Suppression of Conidial Germination of *Helminthosporium victoriae* in Soil and in Model Fungistatic Systems

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ABSTRACT

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Conidia of *Helminthosporium victoriae* germinated most rapidly in a sterile salts solution at 30 C, ~pH 5, and osmotic potential from −0.2 to −1.2 bar. Of several buffers tested, 2-(*N*-morpholino)ethanesulfonic acid (MES) was the most suitable for germination. In a sterile sandy clay loam soil, germination was most rapid at a matric potential of 0 bar. Germination of conidia incubated at 20 C on sterile sand while being leached with 10% White's salts solution was inversely correlated with flow rate. In contrast, ~85% of the conidia germinated on sand leached at all flow rates with 0.01 M MES (pH 5.0) in 10% White's solution at 30 C. The amount of exudation from ^{14}C-labeled conidia leached for 30 min was positively correlated with flow rate in either a germination-suppressive or germination-conducive environment. Thus, the suppression of germination in soil or in the leaching system cannot be attributed solely to the loss of exudate. The environmental conditions (pH, temperature, and water potential) that allowed germination in the leaching apparatus were largely ineffective in promoting germination in nonsterile soil.

Conidia of *Helminthosporium victoriae* Meehan and Murphy (=*Cochliobolus victoriae* Nelson, =*Drechslera victoriae* (Meehan and Murphy) Subram. and Jain) germinate in water in the absence of any organic or inorganic compounds, if the environment is free of other microbes. However, these spores do not germinate in the presence of other microorganisms unless a carbonaceous energy source is available (15). This inhibition of germination in soil is a type of fungistasis that can be simulated in an axenic model system in which conidia are incubated on a sand substrate through which water, phosphate buffer, or a dilute salts solution is continuously percolated (13,15). In addition, the germination rate is delayed when conidia are floated on relatively large volumes (40 or 80 ml) of a dilute salts solution containing 0.05 M 2-(*N*-morpholino)-ethanesulphonic acid (MES) buffer, pH 6.1 (5).

Since fungistasis occurs in nonsterile soil under conditions of pH, temperature, and moisture which in a sterile environment favor germination, the question arises whether germination would be similarly suppressed in a model system providing conditions optimum for spore germination. Thus, the purpose of this research was (i) to identify the optimal pH, temperature, and water potential for germination of conidia of *H. victoriae* in axenic conditions and (ii) to determine if specifically selected "optimal" or "suboptimal" conditions determined in (i) would affect fungistasis in nonsterile soil or in the axenic model systems used to simulate soil fungistasis.

MATERIALS AND METHODS

Conidia were produced, collected, dispersed, and stained as described previously (4).

Effect of environmental conditions on germination in axenic conditions. Conidia on Nuclepore (Nuclepore Corp., Pleasanton, CA 94566) membrane filters (0.4-μm pores) were floated either on 1 ml of various aqueous solutions in 20-ml glass scintillation vials, or on 40 or 80 ml of the 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 in incubators maintained at 5-C increments from 10 to 35 C. For pH studies, conidia were incubated at 21 ± 1 C for 6 hr in 1 ml of six different buffers ranging from pH 3.0 to 8.5. The buffers, concentrations of solutions used, and specific pH range tested (in 0.5-pH unit increments) were as follows: citrate buffer made with various combinations of 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 made with 0.1 M acetic acid and 0.1 M sodium acetate for pH 4–5, 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer made with 0.1 M MES and 3 N NaOH or KOH for pH 5–7, phosphate buffer made with 0.1 M monobasic 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 (9,10,17) were prepared in 10% White's solution and pH was verified with a pH meter.

To determine the effects of osmotic potential (ψ_s) on germination, conidia were incubated for 8 hr at 21 ± 1 C on membrane filters floated on solutions with five different osmotic potentials. The concentration ranges of the osmotica tested and the volumes of solution were as follows: 0 to 512 g polyethylene glycol (PEG) 4000/kg H_2O (80 ml), 0 to 512 g of PEG 6000/kg H_2O (40 ml), 0 to 1.0 molal KCl (1 ml), 0 to 0.2 M MES (pH 5.6) (40 ml), 0 to 1× Hoagland's salt solution (40 ml) (11), and 0 to 10× White's salt solution (40 ml). The ψ_s values for the PEG 4000 and 6000 solutions were determined from a standard curve for values $\leqslant -1$ bar generated on a dew point microvoltmeter (HR33T Wescor Inc., Logan, UT 84321). The ψ_s for KCl was interpolated from a published table (18) and the ψ_s of White's and Hoagland's solutions were approximated by using the formula ψ_s = (sum of milliequivalents of cations or anions per liter) × (−0.036) (1).

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 1-cm layer of soil with an adjusted ψ_m 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 (condensed) moisture was wiped from inside the lid of the dish with a sterile paper towel. Spores were deposited on one side of 2.5-cm square membrane filters; these were then folded in half and buried in sterile or nonsterile soil at 22 C for 4 or 6 hr, respectively. At the end of each experiment, the soils were weighed, dried at 90 C to a constant weight, and then reweighed to determine percent moisture. Equivalent matric potentials (of the same nonsterile soil) were determined from a standard curve generated by plotting measurements made with a ceramic pressure

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plate apparatus (18).

Effect of environmental conditions on fungistasis in two model systems. The leaching system used has been described elsewhere (15,16). In previous studies, germination of fungal propagules incubated in this system was inversely correlated with the flow rate of the leaching solution (6,19). In the present study, conidia were incubated for 4 hr at variable flow rates with 0.01 M MES (pH 5.0) in 10% White's solution at 30 C (optimal conditions) or in 10% White's solution, pH 5.8 at 20 C (suboptimal conditions).

In the "static volume system," conidia borne on Nuclepore filters were floated on variable volumes of an aqueous solution. Solutions and incubation temperatures used were the same as those indicated above for the leaching system. Germination was assessed at 2-hr intervals.

Exudation from conidia. To determine the effect of optimum environmental conditions for germination on exudation from spores, ^{14}C-labeled conidia were produced as described previously (4). Conidia from 3-wk-old agar cultures containing ^{14}C glucose were removed dry from the agar with a vacuum collector and then 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 of water, refrozen in scintillation vials, lyophilized again, suspended in 10 ml of a scintillation cocktail containing toluene:Triton X-100 (Rohm and Haas, Philadelphia, PA 19105) (2:1, v/v) and 4 g of Omnifluor (New England Nuclear, Boston, MA 02118) per liter, and then counted in a liquid scintillation spectrometer. Counting efficiency was determined by using a ^{14}C-toluene standard.

Effect of environmental conditions on soil fungistasis. Ten grams of an air-dried sandy clay loam soil (pH 5.9; 0.01 M $CaCl_2$) contained in glass petri dishes (5 cm i.d.) was wetted to approximately −0.05 bars ψ_m with water, 0 to 0.01 M MES (pH 5.1), 0 to 10× White's solution, or 0.01 M MES (pH 5.0) in 10% White's solution, and incubated for 24 hr in the dark at 21 ± 1 C or 30 ± 0.5 C. The conidia on Nuclepore membrane filters were incubated on these soil surfaces for 6 hr at the same temperatures.

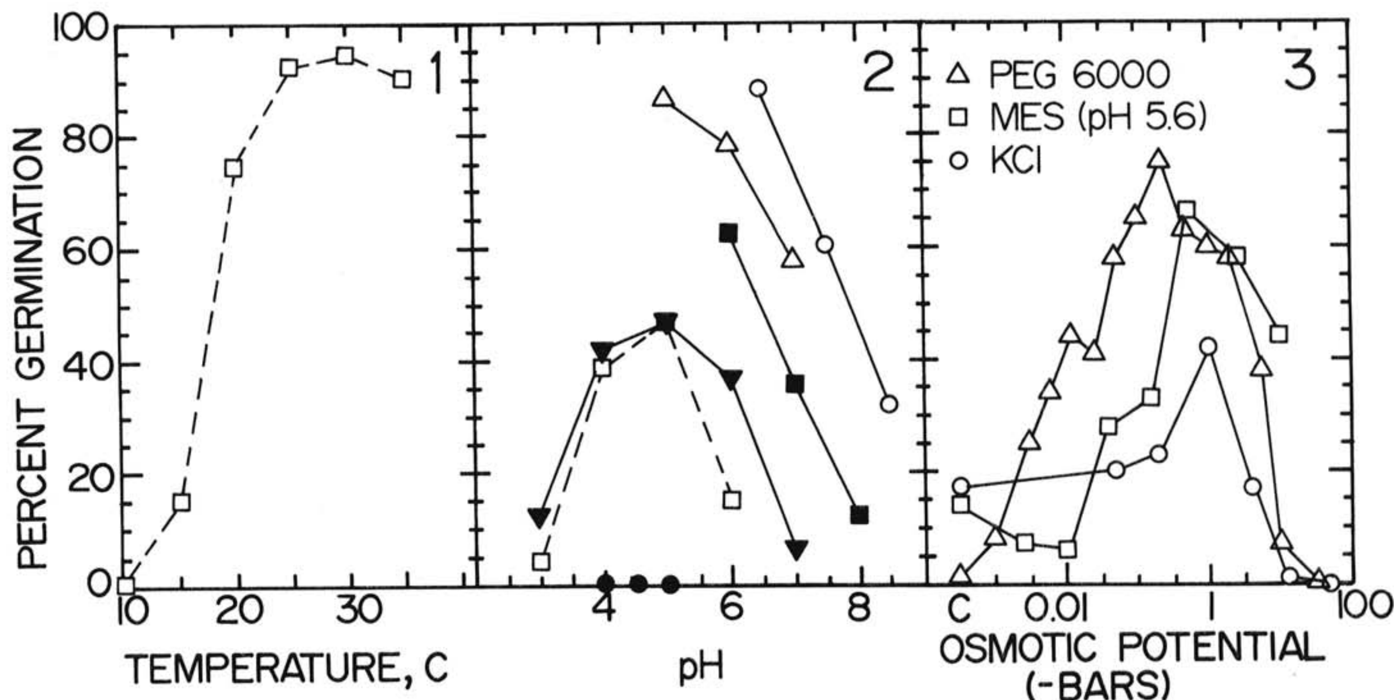
To detect relatively small decreases in soil fungistasis, it was necessary to reduce fungistasis by "diluting" the soil. This was done by saturating sand with an aqueous suspension of soil, and then amending the soil suspension with a series of concentrations of yeast extract or sucrose-peptone solutions (5:1, w/w). One-hundred-gram aliquants of air-dried sandy clay loam soil were wetted to approximately −0.1 bar ψ_m with water and incubated for 16–24 hr. One hundred milliliters of either water (suboptimal conditions) or 0.1 M MES (pH 5.0) in 10% White's solution (optimal conditions) was added and the suspensions were shaken for 1 or 24 hr. The suspensions contained 43% soil. Four milliliters of the suspension, prepared as described above, 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 cc of sand. Thus, the sand-soil mixture contained 1.7 g soil. Fungistasis was assayed by incubating conidia on membranes in darkness on the surface of the soil-sand mixture at 21 ± 1 C (suboptimal conditions) or 30 C (optimal conditions).

Experimental design and statistical analysis. There were three replicates per aqueous treatment and two or three replicates per soil treatment arranged in a randomized design; each experiment was performed at least twice. Bartlett's (20) test for homogeneity of variance ($P = 0.05$) indicated that variance was homogeneous; therefore, 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 ($P = 0.05$).

RESULTS

Effect of environmental conditions on germination in axenic conditions. The optimal temperature range for germination of conidia of *H. victoriae* incubated for 6 hr in 10% White's solution was 25–35 C (Fig. 1). Significantly fewer conidia germinated at 20 C and no conidia germinated at 10 C.

Germination occurred most rapidly at pH 5 in citrate and citrate-phosphate buffers (Fig. 2). With MES, phosphate, and HEPES



Figs. 1-3. 1, Effect of temperature on germination of conidia of *Helminthosporium victoriae*, incubated for 6 hr on membrane filters floated on 1 ml of 10% White's salts solution; s.d. = 4. **2,** Effect of pH adjusted with selected buffers on germination of conidia of *H. victoriae*. Conidia were incubated for 6 hr on membrane filters floated on 1 ml of the indicated buffers in 10% White's solution; s.d. = 13. ● = acetate, □ = citrate, ▼ = citrate-phosphate, ■ = phosphate, △ = 2-(*N*-morpholino)ethanesulfonic acid (MES), O = HEPES. **3,** Effect of osmotic potential on germination of conidia of *H. victoriae*. Conidia were incubated for 8 hr on membrane filters floated on 40, 40, or 1 ml of various concentrations of PEG, MES, or KCl, respectively; s.d. = 6, 14, and 4, respectively. C = water control.

buffers, germination decreased as pH increased above 5, 6, and 6.5, respectively; these buffers could not be tested at lower pH values. No conidia germinated in acetate buffer at pH 4–5. Since germination at pH 5.0 was greater in MES than in citrate or citrate-phosphate, MES buffer with NaOH (pH 5.0) was used in subsequent experiments to maintain an optimum pH for germination.

Osmotic potentials optimal for germination of *H. victoriae* were about −0.2 bar in PEG 6000, −0.5 bar in MES (pH 5.6) and −1.0 bar in KCl (Fig. 3). More conidia germinated in 10× White's solution (71%) (~−0.9 bar) or full-strength Hoagland's solution (86%) (~−1.2 bar) than at lower concentrations tested during the 8-hr germination period, or in water (20%). Germination was detected at −9 bars, but not at −50 bars, in KCl and at −10 bars, but not at −34 bars, in either type of PEG. Since 0.01 M MES provided a ψ_s near the mean of the optimum for the various osmotica (~−0.5 bar), and allowed good 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.

In sterile soil, conidia incubated for 4 hr at 0 bar or −0.05 bar ψ_m germinated significantly more (84 and 70%, respectively) than conidia incubated at −0.3 to −11 bars (60 and 44%, respectively). None of the conidia germinated at −15 bars.

After we determined the above described optima of pH, temperature, and water potential, we considered 0.01 M MES (pH 5.0) in 10% White's solution at 30 C as optimal conditions for germination and 10% White's solution (pH 5.8) at 20 C as suboptimal. This classification was confirmed experimentally; conidia incubated on 2 ml of 10% White's solution (pH 5.8) germinated 40% at 20 C and 74% at 30 C, while conidia incubated in 0.01 M MES buffer (pH 5.0) in 10% White's solution germinated 76% at 20 C and 91% at 30 C. It was not determined whether the stimulation by MES was due to a decrease in pH or water potential, a combination of both, or possibly some other factor. The ψ_s of 10% White's salts and 0.01 M MES (pH 5.0) in 10% White's salts are approximately −0.01 and −0.5 bars, respectively.

Effect of environmental conditions on fungistasis in the leaching system. When conidia were incubated in the leaching apparatus for 4 hr with the suboptimal 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 (Fig. 4). Conversely, when conidia were leached with the optimized 0.01 M MES buffer (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 up to 208 ml/hr. Exudation of carbon compounds 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

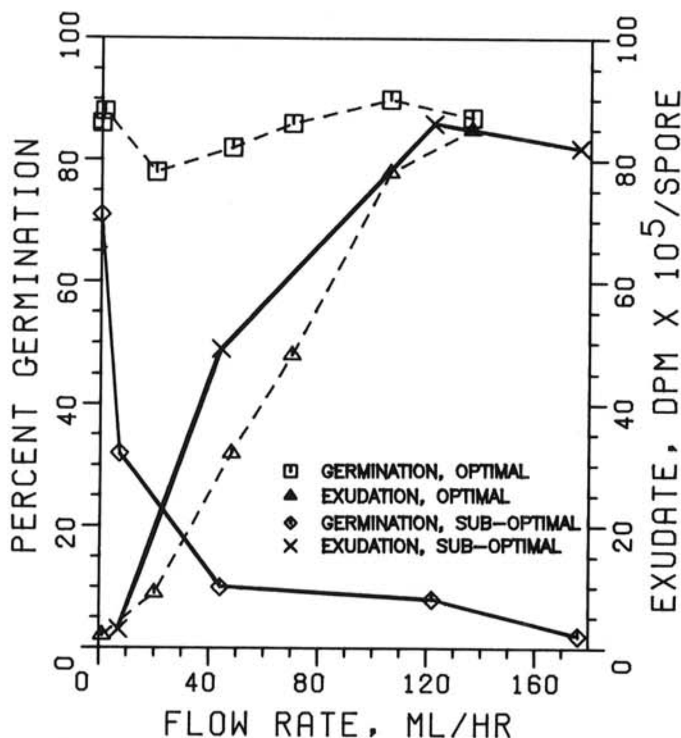


Fig. 4. Effect of environmental conditions on exudation by conidia of *Helminthosporium victoriae* and on their germination in the leaching system. Under "optimal" germination conditions, conidia were leached with 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.0) in 10% White's solution at 30 C; in "suboptimal" conditions, conidia 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 had operated for 30 min.

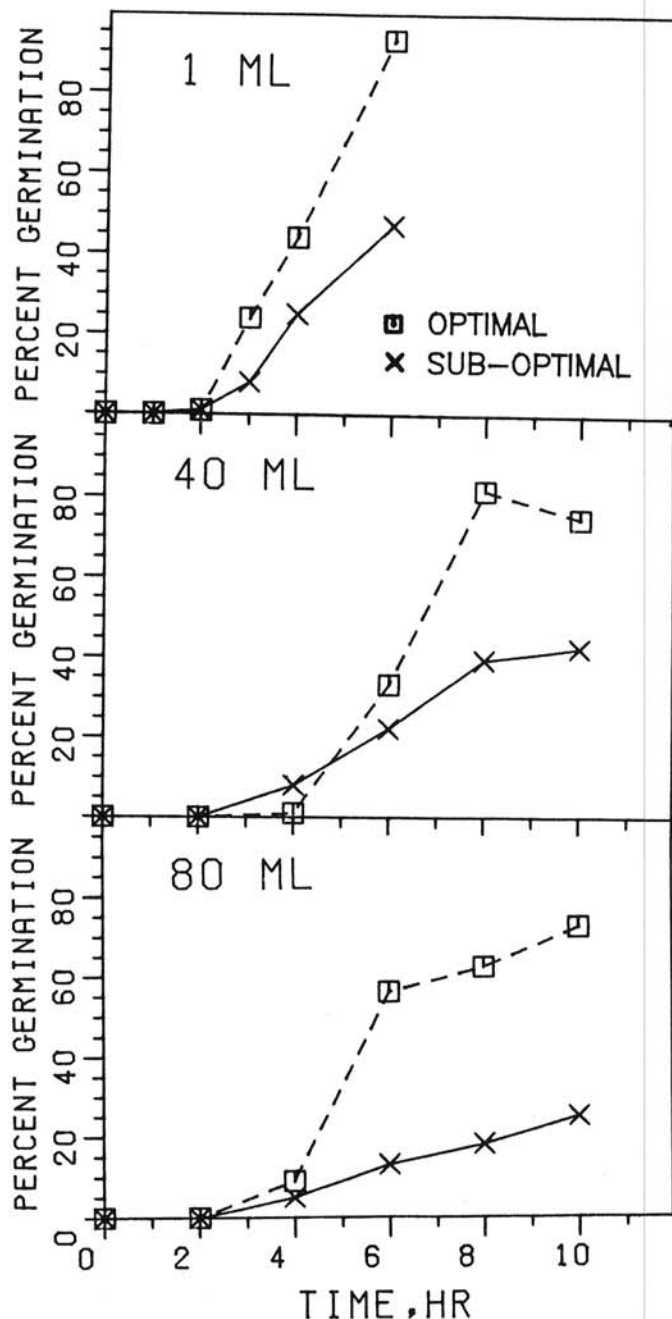


Fig. 5. Effect of environmental conditions on germination of conidia of *Helminthosporium victoriae* incubated on membrane filters floated on 1, 40, or 80 ml of 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.0) in 10% White's solution at 30 C ("optimal" conditions) or in 10% White's solution (pH 5.8) at 20 C ("suboptimal" conditions); s.d. = 10, 11, and 13 for 1, 40, and 80 ml, respectively.

increased; this occurred in leaching conditions which either inhibited or permitted conidial germination.

Effect of environmental conditions on fungistasis in the static volume system. Conidia were incubated on 0.01 M MES (pH 5.0) in 10% White's solution at 30 C or 10% White's solution at 20 C on 1, 40, or 80 ml of solution. The rate of germination decreased as the volume increased (Fig. 5). In all volumes, MES buffer, pH 5.0, at 30 C increased the rate of germination. The difference between rates of germination with the two solutions increased as volume increased. There was no apparent difference in the time of appearance of the first germ tubes in the two solutions at any volume.

Effect of environmental conditions on soil fungistasis. Germination of conidia of *H. victoriae* incubated on nonsterile soil at 21 C ranged from 0–2% at matric potentials of 0 to −5 bars. Since the rate or amount of germination in vitro was greater under optimal than under suboptimal conditions, we tested the effect of

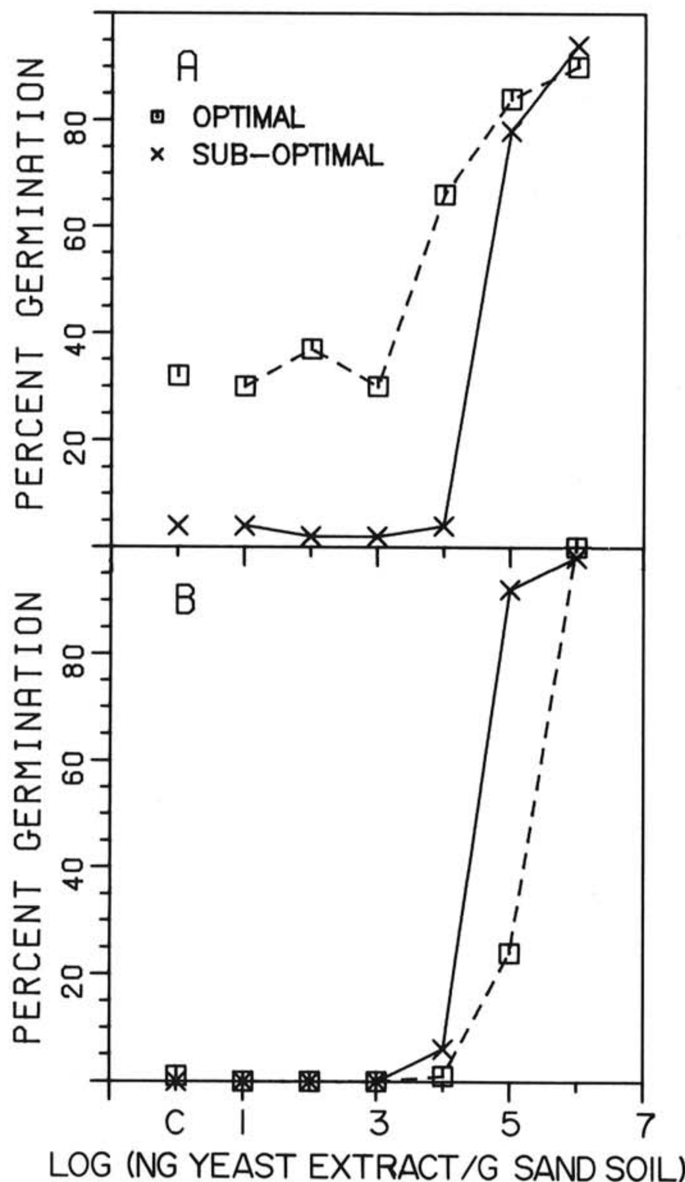


Fig. 6. Effect of environmental conditions on the annulment of soil fungistasis by yeast extract. The soil suspension was prepared by prewetting 100 g of air-dried soil to −0.1 bar, incubating for 24 hr, then shaking with either 100 ml 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.0) in 10% White's solution ("optimal" conditions) or 100 ml of H_2O ("suboptimal" conditions) for either **A,** 1 hr or **B,** 24 hr. Four milliliters of the resulting soil suspension was mixed with $0–10^7$ ng yeast extract and used to saturate 12 g of 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.

optimizing conditions on fungistasis in soil. Only 1% of conidia of *H. victoriae* germinated in soil with optimized conditions of 30 C and pH adjusted to 5.1 with 0.1 M MES (pH 5.1). Germination was similarly unaffected when soil incubated at either 21 C or 30 C was wetted to −0.05 bars ψ_m with 0–10× White's solution or with 0.01 M MES (pH 5.0) in 10% White's solution.

Since optimizing ψ_m, ψ_s, temperature, and pH for conidial germination did not appear to affect soil fungistasis, germination was assayed on a diluted soil with reduced fungistasis. Since 0.01 M MES at pH 5.0 has weak buffering capacity, 0.1 M MES (pH 5.0) was required to reduce the pH. Thus, the ψ_s of the soil was lower than optimal. The pH values of the soil suspensions shaken at 30 C with 0.1 M MES (pH 5.0) (optimal conditions) for 1 or 24 hr were 5.2 and 5.1, respectively; the pH of the soil suspension shaken with water at 21 C was 5.7 (suboptimal conditions). When the soil suspension was prepared by shaking for 1 hr prior to application of spores (Fig. 6A), germination was significantly greater with $\sim 0-10^3$ ng of yeast extract per gram of the soil-sand mixture at optimal than at suboptimal conditions. However, similar quantities of yeast extract ($\sim 10^5$ or 10^6 ng per gram of mixture) were required to completely annul fungistasis under optimal and suboptimal germination conditions. When the soil suspension was prepared by shaking for 24 hr (Fig. 6B), there was little or no germination of conidia, whether incubated at optimal or suboptimal conditions with $\sim 0-10^4$ ng yeast extract per gram of mixture or with $\sim 0-10^4$ ng of sucrose-peptone (5:1, w/w) per gram of mixture (*unpublished*). At $\sim 10^5$ ng of yeast extract per gram of mixture, or $\sim 10^5$ and 10^6 ng sucrose-peptone (5:1, w/w) per gram of mixture, germination was greater in suboptimal than in optimal conditions. At ~1 mg of yeast extract per gram of soil:sand mixture, germination was complete in both conditions.

DISCUSSION

Lockwood and his associates (2,12,13) have demonstrated a correlation between the inhibition of germination of nutrient-independent fungal propagules incubated on soil and in a nutrient-deficient leaching system. In both cases, added nutrients annul the fungistatic effect. Of 17 species of nutrient-independent propagules, 14 responded similarly on soil and when continuously leached (12). In addition, the following processes were similar on soil and on the leaching system: the conversion of *H. victoriae* and *Helminthosporium sativum* from nutrient independence to nutrient dependence (2,8); the reversion in progress toward germination of conidia of *Penicillium frequentans* incubated on sterilized soil for a period less than that required for germ tube emergence (21); the lysis of mycelia (14); and the germination of *H. victoriae* conidia that had been preincubated in a nonfungistatic environment before incubation on soil or the leaching system (3). Furthermore, exudation from ^{14}C-labeled fungal propagules incubated on the leaching system was positively correlated with flow rate and inversely correlated with germination (6,7,19). The ^{14}C-labeled exudate contained sugars (primarily glucose) and amino acids (2). In addition, conidia (previously made nutrient dependent) germinated when leached with spore exudate or a dilute nutrient solution (2). For these reasons, Lockwood (15,16) has suggested that soil fungistasis of nutrient-independent propagules may result from depletion of the nutrient reserves of the propagules, or that the increased exudation may provide a more subtle germination-suppressing message to the propagule.

In the present work, exudation from conidia incubated in the axenic leaching system in both optimal and suboptimal conditions was correlated with flow rate but not with suppression of germination, since germination occurred at high flow rates under optimal conditions (Fig. 4). These results can be explained in one of two ways: either exudation and germination inhibition in the leaching system are causally related in certain environmentally stressful conditions, but not in conditions which are favorable for germination; or alternatively, inhibition of germination in the axenic systems could be imposed either by the continual renewal, or a sufficient volume, of an aqueous medium of suboptimal environmental conditions, such as temperature, pH, and ψ_s. Here,

the increased loss of ^{14}C-labeled exudate would not be causally associated with fungistasis. Further work will be required to evaluate these possibilities.

We have assumed that the MES buffer used in the leaching system functions only to reduce pH and decrease water potential and that it does not serve as a nutrient. Although Good's buffers (10) were designed to be metabolically inactive, their metabolic inertness has never been established and it is possible that MES buffer was stimulatory to germination. No other buffers were used to confirm the results obtained with the model fungistatic systems because MES has the lowest pK_a (6.1) of any of Good's buffers (10), and organic acid buffers with lower pK_as inhibited germination (Fig. 2). However, other buffers or osmotica demonstrated relatively large effects of osmotic potential and pH per se on germination (Figs. 2 and 3). Our experiments did not distinguish between the effects of MES as an osmoticum and as a regulator of pH, since MES was used only at 0.01 M, pH 5, in the leaching system (Fig. 4).

The results with the model systems raise the question of the extent to which suboptimal environmental conditions might inhibit germination in soil. Adjusting soil temperature and pH to optimum conditions increased germination (reduced fungistasis) in diluted soil of low fungistatic capacity, but did not do so in a similarly diluted soil of a higher fungistatic capacity (Fig. 6). It was not possible to adjust ψ_s to optimum levels, since a concentration of MES greater than optimum was required to adjust soil pH. Nonetheless, since optimizing temperatures and pH did not stimulate germination in undiluted soil, the results indicate that the soil used must have a fungistatic capacity far in excess of that necessary to suppress germination. Therefore, suboptimal conditions must be a minor component of fungistasis in this soil.

Further work will be required to evaluate more fully the role of suboptimal conditions in soil fungistasis and the appropriateness of the axenic systems as models for soil fungistasis.

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