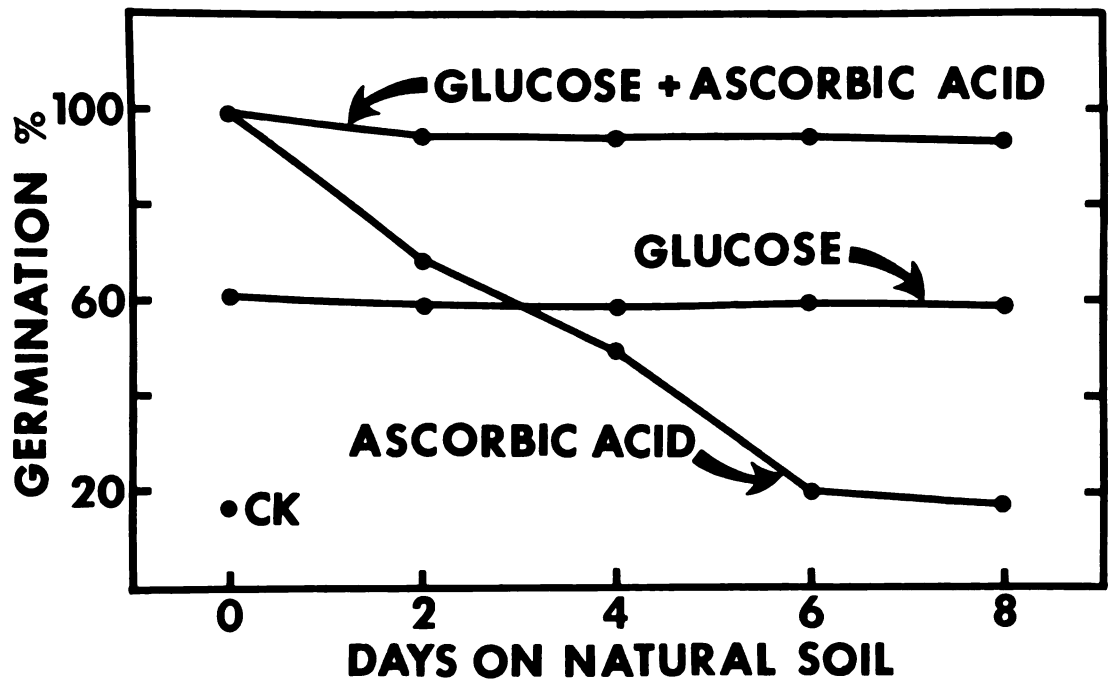

Figure 5. Germination of P. frequentans conidia exposed 12 hr to glucose-amended soil, transferred to natural soil, and subsequently exposed 12 hr to glucose-, glucose and ascorbic acid-, or ascorbic acid-amended soils at the times indicated.

soil, germination was 98-99% after 12 hr either with or without an intervening exposure to natural soil for up to 8 days. When conidia were transferred to ascorbic acid-amended soil without an intervening incubation period on natural soil, germination was 98-99%. However, with increasing time of incubation on natural soil, germination percentages decreased to the level of the control conidia. A subsequent exposure to these conidia to soil amended with glucose resulted in 98% germination.

The maintenance of a constant level of germination in spores exposed to a second glucose treatment following incubation on non-amended natural soil may reflect a capacity of part of the conidial population to respond to glucose even though initial exposure to glucose was adequate for only 17% germination. Since increased time of incubation on natural soil resulted in a progressive reversal of accumulated germination time for A. ustus conidia, and a decreasing response of P. frequentans conidia to ascorbic acid-amended soil, it was concluded that the reversal in the germination process was possibly a result of a gradual loss of an acquired energy-yielding nutrient during the period of incubation on natural soil.

Loss of energy-yielding substrates from germinating spores placed on natural soil.--To test whether the increased time required for germination observed in previous

experiments after incubation on natural soil was due to a loss of an energy-yielding substrate, isotopically labeled spores were used. Washed conidia of P. frequentans were incubated for 7 hr in an extract of autoclaved subsoil containing 0.1% glucose and ^{14}C-labeled glucose at a concentration of 1 µCi/ml. After incubation conidia were washed and pipetted onto membrane filters on natural soil and incubated for 1, 2, 4, 6, or 10 days. Following incubation total radioactivity was determined. GT_{50} on sterilized soil was also determined at the same intervals.

A linear relationship between the percent loss of radioactivity and percent reversal in germination time was found (Figure 6; $P<1\%$). Confidence intervals at the 95% level were not significant. The relationship between time of incubation on natural soil and percentage reversal in germination time and in loss of radioactivity was also plotted (Figure 7). It was concluded that the reversal in time required for germination resulted from a loss of radioactive glucose, or an energy-yielding metabolite, from spores during incubation on natural soil.

Fate of labeled glucose from germinating spores placed under nutrient stress conditions.--The loss of radioactivity from ^{14}C-glucose labeled conidia may be by two possible routes: respiration as $^{14}CO_2$, or exudation of labeled glucose or metabolites other than $^{14}CO_2$.

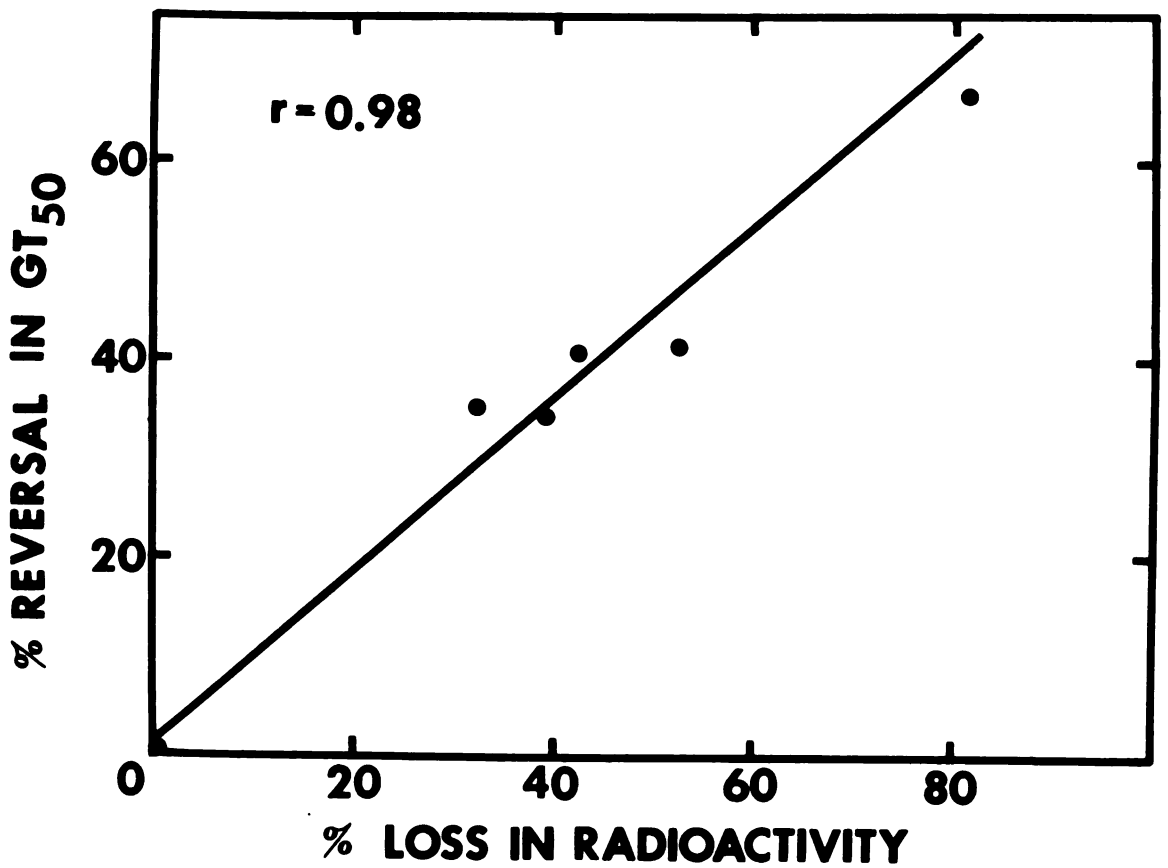

Figure 6. Correlation of percent reversal of germination time of P. frequentans conidia with percent loss of radioactivity during 10 day's incubation on natural soil.

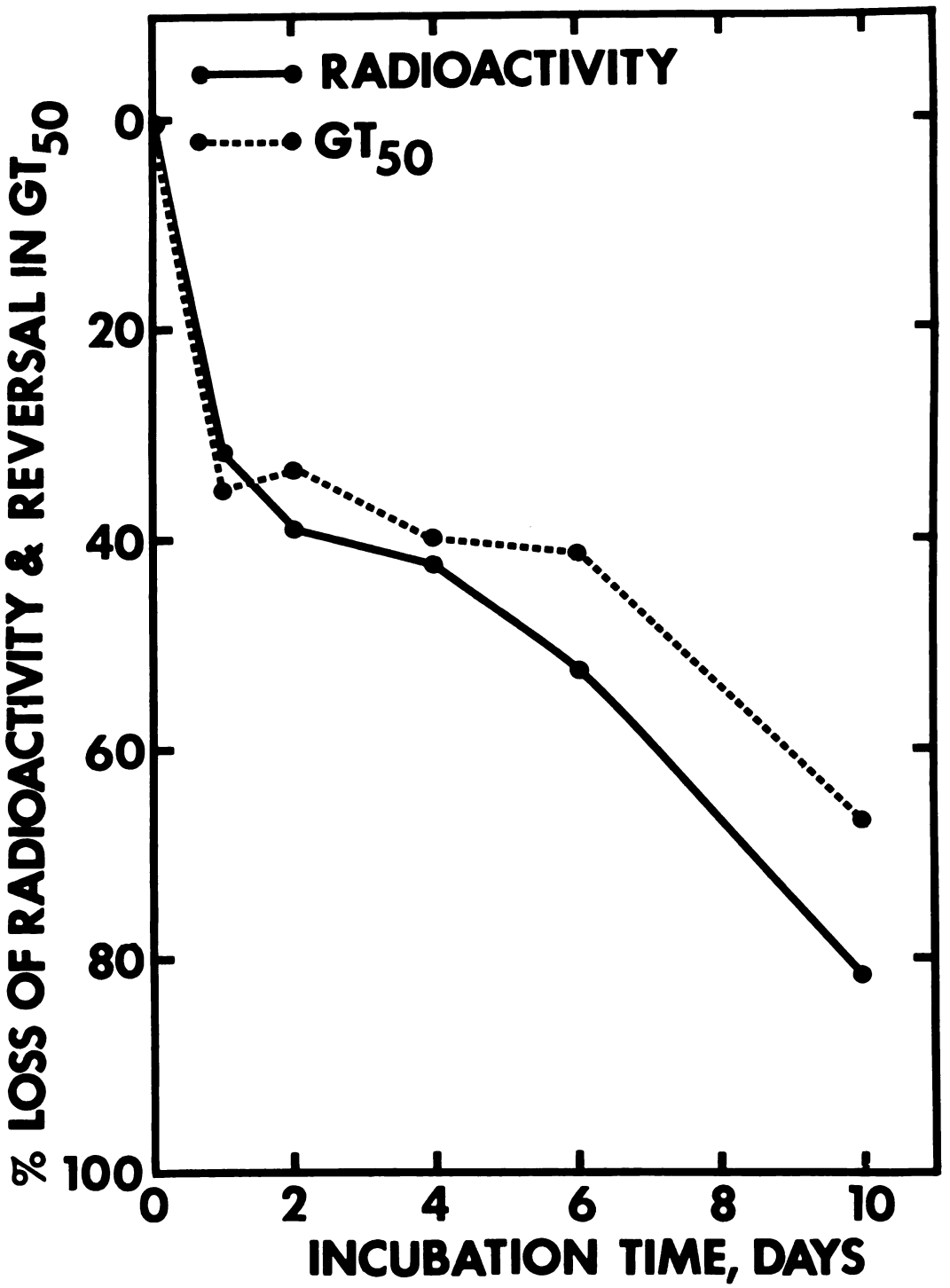

Figure 7. Percent reversal in time to reach GT_{50} and percent loss of radioactivity during 10 day's incubation of ^{14}C-glucose labeled conidia of P. frequentans on natural soil.

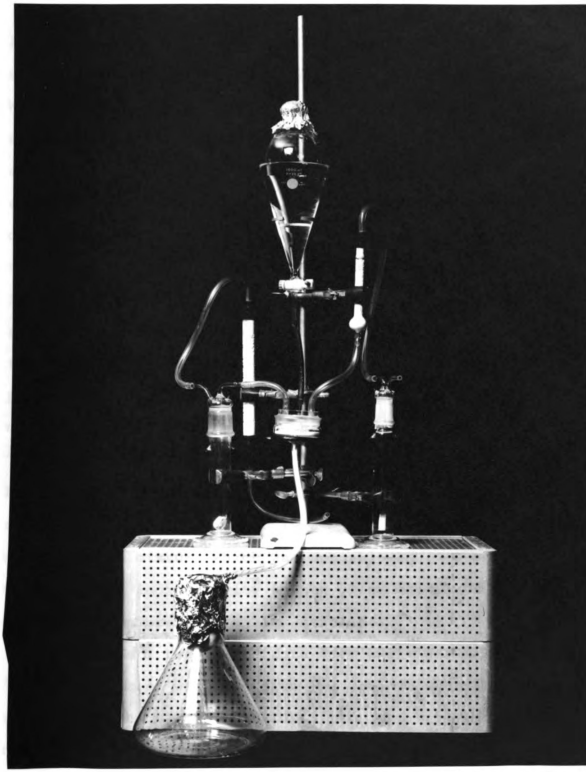

Figure 8. Leaching system designed for collection of $^{14}CO_2$ and spore exudates. CO_2-free, dried air entered chamber at left and exited at right. $^{14}CO_2$ was removed by air scrubber at right. Water, from separatory funnel, dripped to and percolated through glass beads supporting membranes and spores and was collected in flask at bottom.

A model system designed to collect leachings and CO_2 from spores labeled with ^{14}C-glucose was used (Figure 8). Washed conidia of P. frequentans were incubated in an extract of autoclaved subsoil containing 0.1% glucose and ^{14}C-glucose. After 7.5 hr incubation the labeled conidia were removed, washed, and aseptically pipetted onto membrane filters in the leaching chamber. An equal volume of the conidial suspension was pipetted immediately into vials containing modified Bray's scintillation fluid as a control. After incubation in the leaching chamber for 6 days, the membranes bearing the conidia were each placed in a vial containing the scintillation fluid. One ml of the methyl cellosolve-ethanolamine mixture from the $^{14}CO_2$ trap was also dispensed into each of three vials. Five hundred ml of the leachings were boiled for 10 min and the $^{14}CO_2$ was collected in a methyl cellosolve-ethanolamine mixture which was apportioned into scintillation vials. Radioactivity in the glass beads and the large supporting membranes was also assessed.

Table 1 represents data from one experiment. About 50% of the label was lost from the conidia when incubated under nutrient stress conditions provided by the artificial nutrient sink. Of this loss about half appeared as $^{14}CO_2$ and half as some other non-gaseous product leached from the spores. Other experiments of the same design resulted in a higher percentage of the ^{14}C appearing in

Table 1. Fate of ^{14}C in labeled spores incubated aseptically in a leaching system designed for collection of CO_2 and exudates from spores.

Source	Total CPM	CPM/spore	% of total	% of loss
Initial activity	11,856 ± 1724	0.283 ± 0.041		
Spores	5,997 ± 671	0.143 ± 0.016	50.58	
CO_2	3,239 ± 352	0.077 ± 0.008	27.32	54.39
Leachings	2,620 ± 235	0.062 ± 0.006	22.09	43.99
Membrane support	95 ± 0	0.002 ± 0.000	0.08	1.59
Total recovered	11,952 ± 1258	0.284 ± 0.030	100.07	99.97

the CO_2, but total recovery of the label was not as complete. It was concluded that germinating fungal spores, when exposed to starvation conditions, as provided by natural soil or the leaching system, are not able to maintain their position in the germination process acquired during previous exposure to energy-yielding substrates because of (a) a loss of the original substrate or its metabolites in spore exudates, and (b) respiratory activity resulting in oxidative utilization of the newly acquired reserves.

Effect of natural soil on GT_{50} of spores not exposed to energy sources.--Fungal propagules in soil, except in extremely arid locales, are situated in microsites which generally provide conditions of high relative humidity or sufficient moisture forming a film of water around each propagule. The data presented in Figures 1 and 2 suggested that spores of fungi incubated on non-amended natural soil may be able to initiate the germination process although germination did not reach completion due to the low energy-yielding nutrient status of this medium. To determine whether the effect of natural soil in decreasing the GT_{50} of P. frequentans was real, and if other fungi were similarly affected, conidia of P. frequentans, A. ustus, H. sativum, sporangiospores of Mucor ramannianus, and chlamydospores of F. solani f. sp. pisi,

F. oxysporum f. sp. lycopersici, and F. oxysporum f. sp. melonis were incubated on natural soil for 6 days, with the exception of conidia of H. sativum which were incubated 1 day. All conidia were then transferred to sterilized soil for GT_{50} determination. GT_{50} values of control spores on sterilized soil were also determined and data appear in Figure 9.

With the exception of F. oxysporum f. sp. lycopersici and f. sp. melonis incubation on natural soil decreased the germination time by 8-15% of that required for untreated controls. The increased GT_{50} values shown by F. oxysporum had also been observed with conidia of H. sativum following long incubation in natural soil; like H. sativum conidia, low percentages of these chlamydospores germinated in water alone, and they have a low sensitivity to soil fungistasis (60). Chlamydospores of the two form species of F. oxysporum were considerably larger than those of F. solani f. sp. pisi, and incubation on natural soil may result in a significant loss of endogenous reserves resulting in longer periods required for germination.

Germination time of spores pre-incubated on non-nutritive media.--An experiment was designed to determine whether the decreased GT_{50} values which followed incubation on natural soil required the presence of soil itself, or was due to water soluble substances from soil (subsoil

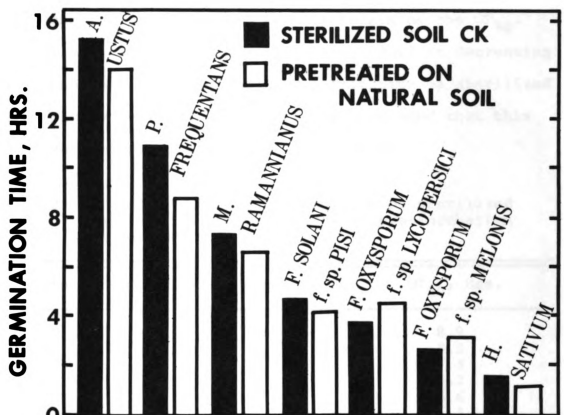

Figure 9. Effect of pretreatment of spores on natural soil on the GT_{50}.

extract was used because topsoil extracts contained anthrone- and ninhydrin-positive materials which stimulated germination), or to water alone. Washed P. frequentans conidia were incubated on natural soil, glass beads saturated with an extract of autoclaved subsoil or water for 24 hr followed by incubation on sterilized soil to determine the GT_{50}. Water alone was as effective as natural soil in decreasing the subsequent time required for germination on sterilized soil (Figure 10; P<5%). Thus it was concluded that this was a water-dependent phase of germination.

Table 2. GT_{50} of P. frequentans conidia on sterilized soil after various periods of pre-incubation on natural soil.

Time on Natural Soil, Hrs.	GT_{50}, Hrs.
0	8.9
3	8.5
6	7.5
9	7.2
15	6.6
24	6.9
48	6.9

Time course experiments indicated that 6 hr incubation on natural soil or water produced a significant reduction in GT_{50} on sterilized soil, and the maximum effect was produced by 15 hr incubation with no further decrease in GT_{50} values observed with increasingly longer incubation times (Table 2; P>5%).

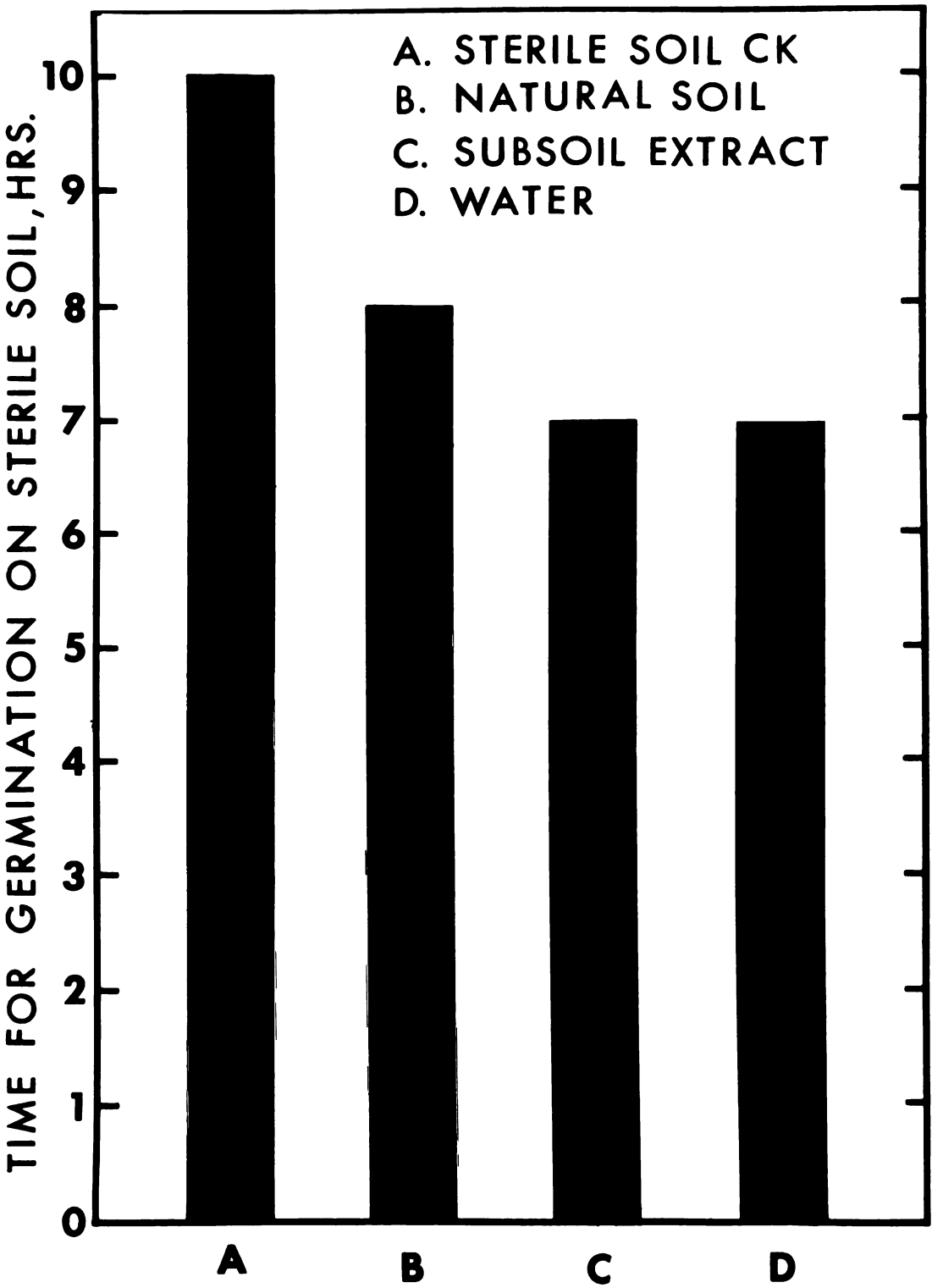

Figure 10. Effect of 24 hr pre-incubation on natural soil, subsoil extract, or water alone on GT_{50} of P. frequentans conidia determined subsequently on sterilized soil.

The effect of temperature on the water-mediated phase of the germination process.--Active metabolism of biological organisms is often temperature dependent. To investigate the role of metabolism in the water-mediated phase of germination, conidia of P. frequentans were incubated on natural soil, or on glass beads saturated with either subsoil extract or water at 24°C which did not restrict metabolic activity, or at 1°C which minimized active metabolism. After 24 hr incubation the GT_{50} was determined on sterilized soil at 24°C for each treatment and control spores. A decrease in GT_{50} of 3-3.5 hr occurred at 24°C, but even at 1°C a decrease of about 1 hr was observed as compared to the controls (Figure 11). Respiratory measurements were made with a Gilson Differential Respirometer at both temperatures; the QO_2 at 24°C was 4.28×10^{-8} µl per spore per hour as compared to 7.67×10^{-9} µl per spore per hour at 1°C. Thus, respiration was not completely inhibited by incubation at 1°C.

A similar experiment involved incubation at 24° or 1°C for 24 hr followed by reciprocal incubation for a further 24 hr period. GT_{50} values were determined on sterilized soil at the end of the incubation and also after the first 24 hr period. Conidia transferred from 24° to 1°C required no longer to germinate than those treated only at 24°C (Figure 12), but when transferred from 1° to 24°C a further decrease in GT_{50} resulted,

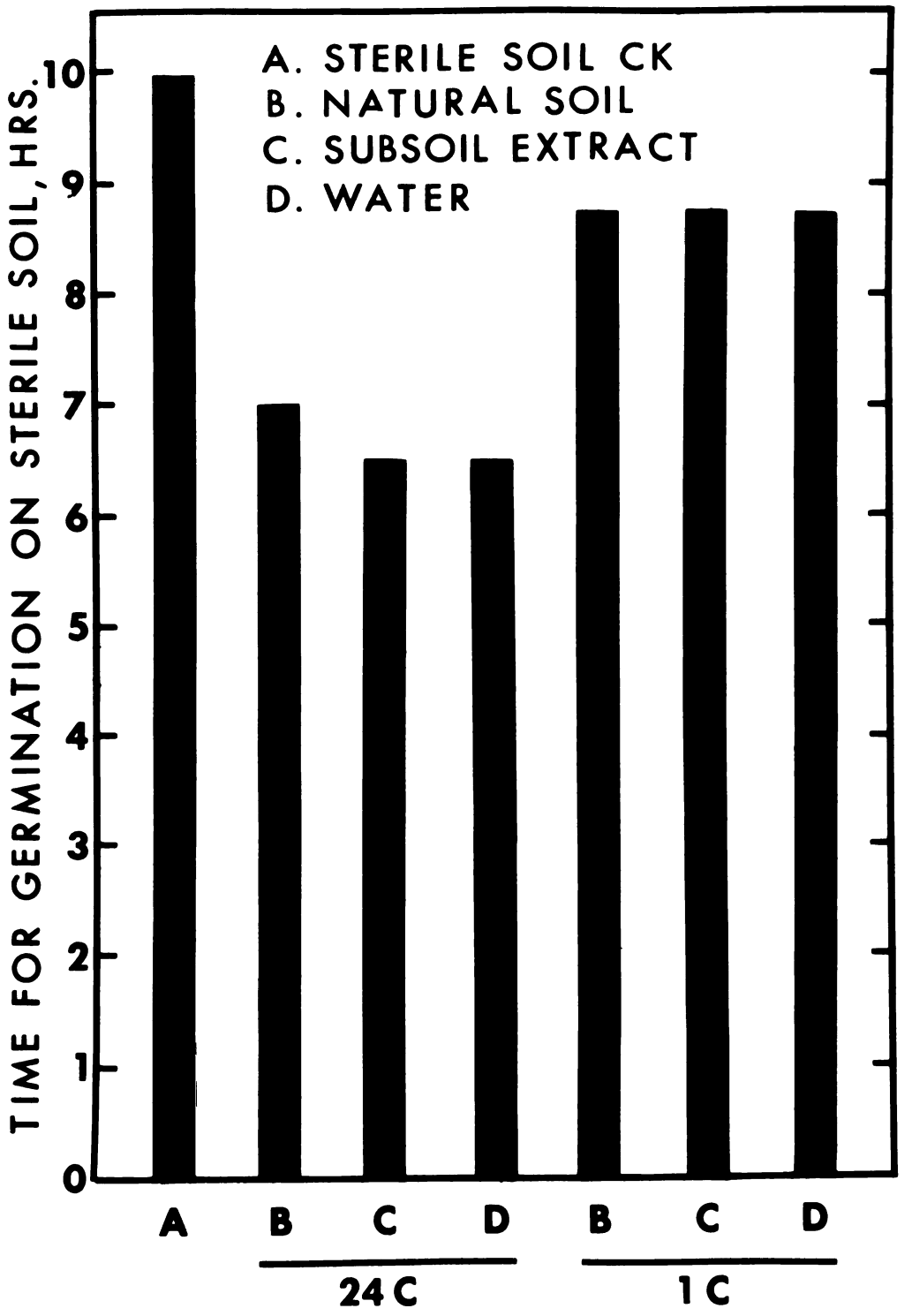

Figure 11. Effect of pre-incubation on natural soil, subsoil extract, or water alone at 1° and 24°C on GT_{50} of P. frequentans conidia determined subsequently on sterilized soil.

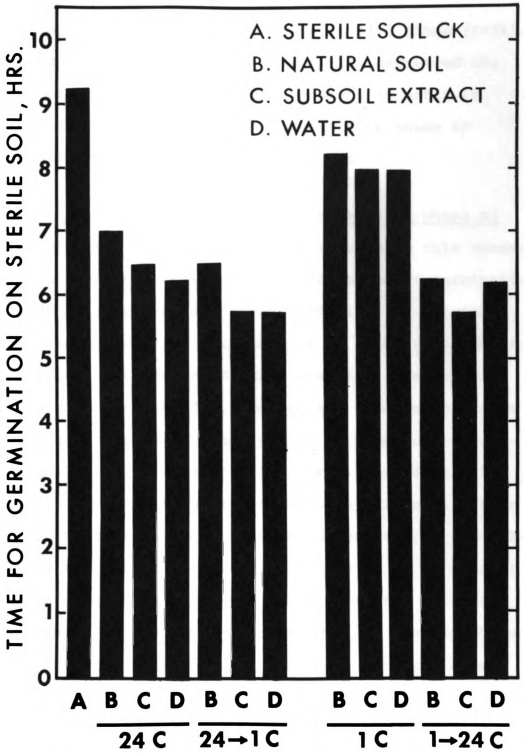

Figure 12. Effect of temperature and reciprocal temperature pretreatments on the GT_{50} of P. frequentans conidia incubated on natural soil, subsoil extract, or water.

equalling the time of those treated at 24°C alone (P>5%). Since suppression of repiratory activity depressed the effect of natural soil, subsoil extract, or water in decreasing the GT_{50}, it appeared that this phase of germination was under metabolic control.

Irreversibility of the water-mediated phase of the germination process.--Previous results in this research indicated that the nutrient-dependent phase of germination was a dynamic process and under conditions of nutrient stress was reversible. It was thus of interest to determine if the water-mediated phase was also a reversible phenomenon. Conidia of P. frequentans were incubated on natural soil for 24 hr, then placed in a desiccator containing 10 g P_2O_5, a dehydrating agent, for 24 hr. Untreated control conidia germinated on sterilized soil in 8.2 hr, and control conidia, first dried over P_2O_5 for 24 hr, required 8.8 hr. Conidia incubated 24 hr on natural soil, and those incubated on natural soil for 24 hr and then dried over P_2O_5 both attained GT_{50} on sterilized soil in 7.2 hr. Thus this phase of the germination process appeared to be irreversible.

Water-uptake investigations.--Two approaches were used to answer the question whether water was taken up by spores during incubation on non-amended natural soil or

in water alone. Measurements of 100 P. frequentans conidia were made prior to and after 24 hr incubation periods on water-saturated glass beads at 24° and 1°C. At zero time conidial diameters were 2.07 $\pm$ 0.17 μ. After 24 hr at 1°C diameters were 2.3 $\pm$ 0.17 μ, whereas at 24°C conidia enlarged to 2.83 $\pm$ 0.17 μ diameter. Thus, conidial volumes increased 0.32 μ^3 at 1°C and 7.23 μ^3 at 24°C.

Secondly, conidia were incubated on membrane filters on glass beads saturated with tritiated water (5 μCi/ml) for 24 hr at both 24° and 1°C. After incubation the spores were washed and the tritium content determined. The radioactivity was 1.75 X 10^{-3} CPM/spore at 24°C and 3.50 X 10^{-4} CPM/spore at 1°C. If there was no discrimination in uptake of unlabeled water over uptake of labeled water, the water uptake per spore at 24°C was 4.37 X 10^{-10} μl (4.37 X 10^{-1} μ^3 volume) and 8.75 X 10^{-11} μl (8.75 X 10^{-2} μ^3 volume) at 1°C. However, the spore volumes at 24°C increased 7.23 μ^3, which suggested either that there was a discrimination in uptake of unlabeled water over uptake of labeled water, or that the labeled water was largely exchanged with unlabeled water during the washing process. The latter seems to be the more plausible explanation.

It was of interest to determine the kinetics of water uptake and incorporation, and also to determine if during the period of incubation labeled spore materials

were lost into the medium. Conidia of P. frequentans were incubated on glass beads saturated with tritiated water for 0, 1, 2, 4, 6, 8, and 24 hr at both 24° and 1°C. Spore samples were collected for determination of radioactivity at each interval. After 24 hr all spores were removed and the water was collected and evaporated to dryness. The residues were washed with unlabeled water and dried 3X to remove any contaminating tritiated water. The dried residues were then placed in scintillation vials and tritium determination was made.

Maximum tritium concentration in the spores was found after 1 hr incubation at both 24° and 1°C, after which the label was rapidly lost (Figure 13). The residue which resulted from incubation of 1.26×10^5 spores on glass beads at 24°C contained 2783 CPM or 2.21×10^{-2} CPM/spore. At 1°C the residue contained 496 CPM or 3.72×10^{-3} CPM/spore. The occurrence of labeled residues indicated that water was involved in some metabolic process even in the absence of energy-source nutrients. The total radioactivity of the spores after 24 hr (9.0×10^{-4} CPM/spore) plus the residue (2.21×10^{-2} CPM/spore) was less than the maximum radioactivity of the spores after 1 hr incubation (7.5×10^{-2} CPM/spore). This again suggested either that the spores preferentially exchanged tritiated water for unlabeled water, or that the label on the radioactive metabolites, and bound and unbound water,

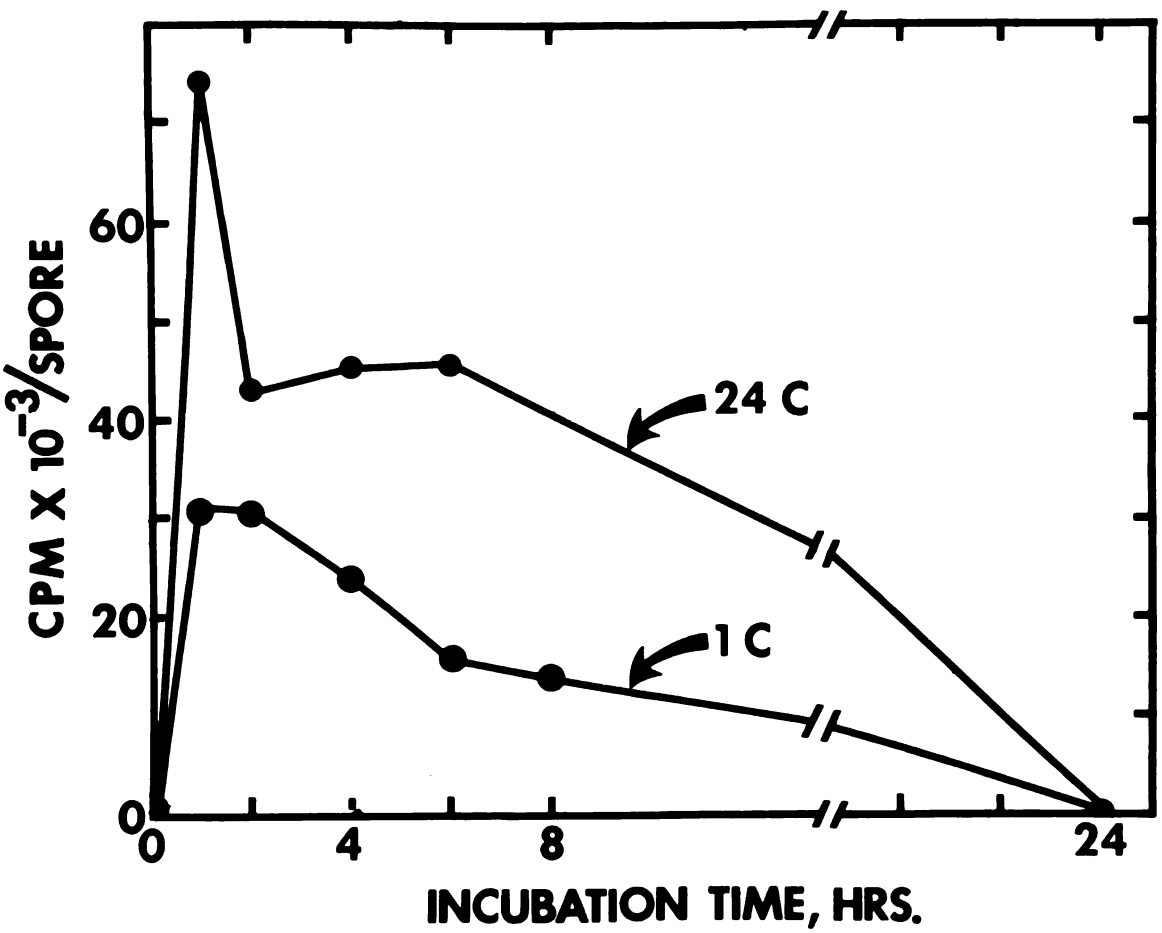

Figure 13. Total radioactivity of P. frequentans conidia incubated in tritiated water at 24° and 1°C for the times indicated.

was freely exchangeable and replaced by non-radioactive hydrogen, or water, during the washing process.

To test uptake of water during the germination process, conidia were incubated in a liquid germination medium consisting of the extract of autoclaved soil containing glucose (0.1% w/v) and tritiated water. In these experiments inconsistent germination occurred. In those cases when germination percentages were low after 24 hr incubation, the pattern of uptake and release of tritium was the same as observed in water alone (Figure 13). However, when germination percentages were high at the end of the incubation period, the uptake and release pattern was similar with the exception that after 24 hr a reappearance of high tritium content was observed which was greater than that found at 1 hr.

These results suggest that water uptake and incorporation occurred very early in the incubation period. Permeability alterations allowed freer exchange of labeled water and unlabeled water, and exudation of labeled metabolites after 1-2 hr incubation. In addition, free exchange of tritium, incorporated into metabolites, with non-radioactive hydrogen could account for the decreased radioactivity of the spores found between 2 and 24 hr incubation. The increased level of radioactivity found when germination percentages were high indicated an increased incorporation of the tritiated water into metabolites or other

non-exchangeable forms. This in turn is probably but an expression of greatly increased metabolism.

The role of water in the total germination process.--Apparently water mediates an initial phase in the germination process which, in addition to decreasing the time required for germination, may prepare the spore for utilization of energy substrates. To test this possibility the rates of energy-source uptake by spores pretreated and not pretreated in water were compared.

Conidia of P. frequentans were incubated in Petri dishes (50 X 15 mm) containing 1.5 ml water for 24 hr. After treatment these conidia and untreated conidia were incubated in 1.5 ml soil extract containing 0.1% glucose and ^{14}C-glucose (1 μCi/ml). After incubation for 0, 1, 2, 4, 6, and 8 hr the conidia were washed and dispensed in vials containing modified Bray's solution. Total ^{14}C activity was determined (Figure 14). Although excessive variability masked differences which may have occurred early, by 8 hr spores pretreated in water had taken up ca. 4X more ^{14}C-glucose than did spores not so pretreated. The results indicated that water uptake preceded, and prepared the spores for, energy source uptake.

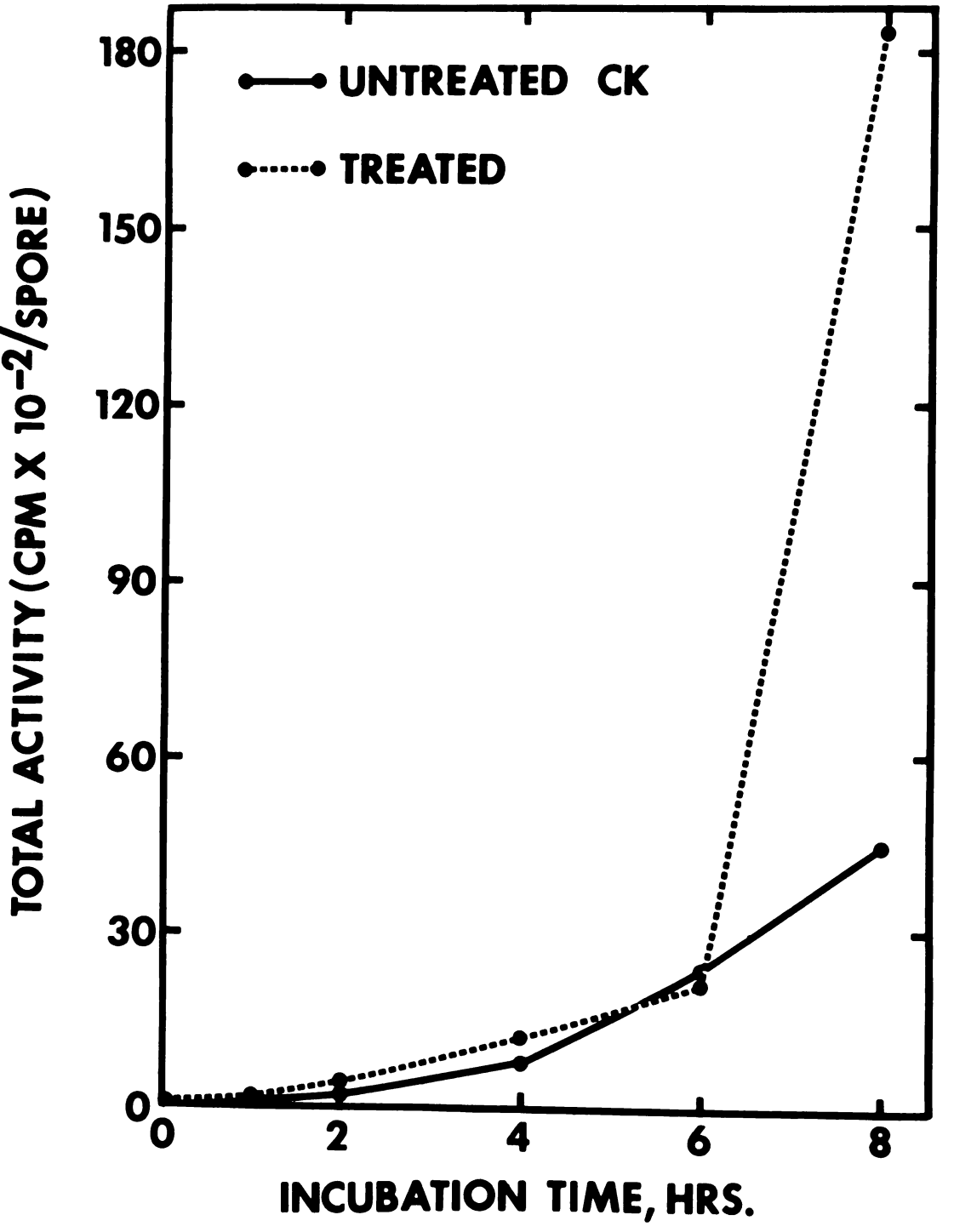

Figure 14. Effect of 24 hr water pretreatment on uptake of ^{14}C-labeled glucose by P. frequentans conidia.

Germination initiation and reversal of the nutrient phase using model systems in an integrated experiment.--Data presented in Figure 10 indicated that non-amended natural soil or subsoil extract both reduced the GT_{50} of quiescent conidia of P. frequentans. In addition, data in Figure 3 showed that incubation of germinating spores on natural soil following previous incubation under favorable germination conditions resulted in an increased GT_{50}, or a reversal in the germination process. An experiment was designed to relate these two phenomena and to compare the effect of natural soil with that of an artificial nutrient sink on the reversal after pretreatment which effected the water phase of the germination process. The flow chart (Figure 15) illustrates the procedure followed. Washed conidia of P. frequentans were used as the test organism.

Untreated control conidia germinated 50% after 9.4 hr on sterilized soil (Figure 16). Control conidia pre-incubated 6 days on natural soil or on glass beads saturated with autoclaved subsoil extract required 6.9 and 6.8 hr, respectively, for germination on sterilized soil. Thus, the water phase reduced the GT_{50} values by ca. 25%.

Conidia incubated on natural soil both before and after a 6 hr period on sterilized soil required 5.5 additional hr, or 11.5 total hr on sterilized soil to reach the GT_{50}. Thus, ca. 75% $(\frac{11.5 \text{ hr} - 6.9 \text{ hr}}{6 \text{ hr}})$ of the nutrient

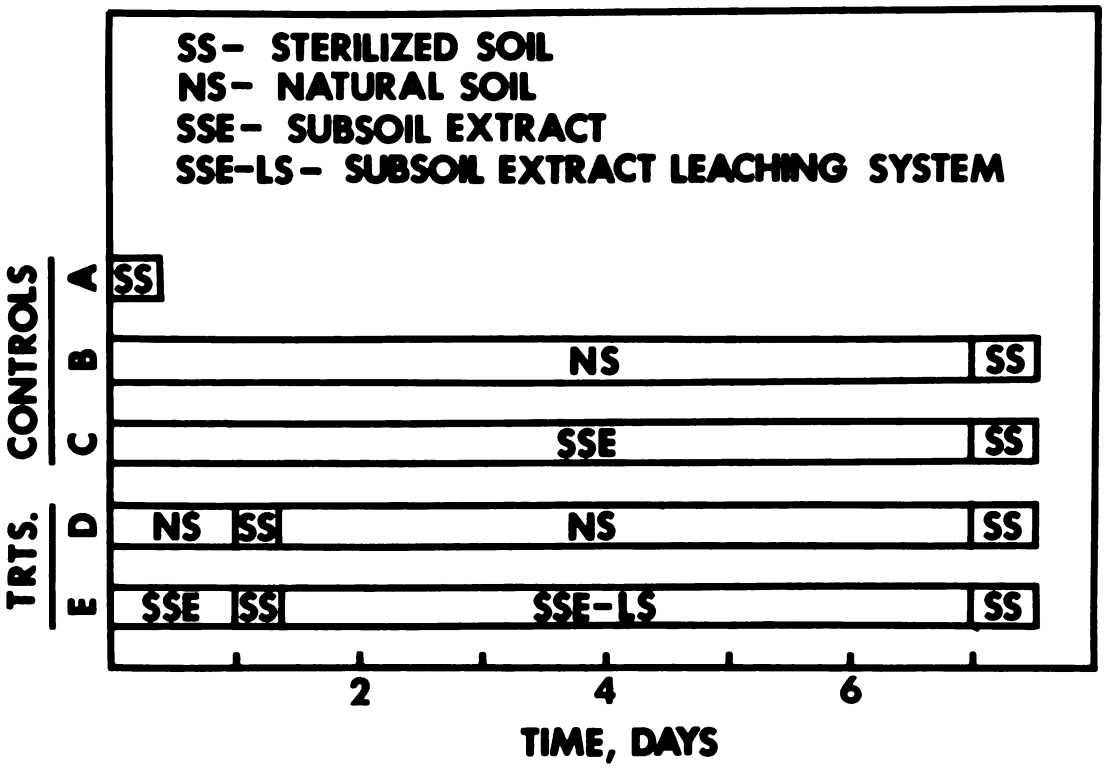

Figure 15. Flow Chart: The effect on reversal of the nutrient-dependent phase of germination of pre- and post-treatment under non-nutritive conditions.

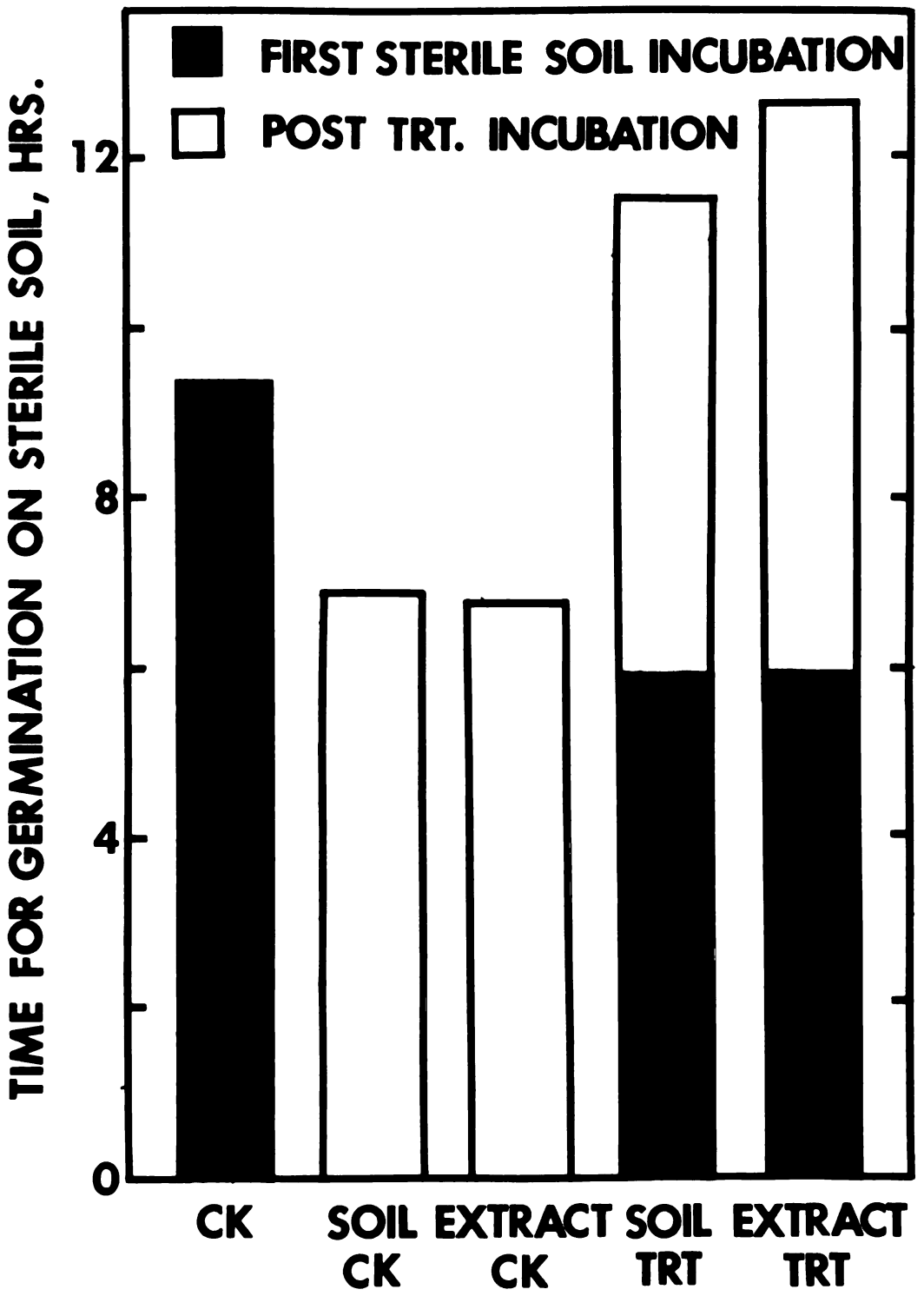

Figure 16. GT_{50} of P. frequentans conidia determined on sterilized soil following incubation on (A) no prior treatment, (B) natural soil for 6 days, (C) autoclaved subsoil extract for 6 days, (D) natural soil for 24 hr, then sterilized soil for 6 hr, followed by natural soil for 6 days, and (E) subsoil extract for 24 hr, then sterilized soil for 6 hr, followed by subsoil extract in the leaching system for 6 days.

phase gained during the 6 hr on sterilized soil was lost during the subsequent incubation on natural soil.

The conidia pre-incubated on glass beads saturated with the autoclaved subsoil extract then transferred to sterilized soil for 6 hr were subsequently incubated in a leaching chamber using subsoil extract as the leaching medium. After 6 days the GT_{50} was determined on sterilized soil and was found to be 6.5 hr. The total exposure to sterilized soil was thus 12.5 hr. In this instance ca. 95% $(\frac{12.5 \text{ hr} - 6.8 \text{ hr}}{6 \text{ hr}})$ of the nutrient phase gained in the 6 hr period on sterilized soil was lost during the 6 day period in the leaching system.

It was concluded that the nutrient phase of germination was a dynamic, transient phase dependent on the nutrient condition of the medium in which the spores were placed. The pretreatment of spores on natural soil or on the autoclaved-subsoil extract permitted a quantitative determination of the reversal under these conditions. Under conditions of extreme nutrient stress, such as imposed by the artificial nutrient sink, near total reversal of the advance towards germination made by the previous exposure to favorable nutrient conditions occurred. The leaching system imitated natural soil qualitatively, by reversing the germination process, and quantitatively in reversing this phenomenon to nearly the same degree.

CHAPTER V

DISCUSSION

Fungal spore germination in soil was found to be a dynamic process sensitive to the competitive conditions of the soil environment. The germination process of nutrient-dependent fungi, and those fungi which germinated to a limited degree in water, was initiated in natural soil, a medium deficient in energy-yielding substrates. This phase of germination was dependent on water in soils and was irreversible. In soil, uninterrupted progress towards completion of the germination process was dependent on a continuous supply of exogenous energy-yielding nutrients. However, in conditions of energy source deprivation, progress towards germination ceased and those energy-yielding nutrients taken up by the spores were lost and the germination process reverted towards the original irreversible water phase.

All fungal species tested in this study, with the exception of those having large spores, showed a small, but consistently shortened GT_{50} after exposure to natural soil. Those fungi possessing large spores also germinated to a low percentage in water alone, and were relatively

insensitive to soil fungistasis (60). H. sativum conidia were found to germinate most rapidly following brief exposures to natural soil, but longer incubation on natural soil increased the time required to reach GT_{50}. Loss of endogenous nutrients from conidia in soil has been reported (46). Possibly loss of endogenous reserves from H. sativum conidia and chlamydospores of the form species of F. oxysporum may mask the opposing effect of water in advancing germination, and explain why prolonged incubation of such fungi on natural soil increased their GT_{50}.

Water was as effective as natural soil or autoclaved-subsoil extract in reducing the requisite time for germination. Mineral contaminants in water cannot be ruled out completely, but triple glass-distilled water contained in acid-washed glassware was as effective as natural soil in decreasing the GT_{50} of P. frequentans conidia. GT_{50} of spores of P. frequentans was reduced 20-30% by 24 hr incubation on water-saturated glass beads. Steiner and Lockwood (60) recently reported that pretreatment of conidia of Botrytis cinerea and P. frequentans in water had no effect on sensitivity to soil fungistasis or on germination time. Data in the present research would suggest that their observations resulted from a too brief water pretreatment which did not allow expression of the effect. An exposure of 6 hr was required to detect any shortening of germination time, and 15 hr were required

for the maximum effect, in this research.

Previous reports on spore germination have stated that spore swelling, which preceded germ tube emergence, was a result of water uptake (35, 44, 63). Studies with Aspergillus niger (68), Fusarium culmorum (49), Penicillium atrovenetum (32), P. notatum and Trichoderma lignorum (51), Rhizopus arrhizus (23), and Trichoderma sp. (3) showed that no swelling occurred in the absence of a carbon and/or nitrogen source; however, in some cases diameter increases less than 50% were discounted. In the present study limited spore swelling occurred on incubation in water alone.

Uptake and incorporation of tritiated water occurred very early during incubation in water alone and during germination. Exudation of labeled metabolites indicated that the water was actively involved in metabolic processes and suggested that spore permeability increased during the incubation period. Uptake and incorporation of water possibly occurred throughout the incubation period. Although the data showed an apparent loss in label from within the spores as incubation time progressed, it is more likely that uptake and incorporation of water increased. The loss of label is probably due to increased permeability during the incubation period, allowing free exchange of tritiated water with unlabeled water, and loss of labeled

metabolites during washing. Immediately prior to and during germ tube formation increased respiratory activity, as indicated by Cox and Sisler (18), may have stimulated the increased uptake and incorporation of water found in the present study. Studies with deuterated water and tritiated water have shown that quiescent spores are freely permeable to water, but that water uptake by these spores did not reach the theoretical maximum based on spore volumes (24, 26, 50). However, increased membrane permeability and exchangeable label on metabolic products in these studies could also result in marked discrepancies between the volume of water actually taken up and the theoretical volumes calculated.

Active metabolism appeared to be involved in the water-mediated phase of germination. Several studies have implicated metabolic activity with the swelling phase of the germination process (3, 18, 23, 25, 30, 49, 51, 68). In the present study maximum swelling and reduction in the GT_{50} was observed when incubation occurred at 24°C, but at 1°C, which limited respiration, spore swelling was less and minimal reduction in the GT_{50} was found. This study did not rule out the alterations in water viscosity which may have prevented or limited water uptake at 1°C; because of the bioassay technique employed metabolic inhibitors were not used.

Investigations of the energy-yielding nutrient-dependent phase of germination indicated that spores incubated under favorable germination conditions for short periods, relative to the time required for germination, were capable of maintaining their position in the germination process during subsequent short exposures to natural soil. These results confirm the work of Steiner and Lockwood (60) using 4 hr alternate incubation periods in a nutrient solution and in water. They found no difference in the total time in nutrient solution required for germination whether the incubation was continuous or interrupted by 4 hr alternate periods in water. Steiner and Lockwood (60) proposed that fungus spores advanced stepwise and irreversibly towards germination. However, in the present study, when spores were incubated on sterilized soil for longer periods approaching germ tube emergence, then subsequently transferred to nutrient-deprived conditions of natural soil for long periods, a reversal in the germination process resulted. In contrast, Steiner and Lockwood (60) found that spores treated in the same manner and held on natural soil for periods up to 11 days failed to revert in the germination process. Variability in their experiments, and statistical analysis of the entire set of data rather than comparisons of individual times of incubation on natural soil with control germination times obscured the reversal which is suggested by observation

of their data. Results in the present study indicate that the irreversible, stepwise progression toward germination completion suggested above was too restrictive, in that short periods on natural soil were of insufficient duration to permit detection of the reversal in the germination process.

Additional evidence that prolonged natural soil treatment following the initiation of germination on sterilized soil resulted in reversal of the nutrient-dependent phase of germination was found in the time course study with A. ustus conidia. Increasingly longer periods on natural soil resulted in increased reversal in the nutrient-dependent phase of germination. Further, decreased sensitivity of P. frequentans conidia to ascorbic acid-amended soil, following increased periods on natural soil, indicated that a progressive reversal occurred as incubation on a nutrient-deprived medium continued. This response confirmed the contention that glucose, or metabolites thereof, were progressively lost from the spores. The fact that reversal on nutrient-deprived natural soil required considerably longer to occur than did the initial advance made towards germination completion on sterilized soil was made clear when conidia were initially exposed for relatively long periods on sterilized soil, then transferred to natural soil for 3 and 6 days. No reversal was evident after 3 days on natural soil but was obvious after 6 days.

When conidia were incubated initially on sterilized soil for short periods then on natural soil for short or long periods no reversal was observed; in fact, an apparent decrease in total time required for germination was observed. In these experiments and those involving a long initial period on sterilized soil followed by a 3 day incubation on natural soil, a partial reversal probably occurred but was not expressed because of a cancellation of this effect by the water-mediated phase of the germination process.

Loss of radioactivity from the ^{14}C-glucose labeled spores during incubation on natural soil coupled with reversal of the germination progress made provided direct evidence that reversal accompanied a concomitant loss of energy-yielding nutrients previously taken up, or their metabolites, from the spores. The reversal was undoubtedly a result of this loss. Further studies in a leaching system designed to imitate the microbial-nutrient sink of natural soil, indicated that ca. 50% of the labeled materials lost from the ^{14}C-glucose labeled spores was $^{14}CO_2$. This was not unexpected since increased respiratory activity has been reported for germinating spores of many fungi (5, 18, 48, 50, 55). No attempt at indentification of the non-gaseous labeled materials lost was made. Loss of amino acids and labeled carbon-containing compounds has been reported from germinating fungus and bacterial

spores (19, 38, 54). Moreover, Ko and Lockwood (39) reported loss of carbohydrates and amino acids from nutrient-independent conidia of Neurospora tetrasperma when leached in a system similar to that used in this research.

The property of natural soil which prevents germination of fungal spores was found by Ko and Lockwood (39) to be a general deficiency of energy-yielding nutrients. Bulpitt and Gunner (10) have suggested that ascorbic acid reverses nitrite toxicity in soil which may inhibit germination of fungal spores. This toxicity was expressed through oxidation of the cytochrome system of soil organisms resulting in inhibited growth and development of these susceptible organisms. There is no evidence in the present study to confirm this suggestion; in fact, conidia which failed to germinate on ascorbic acid-amended soil after 6-8 days on natural soil responded during a subsequent incubation on glucose-amended soil by germinating 98-99%. The fungistatic property of soil was used throughout this study, and by Steiner and Lockwood (60), to stop the sequence of events leading to germ tube emergence and thereby to investigate the germination process. The fact that incubation of spores on natural soil or in an artificial nutrient sink both resulted in an almost total reversal of the nutrient phase of germination provided further evidence that the artificial nutrient sink was a

valid model for the microbial-nutrient sink in soil. Ekundayo (23) reported that sporangiospores of R. arrhizus germinated on water agar, which he assumed to be nutrient-free, after transfer from nutrient agar. Ko and Lockwood (39) demonstrated that water agar discs possess sufficient energy-yielding nutrients to stimulate germination of many fungal spores. Therefore, Ekundayo's conclusion that only an initial exposure to nutrient conditions is sufficient to stimulate germination does not appear valid. The results of Steiner and Lockwood (60), confirmed in the present research, show that the duration of exposure to energy-yielding nutrients throughout the germination process is the critical factor determining germination of fungus spores.

Steiner and Lockwood (60) reported that sensitivity of fungal spores to soil fungistasis was a function of the time required for their germination. Data from the present research indicated that in soil, due to the presence of water, 8-15% of the total germination time would occur in the absence of exogenous energy-yielding nutrients. Therefore, the sensitivity indexes ascribed to different fungal species by Steiner and Lockwood were perhaps exaggerated; however, the relative order of these indexes would probably remain unaltered. Germination in favorable soil microsites may occur more rapidly than predicted from germination studies conducted on artifical media or on sterilized soil.

Germination of fungal spores in soil is dependent on moisture and soluble energy-yielding nutrients. Except in extremely arid conditions soil moisture is generally not a limiting factor. This phase of germination advances the spore a short way towards germ tube emergence, and appears necessary to prepare the spore for the dynamic nutrient phase. In these studies the rate of ^{14}C-glucose uptake was stimulated by pretreatment of spores in water. In rhizospheres or in the vicinity of organic debris, nutrients released would provide adequate stimuli for germination. However, with lesser available quantities of nutrients, possibly resulting from vigorous microbial activity, the small fungal spores, which require longer periods for germination, would be prevented from germination and their advancement towards germination would be reversed as the environment became depleted of nutrients. This would tend to preserve the fungal propagule since germination would present the sensitive mycelium with inadequate nutrients and susceptibility to lysis (40). In conditions of periodic nutrient increments of short duration, fungus spores would appear to progress stepwise towards germination since brief time spans between nutrient increments would not be of sufficient duration to result in the reversal of accumulated germination time. Fungal spores having larger volumes and requiring shorter exposure to energy-yielding nutrients for germination would have an

advantage and possibly could colonize and enzymatically utilize more complex organic materials prior to depletion of water-soluble nutrients. This characteristic may account for the success of the Fusarium species as soil-borne organisms and possibly other species which form chlamydospores and other large resting structures in soil.

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