Roles of Matric and Osmotic Components of Water Potential and Their Interaction with Temperature in the Growth of *Fusarium oxysporum* in Synthetic Media and Soil

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ABSTRACT

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Interactions of water potential, both matric and osmotic, and temperature as they affect radial growth of *Fusarium oxysporum* were studied. Water potential (ψ) was adjusted osmotically with KCl and $MgCl_2$ and matrically in sterile and nonsterile field soil and with polyethylene glycol (PEG) 6000, a suspended matricum. High temperatures were more inhibitory to growth in matrically adjusted than in osmotically adjusted media. Growth decreased steadily with decreases in soil matric ψ; however, growth in osmotica was stimulated as ψ_s declined from −8 to −15 bars. At osmotic potentials <−20 bars, $MgCl_2$ as a solute was more inhibitory than KCl. PEG, for which a method of radial growth determinations is described, affected growth <−10 bars similarly to the osmotica. The effects of temperature and ψ on growth in sterile and nonsterile soil were similar. However, growth responses obtained on any of the synthetic media tested, adjusted either osmotically or matrically, did not adequately represent those obtained in soil. We conclude that inferences concerning the role of soil ψ in fungal ecology cannot be drawn from studies in which ψ is adjusted osmotically or by the use of a suspended matricum such as PEG. Techniques are described for measuring radial growth as a function of matric ψ in sterile and nonsterile soil.

Fusarium oxysporum (Schlecht.) em. Snyd. and Hans., which causes, among other diseases, hypocotyl rot of sugar pine (*Pinus lambertiana* Dougl.), attacks subterranean hypocotyls in seedling nurseries at an average depth of 1.7 cm (2). Because of the extreme fluctuations in temperature and moisture that occur in the soil strata near the surface (1,3), this study was conducted to determine the interactions of these environmental determinants on radial growth of the fungus in synthetic media and soil. We assumed that growth from a soilborne propagule through the soil to the infection court was requisite for disease development.

Temperature and soil water potential have long been recognized as important factors in diseases caused by soilborne *Fusarium* spp. (4). Most of the work on the influence of water potential on fungal growth has been done with osmotically controlled media (6). Comparisons of the influence of osmotic (ψ_s) and matric potential (ψ_m) (the major component of soil water potential) have shown significant differences in effects on growth of soil fungi (6,10,19). Although there are a few reports on the effects of ψ_m on *Fusarium* spp., (8,10,19), little has been reported on the influence of ψ_m on hyphal growth through nonsterile soil (7,16).

In most growth studies in which soil ψ_m was controlled, especially at <−15 bars, soil moisture was adjusted by rewetting dry soil to a level determined by moisture release curves. However, because of soil hysteresis, wetting results in a different relationship between soil ψ and water content than drying even though the final water content is the same (1,24). Additionally, it is extremely difficult to achieve an even distribution of moisture when wetting a dry soil (9). This source of experimental error was eliminated in the present study.

Polyethylene glycol (PEG) of large molecular weight has been used to adjust ψ of liquid media in microbial studies. Its advantages over conventionally used salts, such as KCl, NaCl, and Na_2SO_4, being the inability of the large molecules to pass through membranes, low toxicity, and very slow microbial degradation (20,24). Although PEG was generally believed to affect ψ primarily as an osmoticum, recent evidence (28) indicates that the primary effect is matric. Inasmuch as agar fortified with all but very low concentrations of PEG does not solidify (21), only liquid media have been used in previous growth studies. However, because of our interest in radial growth of fungi, we developed and describe a technique for using PEG as a matricum in which linear growth was determined.

The influences of temperature on *Fusarium* spp. and the diseases they cause are well known (23). The interactions between temperature and ψ, however, have not been adequately investigated. These interactions are especially important in sites where hypocotyl rot occurs, ie, the surface soil levels, where water flux and solar radiation cause large diurnal fluctuations in both temperature and ψ.

In the present study, the effects of interactions of temperature and ψ on the radial growth of *F. oxysporum* in sterile and nonsterile soil are compared with growth on agar media amended with osmotica and PEG 6000, a suspended matricum. Special emphasis is placed on fungal growth through soil. A procedure is described for measuring radial growth from added inoculum propagules in nonsterile soil by using a pigmented mutant of *F. oxysporum*.

MATERIALS AND METHODS

Isolates. A single-spore-derived culture of *F. oxysporum* (isolate M-2), originally recovered from a sugar pine hypocotyl lesion, and an orange mutant (OR-2) derived from the M-2 isolate were used. Additional information on the hypocotyl rot disease and isolate M-2 has been published (2).

The OR-2 mutant was obtained by using the procedures of Puhalla ([26] and J. E. Puhalla, *personal communication*). Briefly, the procedure is as follows. Isolate M-2 was grown on potato-dextrose agar (PDA) in darkness for 1 wk. Harvested spores in sterile water were exposed for 70 sec to ultraviolet radiation at 3.3×10^3 ergs/cm^2. A sorbose medium that restricted colony growth was used to culture irradiated spores. The medium contained 2.0 g of KH_2PO_4, 1.7 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 1.9 g of asparagine, 2.5 g of casein hydrolysate, 5 ml of hydrolyzed nucleic acids (25), 10 ml of a vitamin solution (14), 1.0

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ml of a minor elements solution, and 20 g of Difco-Bacto agar in 800 ml of distilled water. The minor elements solution contained 0.5 g of $ZnSO_4 \cdot 7H_2O$, 1 g of $MnCl_2 \cdot 4H_2O$, 1 g of H_3BO_3, 5 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $CuSO_4 \cdot 5H_2O$, and 0.1 g of $Na_2MoO_4 \cdot 2H_2O$ in 1 L of distilled water. A solution containing 20 g of sorbose and 0.2 g of $Ca(NO_3)_2 \cdot 4H_2O$ in 200 ml of distilled water was prepared separately, and both the 800- and 200-ml portions were autoclaved separately at 121 C for 20 min. After they had cooled to 45 C, the portions were combined, 0.5 ml of Tergitol NPX (Sigma Chemical Co.) was added, and 30 ml was dispensed into each 9-cm-diameter petri plate.

Spores were plated onto the sorbose medium from which orange mycelial mutants were recovered at the rate of 1 in 4,000 surviving colonies. The OR-2 mutant was identical to the wild type in virulence on sugar pine and growth rates on PDA, osmotically adjusted media, and matrically adjusted sterile soil.

Inoculum disks for radial growth studies were prepared as follows. Sterile 3-mm-diameter Whatman No. 1 filter paper disks were soaked in a macrospore suspension (10^6 spores per milliliter) for 1 min. The excess solution was decanted, and the disks were dried in sterile air and stored at 2 C. Growth studies were conducted in incubators at 15, 21, 24, 30, 33, and 36 ± 1 C for 4–7 days as indicated.

Media. Eckert's broth, the osmotica KCl and $MgCl_2$, and the matricum PEG 6000 were prepared as described by Ioannou et al (15). Water potential of all media was determined with an isopiestic thermocouple psychrometer. Media containing KCl and $MgCl_2$ were solidified for radial growth studies with 15 g of Difco-Bacto agar per liter and poured into 9-cm-diameter plastic petri plates at 30 ml per plate. Following unsuccessful attempts to solidify media containing PEG 6000, a method was developed to permit radial growth measurements in liquid media. Polyester batting 2 cm thick and a thin, tightly woven black polyester cloth were cut into 9-cm-diameter disks. The black cloth was placed on top of the batting and both pieces were placed into 30 ml of Eckert's medium, with or without added PEG 6000, in glass petri plates and lightly compressed to moisten all pieces. The polyester and medium were then autoclaved at 121 C for 20 min. The fungus grew on the surface of the black cloth which was covered with a thin film of liquid medium.

Hyphal tips were cut from 3-day-old M-2 cultures on PDA and used to inoculate the center of the black polyester in plates containing PEG 6000. Agar plates were inoculated centrally with inoculum disks. All plates were sealed separately in plastic bags before being placed in constant temperature incubators. After 5 days, radial growth was determined by measuring colony diameter minus the diameter of the inoculum disk on two perpendicular axes. The experiment was repeated three times.

Soil used for growth studies was an Aken clay-loam collected from a forest nursery that had been fumigated 18 mo previously and found to be free of the hypocotyl rot *Fusarium* in a greenhouse bioassay (2). The soil was sieved through a 1-mm screen, thoroughly mixed, and stored at 9% moisture at 21–23 C.

Sterile soil. Soil was placed in large beakers and brought to saturation with dilute Eckert's broth at a concentration equivalent to 15 ml of broth per 50 g of oven-dry soil. The beakers were sealed inside paper bags and autoclaved for 1 hr immediately and again after 24 hr. Soil samples to be tested at saturation were prepared similarly, except that the soil and dilute medium were added directly to petri plates and used 1 day following the second autoclaving. The sterile soils that were to be used at less than saturation were allowed to dry for several days at 21–23 C until previously calculated weights equivalent to 11–16% soil moisture were attained. The soil in each beaker was aseptically mixed, amounts equivalent to 50 g dry soil were dispensed into petri dishes, and the surfaces lightly compacted. Samples of soil from each beaker were tested psychrometrically for ψ and oven dried to determine percent water content. The water contents of the soil including added medium at ψ values of −1.1, −2.3, −6.1, −14.5, −27, −35, and −57 bars were 0.40, 0.165, 0.152, 0.142, 0.130, 0.123, and 0.115 g of water per gram of oven-dry soil, respectively.

Each soil-filled plate was inoculated in the center with a paper inoculum disk which was pressed lightly into the soil to ensure adequate contact. Six plates at each ψ were sealed individually in polyethylene bags and placed in constant temperature incubators. At 9 days, the diameter of the surface mycelium minus the diameter of the inoculum disk was measured on two perpendicular axes with the aid of a dissecting microscope. The experiment was run a total of four times.

Nonsterile soil. OR-2 was grown on ground barley straw as previously described (2). To produce a more uniform particle size, the ground straw was dry-sieved through a 0.5-mm-mesh screen

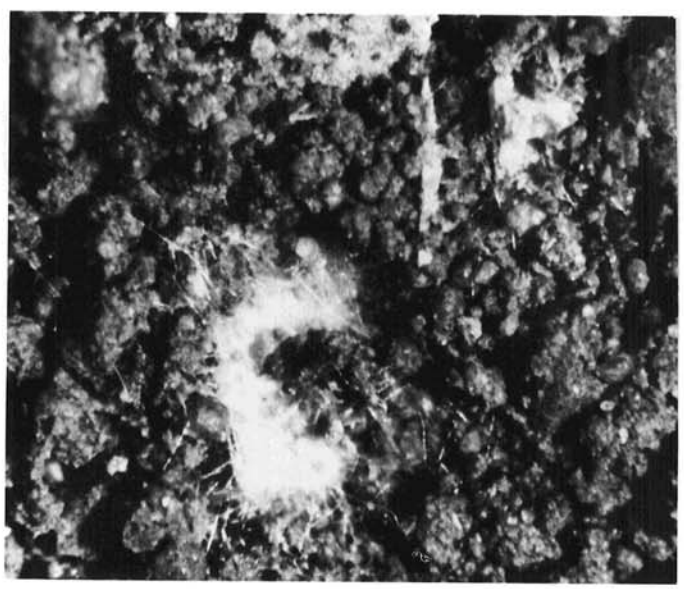

Fig. 1. Pieces of barley straw colonized with an orange mutant (OR-2) of *Fusarium oxysporum* in nonsterile soil incubated at 30 C for 3 days at −1 bar.

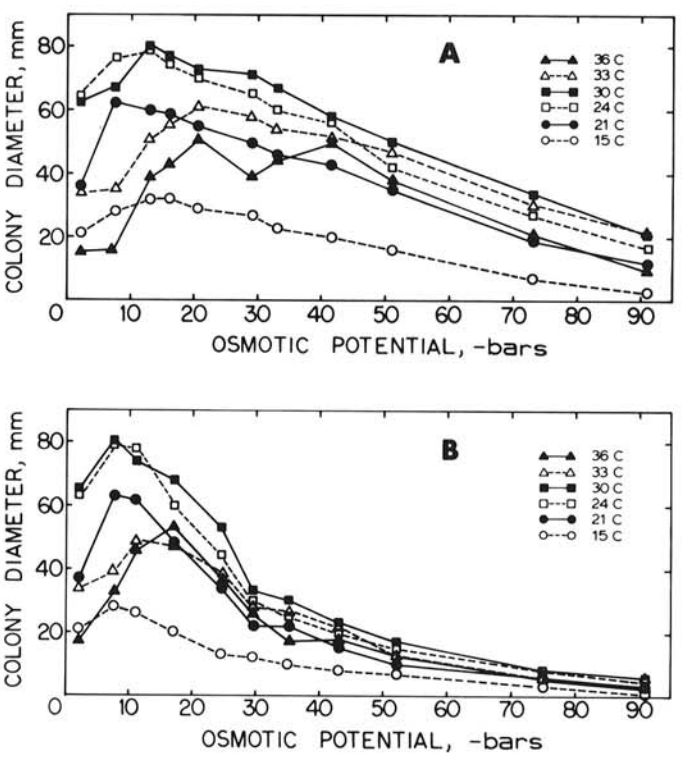

Fig. 2. Effect of osmotic potential of an agar medium, adjusted with either **A**, KCl or **B**, $MgCl_2$, on the radial growth of *Fusarium oxysporum* incubated for 4 days at six temperatures. Mean standard error is 3 mm.

and arrested on a 0.3-mm-mesh screen. The final population in the barley straw inoculum was 1.25×10^5 propagules per gram.

The sieved OR-2 inoculum was added to the sieved nursery soil at 0.1 g/L. Similarly ground and sieved uninfested straw was autoclaved at 121 C for 20 min on 2 consecutive days, added to the same soil at 8 g/L, and mixed thoroughly. Samples (30 g) of the soil-straw mixture were wet to saturation, placed on a ceramic plate moisture extractor, and adjusted to ψ_m values of −0.9, −4.5, and −14 bars. Final ψ was determined psychrometrically. The soil samples were transferred from the ceramic plates to 5-cm-diameter petri plate bottoms with only minimal disruption of soil structure, covered with polyethylene, and placed in constant temperature incubators. Two plates were immediately tested for colonization of the uninfested straw.

The soil plates were removed after 72 hr. Microscopic observations revealed hyphae growing in and between soil aggregates and straw pieces. A random sample of 60 hyphal segments from the −1-bar soil plates were removed with sterile forceps and plated on acidified PDA. Thirty-two percent of the hyphal segments were the OR-2 isolate. A piece of the added inoculum straw was occasionally seen near the surface of the soil with abundant hyphae growing from it and to nearby added sterile straw (Fig. 1).

Soil plates were assayed for straw colonization as follows. The surface soil to a depth of 5 mm was removed from each plate, and 50 randomly selected straw pieces were picked out with the aid of a dissecting microscope and fine forceps. Care was taken to remove the straw piece with a minimum of attached soil. Another 5 mm of soil was scraped from the plate and 50 additional straw pieces were removed. All straw pieces were plated on acidified PDA. After 6 days, pieces were checked for the presence of orange colonies characteristic of the OR-2 isolate. The experiment was run a total of three times.

Assuming random distribution of straw pieces and equal radial growth in all directions from the infested pieces, and given the density of the added sterile straw pieces (one piece per cubic millimeter), the ratio of added preinfested to added sterile straw (0.01205), and the formula for the volume of a sphere, the percent colonized pieces was converted to mean radial hyphal growth for each treatment as follows:

$$\text{Radial growth (mm)} = \left\{3/4[(A - B)/B]C\right\}^{1/3}$$

in which A = percent infested straw pieces, B = percent added preinfested straw pieces, and C = soil volume (mm^3) occupied by each sterile straw piece.

RESULTS

Effects of osmotic potential and temperature on radial growth in synthetic media. Maximum radial growth in agar adjusted with both KCl and $MgCl_2$ occurred at water potentials below that of the nonamended basal medium ($\psi_s = -2$ bars) at all temperatures (Fig. 2). The lowest optimal ψ_s for growth in both osmotica occurred at 33 and 36 C. However, growth at <−20 bars at all temperatures in KCl-adjusted media declined more slowly with decreasing ψ than in $MgCl_2$. Growth was reduced 50% at −60 and −27 bars in media adjusted with KCl and $MgCl_2$, respectively. Differences in response were particularly evident at −90 bars where growth almost ceased in $MgCl_2$, whereas growth in KCl was 27% of maximal.

Mycelial growth on the polyester-stabilized liquid medium with PEG 6000 was circular and similar to growth on agar plates with the exception of slightly reduced aerial mycelium. Only at 33 C was there a significant increase in growth over the basal medium as ψ declined to −10 bars (Fig. 3). Growth at the other temperatures was essentially unaffected down to −15 bars below which growth at all temperatures declined to 50% of maximum at −33 bars. Unlike the osmotic media, growth on the suspended matricum was severely reduced at 36 C at all levels of ψ tested.

Effects of soil water potential and temperature on mycelial growth in sterile and nonsterile soil. Maximal growth in sterile soil occurred at −1.1 bars at all temperatures except 33 C (Fig. 4) at which the maximum occurred at −6.1 bars; this was similar to the increased growth seen at 33 C in PEG and at all temperatures in osmotically adjusted agar. As with PEG, little growth occurred at 36 C. However, unlike the results from PEG or salts, growth ceased below −35 bars at both 18 and 36 C.

Maximal radial growth in nonsterile soil, calculated from percent colonization of barley straw pieces, occurred at the highest ψ_m tested (−0.9 bars) (Fig. 5). Growth at 36 C is not included as it was not statistically different from zero. Mean maximal radial growth through the nonsterile soil calculated as described above was 1.7 mm in 4 days.

Comparisons of growth in synthetic media and soil. The effect of ψ on relative growth at 24 C as determined by all five methods is illustrated in Fig. 6 for comparative purposes. Below −15 bars growth was far less sensitive to reduced water potentials in KCl-amended media; and below −38 bars growth was over twice that of the other methods. Responses to $MgCl_2$ and PEG 6000 were very similar despite some divergence at high (−2 bars) and moderate (−20 to −30 bars) ψ. Fig. 6 clearly illustrates the increased sensitivity of fungal growth to decreases in soil matric water potentials below −10 bars. This is in contrast to the increases in growth seen with salts and PEG.

The average 50% growth reduction in sterile soil at −16 bars illustrates the rapid decline in growth with decreasing ψ_m, as contrasted with both salts and PEG 6000. Responses were similar in both the percent and rate of growth reduction between −40 and −50 bars (Fig. 6). At <−50 bars responses again diverged as growth in soil reached an extrapolated extinction at −65 bars while at least minimal growth continued in the other media to <−90 bars.

Although determined by different methods, the growth responses in sterile and nonsterile soils are very similar. The differences in mean percent reduction in growth between the two

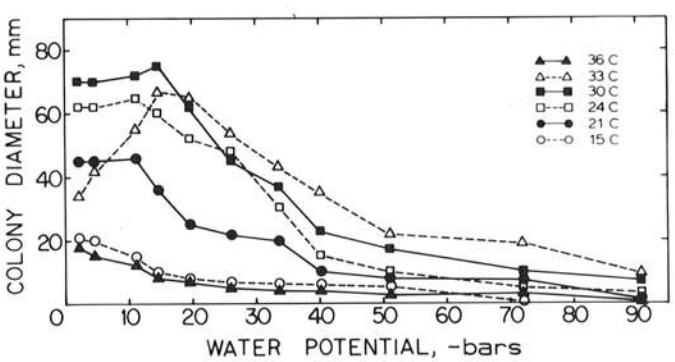

Fig. 3. Effect of matric potential of a synthetic medium, adjusted with the suspended matricum polyethylene glycol 6000, on the radial growth of *Fusarium oxysporum* incubated for 5 days at six temperatures. Mean standard error is 2 mm.

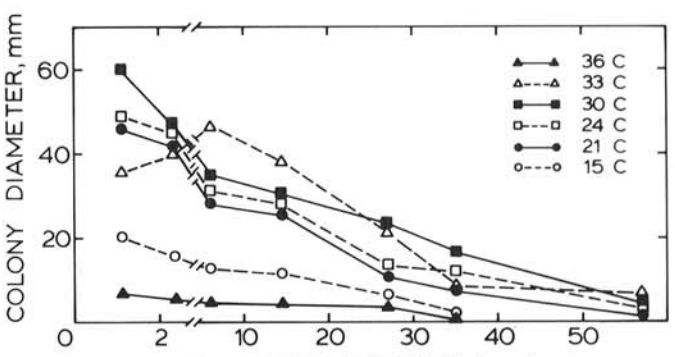

Fig. 4. Effect of soil water potential on radial growth of *Fusarium oxysporum* incubated for 9 days at six temperatures in sterilized field soil. Mean standard error is 6 mm.

methods from −1 to −4.5 and −4.5 to −14 bars at all temperatures (21–33 C) (Figs. 4 and 5) are within 5% for each range.

DISCUSSION

In studying the role of ψ in the ecology of soilborne fungi, many investigators have made inferences from experiments conducted with osmotically adjusted media (17,22,29). Such studies are of limited value inasmuch as the primary component of soil ψ in nonsaline soils is matric (1), and the physiological mechanisms for coping with osmotic and matric forces are different (6,13).

Recent research revealed that the primary influence of PEG 6000 on ψ is matric, with the solute component accounting for a maximum of 10% of the total ψ in dilute suspensions and even less in more concentrated suspensions (28). The virtual impermeability of both cell walls and membranes to PEG 6000 (13) permits physiological studies on the effects of matric potential on fungal growth without the complicating indirect effects of a solid matrix (soil), such as decreasing area for nutrient absorption, increased requirements for hyphal transport of nutrients, and problems in measuring linear growth. Therefore, the development of a method for measuring radial growth in a matricum such as PEG 6000, as described in this study, allows for a more direct comparison to radial growth in soil than by using osmotically adjusted media.

It is generally believed that increases in growth caused by small reductions in ψ, as confirmed in the synthetic media used in this study, result from the establishment of more favorable internal ψ for cellular functions (4,6). This may be accomplished in matrica or nonabsorbed salt solutions by induction of solute production within the cell (10,13) or, in osmotica, by actively accumulating external compatible solutes, both processes resulting in favorable cytoplasmic ψ_s.

In light of these points, useful interpretations of the growth responses in PEG 6000, salt solutions, and soil can be made. Although the initial increase in growth with decreasing ψ from −1 to −10 bars is not as large as with the two salts, the growth response in PEG 6000 closely follows $MgCl_2$. In this case, it appears that microbial responses to osmotic and matric forces are similar. The ψ_s of synthetic media is commonly adjusted with $MgCl_2$ and especially KCl. Because of the similarities in growth responses in the presence of $MgCl_2$ and PEG 6000, also observed with *Phytophthora cinnamomi* (27), it seems likely that $MgCl_2$ either is not absorbed by hyphal cells as a compatible osmoticum or accumulates to toxic concentrations. However, KCl permits the fungus to grow at relatively low ψ_s. It is well known that potassium ions are easily accumulated by microbial cells and can serve as a compatible cytoplasmic osmoticum of low toxicity (13).

The increase in growth as ψ declined from −1 to −6 bars in sterile soil and the large increase from −2 to −14 bars in PEG at 33 C appear to be anomalous when compared to those at the higher and lower temperatures. It is known that some *Fusarium* spp. have lower optimal ψ at higher temperatures (6), and that growth reductions at low ψ are alleviated at above-optimum temperatures (5,15). In both cases, 33 C approached the limiting temperature for growth as seen by the major overall growth reduction at all water potentials at 36 C. The absence of this effect and significant growth at 36 C in the osmotic media represents a differential response of the fungus to osmotic and matric forces at this repressive temperature.

Interpreting the differential responses of growth in soil and PEG 6000 provides an interesting insight into fungal water relations. Because previous studies with PEG were done in liquid media (15,21), the stimulation of linear growth by a small reduction in ψ_s has not heretofore been duplicated with ψ_m. Inasmuch as radial growth was stimulated by small decreases in ψ_m in a suspended matricum, why then was this same phenomenon not seen in either sterile or nonsterile soil (solid matricum)? It is likely that as water content decreased, factors such as decreased solute diffusion, decreased area for solute absorption, and increased requirements for transport of metabolic products along hyphae (11,24) may have exceeded the benefit of a more favorable cytoplasmic ψ. These soil factors appear to be dominated by ψ in the −35 to −50 bar range as growth through soil approached that of both PEG 6000 and $MgCl_2$.

The calculated radial growth distances in nonsterile soil (1.0–1.7 mm) agree with other estimates of the distance through soil which must be traversed by hyphae to infect a seedling hypocotyl. The zone of influence (laimosphere) exhibited by squash hypocotyls on the surrounding microflora was about 3 mm (18). Additionally, peanut roots were found to stimulate germination of spores of *F. oxysporum* to a distance of 2 mm (12).

The similarity between the growth responses in sterile and nonsterile soil indicates that differences in nutrient and energy source availability and biological interactions, such as bacterial repression above −15 bars (4,10) which has been shown with other *Fusarium* spp. (7), are not a major factor under the conditions tested here. We conclude, therefore, that the effects of ψ_m on growth in sterile soil adequately represent the response in nonsterile soil at relatively low ψ_m.

Clearly, much remains to be done concerning mechanisms of osmoregulation in fungi; however, our results indicate that behavior of fungi in synthetic media, even those adjusted with a suspended matricum such as PEG 6000, cannot be used to make inferences concerning the role of ψ in the ecology of soilborne fungi.

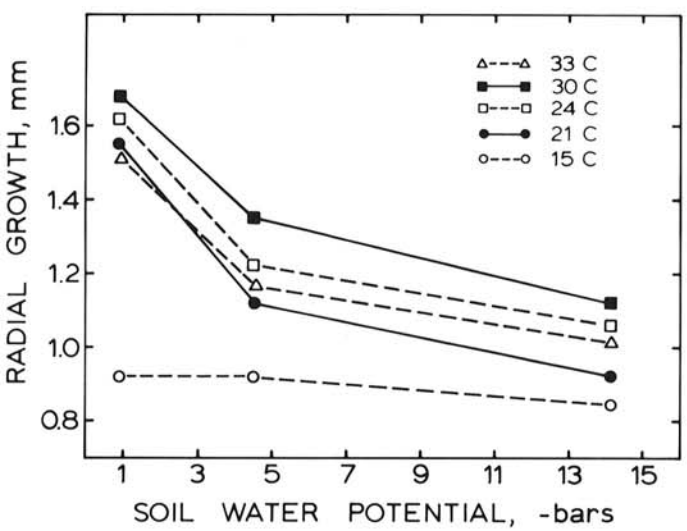

Fig. 5. Effect of soil water potential on estimated radial growth of *Fusarium oxysporum* incubated for 3 days at five temperatures in nonsterile field soil. Mean standard error is 0.3 mm.

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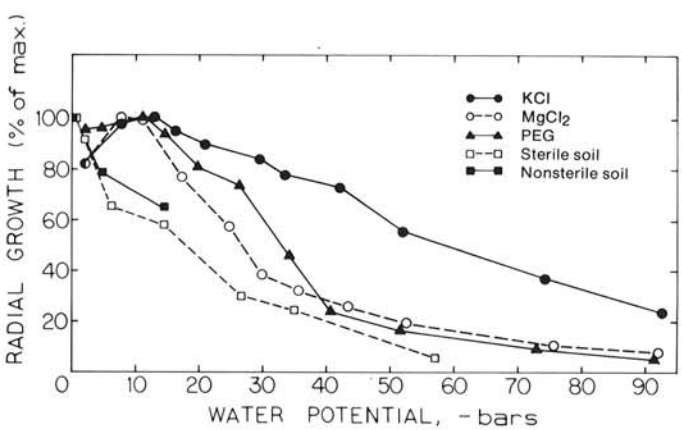

Fig. 6. Effects of osmotic and matric potential on relative radial growth of *Fusarium oxysporum* at 24 C in soil and synthetic media.

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