Effect of Water Potential and Temperature on Growth, Sporulation, and Production of Microsclerotia by Verticillium dahliae

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ABSTRACT

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The minimum osmotic potential (ψ_s) which allowed conidial germination, mycelial growth, and sporulation of *Verticillium dahliae* was −100 to −120 bars. Radial growth on agar medium was maximal at ψ_s values between −10 and −20 bars, but growth measured as increase in dry weight decreased linearly with decreased ψ_s of liquid medium from −2 to −120 bars. Likewise, the rate and final percentage of conidial germination decreased progressively with each decrease in ψ_s below −2 bars. The production of conidia in liquid medium increased logarithmically as ψ_s decreased from −2 to about −20 bars and was maximal at ψ_s values as low as −50 bars. No (or very few) microsclerotia (MS) were produced at ψ_s values between −70 and −80 bars, even though radial growth and production of dry weight were reduced by only 60% at these same ψ_s values. The production of MS either was increased or remained unchanged by reduction of ψ_s from −2 to about −20 bars and it decreased progressively with greater reductions in ψ_s. The production of MS in infected tomato stems buried in nonsterilized soil was maximal at a water potential (ψ) of −32 bars and at 24 C. Appreciable numbers of MS also were produced at 18, 27, and 30 C in soil at ψ = −5 or −32 bars. However, in saturated soil and soil at ψ = −100 bars the production of MS was greatly inhibited at all temperatures tested. When infected stems were adjusted by air-drying to ψ values ranging from −5.8 to −98 bars and buried in soil at ψ = −27.5 bars and 24 C, maximal numbers of MS were produced in tissue dried to ψ = −18.7 bars. When similar tissues were buried in soil at ψ = −0.8 bars only small numbers of MS were produced, regardless of the initial water status of the tissues.

Additional key words: tomato, vascular wilt disease, soil microbiology.

Several reports (19, 22, 23) have presented information on different aspects of the water relations of *Verticillium dahliae* Kleb. Manandhar and Bruehl (19) reported that the fungus can grow at water potential (ψ) values as low as −100 bars, controlled either osmotically [osmotic potential (ψ_s)] or matrically [matric potential (ψ_m)]. The ability of the fungus to grow at low ψ_s values also was reported by Mozumder and Caroselli (22). The ecological and epidemiological significance of this phenomenon is not known. In the presence of living host plants, which usually require available soil moisture at ψ values higher than −15 bars, ability of the fungus to grow at lower ψ values probably would not be required or confer any particular advantage for parasitism. In the absence of susceptible hosts, the fungus survives in the soil as microsclerotia (MS) (26). Because it is a soil invader (29) it probably is unable to grow saprophytically regardless of the soil moisture. The only stage in the life cycle of *V. dahliae* in which significant saprophytic growth occurs in soil is at the end of the growing season when infected moribund plants are returned to the soil. Given the advantage of the pioneer colonist (4) the fungus grows throughout the infested debris and eventually forms numerous MS (10, and N. Ioannou, *unpublished*). Soil-environmental factors that may affect the production of MS in moribund tissue have received little attention. Reports dealing with the water relations of *V. dahliae* in artificial culture (19, 22, 23) and in soil (19) do not refer to MS production. The effect of temperature on the production of MS in vitro (3, 13) and in infected host tissues (3) was studied, but no quantitative data were presented.

In this study the effects of ψ_s, ψ, and temperature on the production of MS in vitro and in infected tomato tissues are examined quantitatively. The effects of ψ_s on the germination of conidia, mycelial growth, and sporulation also are examined. Particular emphasis, however, is given to the influence of ψ and temperature on the formation of MS in host tissue buried in soil. A preliminary report of this study has been published (15).

MATERIALS AND METHODS

Isolates.—Two single-spore-derived isolates of *V. dahliae* (45-1 and 511-4), originally isolated from naturally infected tomato plants and maintained on potato-dextrose agar (PDA) slants at 22-24 C, were used

in the in vitro experiments. Isolate 45-1 was used in all of the experiments, whereas 511-4 was used only as mentioned in the appropriate Results section.

Media and osmotica.—The basal medium used most often was modified Eckert's broth (9) which contained 5.5 g glucose, 1.2 g NH_4NO_3, 1 g Bacto-malt extract, 1 g Bacto-yeast extract, 0.75 g Na_2HPO_4, 0.75 g KH_2PO_4, 0.1 g NaCl, plus the minor salts used by Hall and Ly (12) in 1 liter of distilled water. If solid medium was required, 15 g of Difco-Bacto agar was added. The pH of the medium was 6.8 after autoclaving at 121 C for 15 min. In a number of experiments dealing with the effect of ψ_s on MS production, potato-dextrose broth (PDB) also was used as basal medium (pH 6.5). The ψ_s of liquid or agar media was adjusted to desired values by adding appropriate molal concentrations of sucrose, NaCl, KCl, or a salt mixture comprised of NaCl, KCl, and Na_2SO_4 in a molal ratio of 5:3:2 (27). In some experiments dealing with MS production, polyethylene glycol with average molecular weight of 6,000 (PEG-6000) was substituted for sucrose. Some of the osmotica, when added to the basal media, resulted in considerable volume expansion; in these cases the concentration of nutrients in the basal media was adjusted so that dilution of nutrients was prevented. The molalities of NaCl, KCl, and sucrose required for obtaining different ψ_s values were determined from the water activities (a_w) given by Robinson and Stokes (24). Values of a_w for solutions of the salt mixture used are given by Scott (27). The required concentrations of PEG-6000 were calculated from the formula developed by Michel and Kaufmann (21). In all cases the actual ψ_s values were determined psychrometrically after the media were prepared in the final form by use of the isopiestic thermocouple psychrometer described by Duniway (8).

Radial growth on agar media.—Plastic petri plates, each containing 25 ml of solidified agar medium adjusted to desired ψ_s values, were inoculated in the center with 6-mm-diameter mycelial disks taken from 5-day-old cultures of the fungus on basal agar medium (16). Plates were incubated in closed plastic containers at 24 C for 12 days and radial growth was determined by measuring the colony diameters from five plates per treatment.

Germination of conidia, mycelial growth, and sporulation in liquid media.—Liquid media were prepared in 250-ml Erlenmeyer flasks, each containing 100 ml of medium. A suspension containing 1.5×10^7 conidia per ml in sterile-distilled water was prepared from basal medium slant cultures and 0.5-ml portions were added to each culture flask so that the initial concentration was 7.5×10^4 conidia/ml of medium. The inoculated flasks were incubated at 22-24 C on a rotary shaker at 150 rpm. Conidial germination and production of new conidia were determined in 1-ml samples withdrawn from each flask at prescribed time intervals during incubation. Growth was determined as increase in dry weight after incubation for 10 days. The mycelial mats were recovered on filter papers of known dry weight, rinsed five times with sterile-distilled water, dried for 24 hr at 90 C, and weighed. Sterile-media controls from the respective treatments, also were processed in the same manner.

Production of microsclerotia.—The production of MS was determined in cultures grown on quartz sand moistened with liquid media. Thoroughly washed and dried sand in glass petri plates (90 g per plate) was autoclaved at 121 C for 1 hr. After cooling, the sand in each plate was moistened with 20 ml of liquid medium containing 10^5 conidia/ml and the plates were incubated at 24 C for 15 days. To determine the number of MS that were produced, the contents of each plate were blended in 200 ml of sterile-distilled water in a Waring Blendor jar for 30 sec at high speed. The suspension was allowed to stand for a few seconds so that sand particles would settle, and 10-ml portions were withdrawn and diluted further with 10 to 100 ml of sterile-distilled water (depending on the concentration of MS) with an additional 30-sec blending at high speed in a smaller jar. The MS in at least three 1-ml samples of the final suspension were counted using a nematode-counting chamber and a low-power dissecting microscope. Numbers of MS were expressed as the number per ml in the initial 200 ml of suspension before dilution.

Viability of microsclerotia.—To determine their viability, MS that had been collected on a 38-μm sieve, were treated with 0.5% NaOCl for 5 sec and rinsed with running distilled water for several minutes. The MS then were transferred onto filter paper disks and air-dried for 15 days at 22-24 C in a germ-free transfer chamber with continuous air flow. Rinsing with 0.5% NaOCl and air-drying removed or killed any attached conidia and mycelia. Air-dry MS were suspended in sterile-distilled water at a concentration of approximately 100 per ml and 0.5-ml portions were plated in each of 10 plates containing modified pectate agar media. After about 10 days, viable MS germinated and produced new microsclerotial colonies (14) which were counted with a dissecting microscope. The composition of the pectate agar medium was the same as originally reported by Huisman and Ashworth (14) except that three antibiotics (100 μg/ml streptomycin sulfate, 50 μg/ml chloramphenicol, and 50 μg/ml of chlorotetracycline HCl) were substituted for 200 μg/ml of streptomycin sulfate. The method of preparation was similar to that used by Butterfield (5), but the antibiotics were added to the pectate-salts solution after autoclaving for 15 min at 121 C.

Effects of soil water potential and soil temperature on formation of microsclerotia in infected host tissues.—Soil

TABLE 1. Effect of osmotic potential on the germination of conidia of *Verticillium dahliae* in liquid medium[a]

Osmotic potential (−bars)	Time required for 5% germination (hr)	Time required for maximum percent germination (hr)	Maximum germination (%)
2.0	5.2[b]	11.6	96
6.5	5.6	13.6	97
13.3	5.8	15.4	86
21.5	7.0	17.5	80
29.5	7.9	22.0	72
53.5	11.6	24.0	47
120.0	144.0	144.0	5

[a]Modified Eckert's broth amended with a mixture of salts on a rotary shaker at 22-24 C.

[b]Average values from four replicates per treatment.

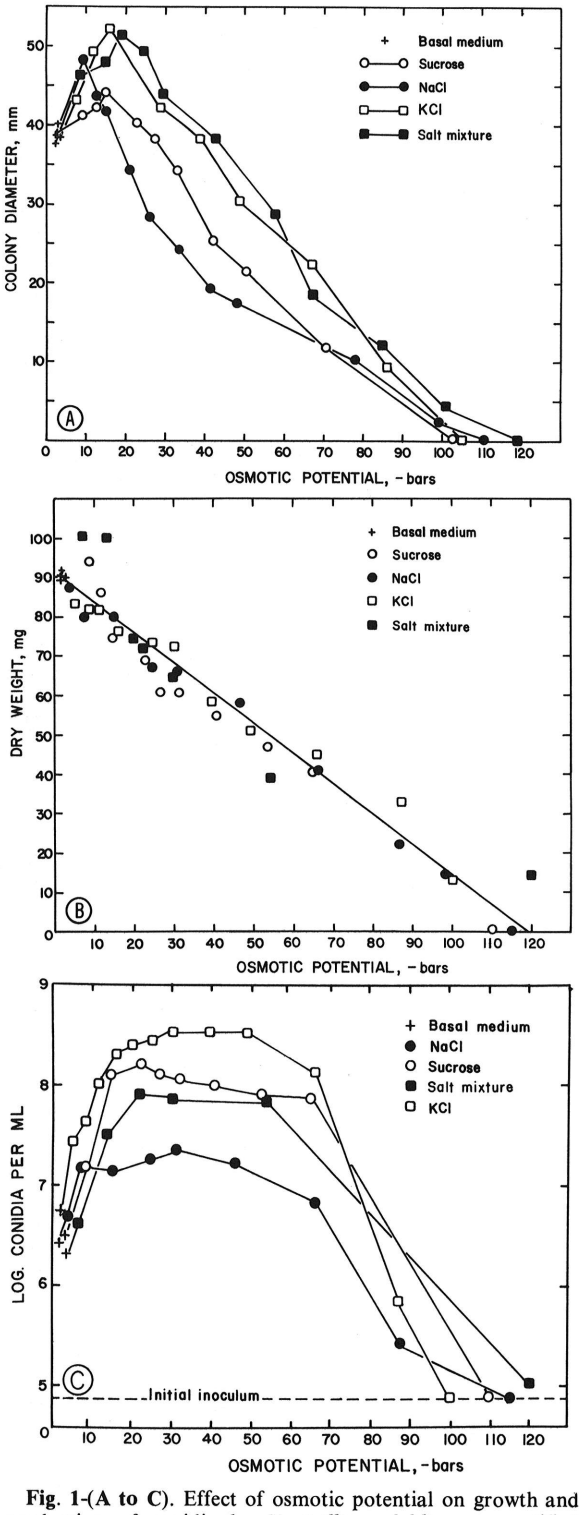

Fig. 1-(A to C). Effect of osmotic potential on growth and production of conidia by *Verticillium dahliae* on modified Eckert's medium amended with four osmotica. **A)** Colony diameter on agar medium after 12-days incubation at 24 C. **B)** Dry weight, and **C)** Production of conidia, both determined in liquid-shake cultures after 10-days incubation at 22-24 C. The solid line in B was drawn according to the regression equation $DW = 91.02 + 0.765\ \psi_s$ ($r = 0.971$).

was prepared by mixing three volumes of unsterilized field soil (Yolo loam, sieved through a 2-mm screen) with one volume of quartz sand. Field soil was collected from a plot that had been fallowed for the previous 5 yr; prior to use, assays for propagules of *V. dahliae* were done using the wet-sieving (2, 14) and the Anderson sampler (5) techniques. Only soil samples that contained no propagules of *V. dahliae* according to both assay methods were used. The quartz sand and soil were mixed thoroughly and samples of the mixture were brought to saturation by adding the required amount of water. One saturated sample was sealed immediately in a plastic bag and used without further alteration. A second sample was drained to field capacity on a pressure plate. Owing to the contribution of ψ_s the saturated sample ($\psi_m = 0$ bar) and the sample at field capacity ($\psi_m = -0.33$ bar) had total ψ values of -0.35 and -0.75 bar, respectively. The remaining samples were air-dried at 22-24 C for various periods of time, until the desired ψ values (-5, -32, and -100 bars) were attained. All ψ values were determined psychrometrically. The water contents of the soil-sand mixture at ψ values of -0.35, -0.75, -5, -32, and -100 bars were 0.363, 0.205, 0.157, 0.084, and 0.051 g water per gram of oven-dry soil, respectively.

The infected host tissues consisted of tomato stems collected from infected plants in the field. Prior to incorporation in the soil, the infected stems were chopped into small pieces in a Waring Blendor, then spread on a piece of cheesecloth and air-dried for 20 min to remove excess moisture and to prevent aggregation of individual pieces. The tissue ψ value after these manipulations was approximately -10 bars and additional water loss was prevented by enclosing the material in plastic bags until it was incorporated into the soil. No MS were detected in three 5-g samples of tissue which were assayed for the presence of MS; however, fresh, infected tissue plated on water agar or PDA yielded numerous *V. dahliae* colonies. Colony counts, obtained by culturing weighed amounts of fresh tissue, revealed that the material was uniformly infected.

One gram of chopped tissue was thoroughly mixed with the soil at various ψ values using the equivalent of 15 g of oven-dry soil. The soil-tissue mixtures were transferred into 20-ml glass vials with caps and then were placed in loosely closed plastic containers and incubated at temperatures ranging from 12 to 33 C. A small opening in the center of the cap of each vial allowed for gas exchange; loss of water through this opening was prevented by maintaining atmospheres in the plastic containers near saturation with a 2-cm-thick layer of moist paper toweling. Controls, which consisted of samples of soil with and without uninfected tissues, were prepared and incubated as described above.

After 10 and 30 days incubation, the numbers of MS formed in three vials of soil at each ψ value and temperature were determined using a modification of the wet-sieving technique (2, 14). The soil samples were spread out and allowed to air-dry for 15 days at 22-24 C and each sample then was suspended in 150 ml of 1% Calgon solution in an Erlenmeyer flask and shaken for 1 hr at 250 rpm on a rotary shaker. The soil suspension then was washed through 125- and 38-μm sieves; the residues retained by the two sieves, as well as the fraction

washed through the 38-μm sieve, were assayed separately for MS of *V. dahliae*. The residue arrested by the 38-μm sieve was treated for two 10-sec periods with 0.5% NaOCl (each followed by a 60-sec rinse with running distilled water) and cultured on 15 plates containing a modified pectate agar medium, with the procedure described by Huisman and Ashworth (14). A modification of the filter paper technique described by Isaac et al. (17) was used to determine the numbers of MS in the residue retained by the 125-μm sieve. This fraction contains only MS embedded in pieces of nondecomposed debris (1, 2). Plant debris was separated from soil particles by flotation, air-dried at 22-24 C for several days, weighed, and ground with a Wiley mill (250-μm screen). Samples (50 mg) of ground material were spread on the surface of a filter paper disk (Whatman No. 2, 9-cm in diameter) on a Büchner funnel. While subjected to a slight vacuum, the material on the filter was rinsed with 0.5% NaOCl and distilled water, as described for the 38-μm residue. The filter paper then was placed on the surface of pectate medium in glass petri plates with the debris-bearing surface in contact with the medium. To determine the numbers of MS washed through the 38-μm sieve, the wash water from each sample was collected in a 2-liter flask and ten 25-ml samples were assayed for MS with the filter paper technique described above. Plates containing the residues from the three fractions were incubated at 22-24 C for 12 days. The soil or filter papers then were washed from the surface of the medium and the characteristic microsclerotial colonies of *V. dahliae* that had formed under the surface of the medium were counted with a dissecting microscope (14).

Effect of tissue water potential on formation of microsclerotia.—Infected tissues were adjusted to different ψ values ranging from -5.8 to -98 bars and buried in soil initially adjusted to a ψ value of -0.8 or -27.5 bars. The materials and methods were essentially the same as those already described for infected tissues except that the infected stems were cut crosswise into 2-mm-thick slices. Desired tissue ψ values were attained by air-drying freshly sliced tissue for periods ranging from 0 to 1 hr. Periodically, samples of tissue were enclosed in plastic bags and ψ values were determined psychrometrically. Portions of tissue equivalent to 2 g of initial fresh weight were mixed with soil and placed in 20-ml vials at 24 C for 15 days, as described before. Each treatment was replicated three times. Soil samples with and without noninfected tissues also were included in the experiment.

To determine whether the air-drying of tissue affected the viability of the fungus, triplicate 4-g (initial fresh weight) samples of tissue were homogenized in sterile-distilled water in a Waring Blendor and assayed for total numbers of propagules of *V. dahliae* after each drying period. Nondried tissue ($\psi = -5.8$ bars) yielded the greatest number of colonies. Numbers of colonies obtained from tissue dried for up to 1 hr were reduced by 2-36%, but the reductions were not correlated with the drying time.

Total bacterial and fungal populations in tissues incubated in the two soils were determined at the end of the incubation period. Samples of tissue (with some adhering soil) were homogenized in sterile-distilled water, serial dilutions were made, and 0.5-ml portions were plated on King's Medium B, acidified PDA, and PDA plus 150 μg/ml streptomycin sulfate and 150 μg/ml of chlorotetracycline HCl.

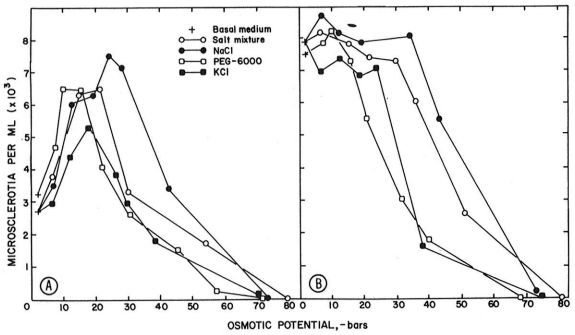

Fig. 2-(A, B). Influence of osmotic potential on the production of microsclerotia by *Verticillium dahliae* grown on quartz sand moistened with potato-dextrose broth amended with four different osmotica. The results of two experiments are shown in which the numbers of microsclerotia produced on nonamended basal medium were relatively **A)** low or **B)** high.

RESULTS

Osmotic potential and radial growth on agar media.—The minimum ψ_s value that allowed radial growth on agar media was between -100 and -120 bars (Fig. 1-A); growth was reduced by 50% at -50 to -70 bars. A marked increase in radial growth occurred when ψ_s was decreased from -2 bars to values of -10 to -20 bars, although the magnitude of the increase and the range of ψ_s values over which the increase was observed varied with the different osmotica (Fig. 1-A).

Osmotic potential and conidial germination, mycelial growth, and sporulation in liquid media.—Germination of conidia was examined in liquid-shake cultures using modified Eckert's broth amended with the mixture of salts to adjust ψ_s. The rate and total percentage of germination observed during the first 24 hr of incubation decreased with decreasing ψ_s value (Table 1). Determinations of germination at ψ_s values equal to or less negative than -53.5 bars were not possible after 24 hr owing to mycelial growth and the production of new conidia. At $\psi_s = -120$ bars, no conidia germinated during the first 3 days of incubation and after 4 and 6 days only about 2 and 5%, respectively, of the conidia had germinated (Table 1).

Germinating conidia produced one, or occasionally two, germ tubes which developed into short, branched hyphae. Hyphae originating from a number of different conidia often were aggregated to form mycelial "balls" which increased in size during the incubation period and produced new conidia at the tips of simple (nonverticillate) conidiophores.

Fungal growth in liquid media was measured as dry weight accumulation after incubation for 10 days. The ψ_s value of the basal medium was adjusted using four different osmotica. The results, summarized in Fig. 1-B, show that dry weight (DW) decreased in a nearly linear manner with decreasing ψ_s values from -2 to -120 bars, regardless of the osmoticum used (regression equation:

DW = 91.02 + 0.765 ψ_s, r = 0.971).

Counts of conidia from the same liquid-shake cultures that were used for dry-weight determinations were made at the end of 10 days of incubation. The results, which are summarized in Fig. 1-C, show that production of conidia increased logarithmically as the ψ_s values were decreased from −2 to about −20 bars and remained nearly maximal down to about −50 bars. Few or no conidia were produced at ψ_s values more negative than −100 bars. The general effect of decreasing ψ_s on the production of conidia was similar for all osmotica tested, although the magnitude of increases induced by changing ψ_s from −2 to −50 bars varied significantly among osmotica (Fig. 1-C). Production of conidia over time was examined in the liquid-shake cultures that were used for determinations of conidial germination (ψ_s adjusted with the mixture or salts). At ψ_s values equal to or less negative than −53.5 bars, maximum yield of conidia was reached by the 4th day and no significant changes occurred during the remainder of the 10-day incubation period. At $\psi_s = -120$ bars only a few conidia were produced by the 10th day.

Concentration of conidial inoculum and production of microsclerotia.—Conidial inoculum was added at logarithmically increasing concentrations, ranging from 10^0 to 10^6 conidia/ml of medium (PDB) used to moisten the sand. After incubation for 15 days at 24 C, the production of MS was assessed in five replications of each treatment. Statistically equal ($P = 0.05$) numbers of MS were produced by inoculum concentrations ranging from 10^2 to 10^6 conidia/ml. The numbers of MS formed at inoculum concentrations of 10^0 and 10^1 conidia/ml were 3.7 and 66%, respectively, of the numbers that were produced at 10^2 conidia/ml. Based on these results, an inoculum concentration of 10^5 conidia/ml was used in all subsequent experiments.

Length of incubation period and production of microsclerotia.—Production of MS over time was examined at selected ψ_s values to determine the length of incubation period required for maximum MS production. The ψ_s of the basal medium was adjusted to −53.5 and −86.5 bars with a mixture of salts. After inoculation with 10^5 conidia/ml of liquid medium, the sand cultures were incubated at 24 C for 30 days. Production of MS was assessed at prescribed time intervals in five replications from each treatment. In unamended basal medium at $\psi_s = -2$ bars, formation of MS had begun after 3-4 days; numbers of MS increased until the 8th day, and from then on remained essentially unchanged. At −53.5 bars, formation of MS had begun after 5-7 days and was maximal after 13 days; no significant changes occurred during the remaining 17 days. No MS were formed at −86.5 bars, even after 30 days of incubation. Based on these results an incubation period of 15 days was used in the subsequent experiments.

Osmotic potential and production of microsclerotia.—The numbers of MS produced on sand moistened with basal medium varied greatly (within the range of 10^3 to 10^4 MS/ml) in experiments performed at different times under the same conditions, even though inoculum was derived from the same culture. Likewise, the effect of ψ_s on MS production varied between experiments and appeared to be correlated with the number of MS produced on the basal medium. This is illustrated by the results from two experiments in which the isolate 511-4 was used. In one experiment (Fig. 2-A), MS production on the unamended basal medium ($\psi_s = -2$ bars) was low, whereas in a second experiment (Fig. 2-B), MS production on the same medium was relatively high. In both cases, MS were produced at ψ_s values ranging from −2 to about −75 bars (Fig. 2-A, B). However, in the first experiment (Fig. 2-A), there was a marked increase in MS production as ψ_s was decreased from −2 to about −20 bars; the magnitude of the increase and ψ_s values involved depended somewhat on the osmoticum used. In the second experiment (Fig. 2-B), in which the number of MS produced on the unamended basal medium was higher than in the first experiment, there was no significant change in MS production when ψ_s values were decreased from −2 to about −30 bars by use of the salts as osmotica. When PEG-6000 was used, MS production was reduced

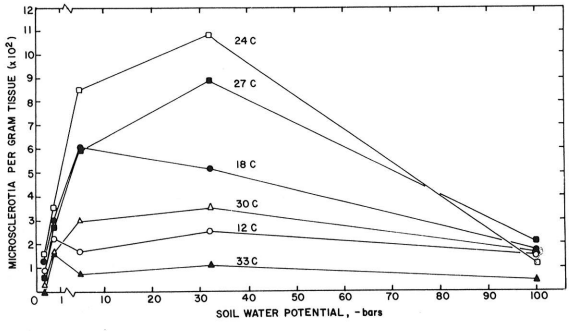

Fig. 3. Effect of soil water potential on formation of microsclerotia by *Verticillium dahliae* in infected tomato tissues buried in unsterilized soil and incubated at six different constant temperatures. The tissues had a water potential of −10 bars when placed in the soil and the production of microsclerotia was determined after 30 days of incubation.

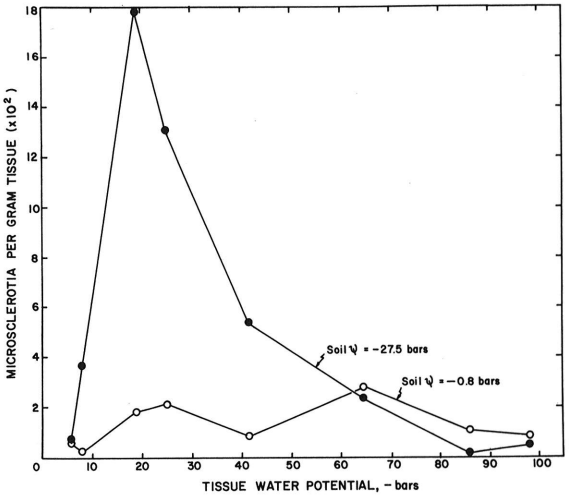

Fig. 4. Effect of the water potential of host tissue on the production of microsclerotia by *Verticillium dahliae*. Infected tomato stems were adjusted to the tissue water potentials shown and were buried in unsterilized soil at a water potential of −0.8 or −27.5 bars. The production of microsclerotia was determined after 15 days of incubation at 24 C.

at ψ_s values of less than −16 bars (Fig. 2-B). Results similar to those shown in both Fig. 2-A and 2-B were obtained using modified Eckert's broth as basal medium and a different isolate (45-1). A hyaline variant of a third isolate (726) produced no MS, regardless of the ψ_s values or the kind of osmoticum used.

The ψ_s value of the substrate on which MS were produced did not affect their viability. However, at ψ_s values of −70 to −80 bars, structures similar in size and morphology to MS occasionally were produced, but they had little or no pigmentation and failed to germinate after air-drying.

Temperature and production of microsclerotia.—The effect of temperature on production of MS was examined with two isolates (45-1 and 511-4) grown for 20 days on sand moistened with unamended PDB, under constant temperatures ranging from 6 to 33 C. Both isolates responded similarly; sparse mycelial growth occurred at 6 and 33 C but no MS were formed. At 9, 12, 15, 18, 21, 27, and 30 C, MS production was 15, 24, 35, 69, 88, 93, and 45%, respectively, of the maximum, which occurred at 24 C.

Influence of soil water potential and temperature on formation of microsclerotia in infected host tissues.—The total numbers of MS determined after 10 days of incubation were essentially the same as those determined after 30 days in all treatments, except in soils incubated at 12 C wherein MS numbers increased slightly between the 10th and 30th day. The results from the 30-day assay, which are summarized in Fig. 3, show that some MS were produced under all temperatures and values of soil ψ tested, except saturated soil (ψ = −0.35 bar) at 33 C. However, relatively small numbers of MS were produced in saturated soil at all temperatures used (Fig. 3). The average number of MS produced at all temperatures in soil at field capacity (ψ = −0.75 bar) was 2.8 times the number produced in saturated soil. The production of MS at ψ values less than −0.75 bar was more temperature-dependent. Temperatures of 12 and 33 C evidently limited the production of MS at all values of soil ψ, whereas at near-optimal temperatures (24 and 27 C) the production of MS was greatly increased at ψ values less than −0.75 bar and was maximal at −32 bars. Increased production of MS at ψ values less than −0.75 bar also occurred in the 18 and 30 C temperature treatments, but production was maximal at ψ = −5 bars. In soil at ψ = −100 bars, the production of MS was low at all temperatures tested (Fig. 3).

The numbers of MS recovered from pieces of undecomposed debris (residue collected on the 125-μm sieve) in the various soil ψ-temperature treatments were always about 20% of the respective total numbers of MS. The residue arrested by the 38-μm sieve invariably yielded the highest numbers of MS; these varied from 60 to 80% of the respective total numbers of MS in each treatment. The numbers of MS recovered in the fraction washed through the 38-μm sieve were relatively low and varied from 0 to 20% of the respective total numbers of MS in the different treatments. More of the MS produced in soil at low ψ values (−32 and −100 bars) and at either low (12-18 C) or high (30-33 C) temperatures passed through the 38-μm sieve as compared with MS produced in soil at higher ψ values and 24-27 C.

No MS were detected in soil samples with or without noninfected tissues. Soil samples containing noninfected tissues also were tested at the end of the 30-day incubation period to determine possible changes in ψ values. No significant changes were detected in treatments with soil initially at ψ = −0.35 to −5 bars. However, there were appreciable changes in treatments with soil initially at ψ values of −32 and −100 bars; these ψ values generally increased during the experiment (i.e., from −32 to an average value of −23 bars and from −100 to an average value of −65 bars). These increases probably were caused by the transfer of water from the relatively wet tissue to the dryer soil.

Influence of water potential of host tissues on formation of microsclerotia.—In this experiment, infected host tissues were dried to different ψ values and buried in soil initially at a ψ value of −0.8 or −27.5 bars. The water status of the tissue had a marked effect on the production of MS when tissues were buried in soil at ψ = −27.5 bars (Fig. 4). Decrease of tissue ψ from −5.8 to −18.7 bars resulted in about a 25-fold increase in the number of MS produced. However, adjustment of ψ of tissue to values lower than −18.7 bars resulted in a marked decrease of MS production and at −86 bars the production of MS was negligible. The numbers of MS produced in tissues buried in soil at ψ = −0.8 bar were low (about 20% of those in soil at ψ = −27.5 bars) regardless of the initial water status of the tissue (Fig. 4). No MS were detected in soil samples with or without noninfected tissues.

Attempts to separate tissue pieces from the soil to determine the ψ of tissues at the end of the experiment were unsuccessful because the tissues were partially decomposed and difficult to separate from the surrounding soil (especially tissues with high initial ψ values). However, from visual inspection, it appeared that relatively moist tissues (ψ = −5.8 and −7.9 bars) buried in dry soil (ψ = −27.5 bars) were surrounded by zones of moist soil. Apparently water had moved out of the moist tissues into the adjacent dryer soil. All tissues in soil at ψ = −0.8 bar were relatively moist at the end of the experiment.

All tissues buried in soil at ψ = −0.8 bar were colonized heavily with bacteria (average about 8.4×10^8 colonies per g of tissue). Equally high numbers of bacterial colonies (about 7×10^8 per g of tissue) were recovered from moist tissues (initial ψ = −5.8 and −7.9 bars) buried in soil at ψ = −27.5 bars. However, in the latter soil, bacterial populations decreased logarithmically with decreasing ψ; for example, in tissues dried to ψ values of −18.7, −25, and −41.5 bars, the bacterial populations were about 5×10^5, 3×10^5, and 4×10^4 colonies/g of tissue, respectively, and almost no bacterial colonies were recovered from tissues dried to ψ values more negative than −64.5 bars. Tissues with high bacterial populations yielded only a few fungal colonies and most of them were *Fusarium* spp. High numbers of fungal colonies (in the range of 10^4 to 10^6 colonies/g of tissue) were recovered only from tissues dried to ψ values less than −18.7 bars and buried in soil at ψ = −27.5 bars. *Fusarium* spp. were isolated most frequently from tissues at ψ = −18.7 to −41.5 bars, but in tissues dried to lower ψ values the fungal microflora consisted primarily of species of *Penicillium* and *Aspergillus*.

DISCUSSION

The results of this study show that *V. dahliae* can grow, sporulate, and form MS under relatively dry conditions. Radial growth and production of conidia were even increased when ψ_s was reduced from −2 to about −20 bars (Fig. 1-A, C). A similar increase in radial growth rate also was observed by Manandhar and Bruehl (19), but not by Mozumder and Caroselli (22). The latter authors (22) reported a linear decrease of radial growth on agar and in growth measured as dry weight increase in liquid media with decreasing ψ_s. Results similar to those of Mozumder and Caroselli (22) were obtained in the present study, but only when growth was measured as dry weight increase in liquid medium (Fig. 1-B).

The decreasing rates of conidial germination observed here with almost all decreases in ψ_s value (Table 1) are similar to those reported by Mozumder et al. (23). However, in the present study there was a low percentage of germination even at ψ_s values as low as −120 bars after incubation for 3-6 days. The lowest ψ_s value used by Mozumder et al. (23) was about −45 bars (calculated from their a_w value).

The role of conidia in the biology of *V. dahliae* is largely unknown. However, in infected plant tissues, the fungus spreads in the vascular system by means of conidia (11). Therefore, the ψ of the xylem fluid may affect the number of conidia produced and thus the rate of systemic invasion and disease development. Although the exact influence of hydrostatic pressure is not known, negative hydrostatic pressures in xylem vessels may have an effect on conidium production similar to that of more negative ψ_s values (Fig. 1-C).

Minimum ψ_s values allowing production of MS in vitro were −70 to −80 bars (Fig. 2-A, B); at these ψ_s values radial growth and production of dry weight were reduced by only 60 to 65% (Fig. 1-A, B). Evidently, production of MS is more sensitive to decreases in ψ_s than is mycelial growth. The occasional production of MS-like structures without pigment at −70 to −80 bars suggests that synthesis of melanin may be inhibited at these low ψ_s values.

The production of MS by *V. dahliae* in vitro is quite variable (25) and the effect of decreasing ψ_s on production of MS appeared to be determined in part by the number of MS produced on the nonamended basal medium (Fig. 2-A, B). When the number of MS produced on the basal medium was relatively low, decreasing ψ_s from −2 to about −20 bars usually resulted in increased MS production. However, if the number of MS produced on the nonamended basal medium was high (6,000-10,000 per ml), reductions in ψ_s usually resulted in relatively little or no increase in MS production. Perhaps in the second case, a near-maximum capability for production of MS had been attained on the basal medium. If so, the potential for enhanced production by alteration of ψ_s would be negligible.

Of the various salts and salt mixtures used, NaCl always induced the greatest increase in MS production and was less deleterious to MS production at the higher concentrations than was KCl. The effect of a mixture of salts containing NaCl, KCl, and Na_2SO_4 on MS production was intermediate between NaCl and KCl. This suggests that the different ions may have specific effects in addition to the osmotic effect common to all the salts used. Addition of PEG-6000 as an osmoticum generally caused a greater inhibition of MS production than the salt solutions at the same ψ_s values (Fig. 2-A, B). Mexal et al. (20) have shown that solubility and diffusion of O_2 is very low in PEG solutions. Thus, reduced availability of O_2 (16) may have contributed to the influence of PEG-6000 on MS production.

Although soil ψ is primarily due to matric forces (6, 7), ψ_s may be a significant component of the ψ of decomposing plant tissues. The range of soil and tissue ψ values over which the production of MS in infected tissues was increased (Fig. 3, 4) was similar to the range of ψ_s values that sometimes greatly stimulated the production of MS in vitro (Fig. 2-A). Thus, decreased ψ_s of tissue may have been primarily responsible for the stimulation in MS production observed at low soil and tissue ψ values (Fig. 3, 4). However, as pointed out by Sewell (28), the responses of soil fungi to changes in soil moisture seldom are attributable solely to the direct effect of water on the fungus of concern. Inadequate gas exchange, resulting in depletion of O_2 and increase in CO_2 concentrations, probably was the primary cause of the inhibition observed in saturated and very wet soils (Fig. 3, 4). Both low O_2 and high CO_2 concentrations have been shown to inhibit the production of MS (16). Slices of moist tissue (−5.8 and −7.9 bars), buried in "dry" soil (−27.5 bars), were surrounded by zones of moist soil. Thus, in this case, too, inadequate gas exchange between these moist spheres and the bulk of the soil may have contributed to the inhibition of MS production (Fig. 4). In soil enclosed in vials with only a small opening, the role of aeration may have been considerably greater than would exist in nonconfined soils of comparable moisture content. Also, the relatively large amount of plant tissue incubated in these soils may have resulted in greater production of CO_2 and depletion of O_2 than would occur under most field conditions.

Another factor that must be considered in the soil experiments is the possible effect of antagonistic or competitive microflora. We reported elsewhere (16) that the numbers of MS produced in infected tomato stems incubated aseptically were four times greater than were those produced during incubation in the presence of soil microflora. Similar results have been reported by Brinkerhoff (3). Although the nature of the interaction with soil microflora was not determined in the experiments on MS production, 13 out of 15 antagonists of *Verticillium* isolated from tomato roots by Kerr (18) were bacteria, mainly *Pseudomonas* spp. Furthermore, an unidentified bacterium has been associated with fungistasis of MS of *V. dahliae* in soil (5). Therefore, the large bacterial population present in the relatively wet treatments (Fig. 3, 4) may have contributed to the inhibition of MS production at high soil and tissue ψ values. Similarly, the increased MS production in the lower ψ treatments (Fig. 3, 4) may be explainable, in part, by the decreased activity of bacterial antagonists under these conditions (6).

The production of even a few MS in soil at $\psi = -100$ bars (Fig. 3) obviously is in contrast with the results from osmotic potential experiments in which the lowest limit for any MS production was −70 to −80 bars (Fig. 2-A, B).

This apparent contradiction is explainable by the fact that the host tissue at the time of incorporation had a ψ value of -10 bars. At the end of the experiment (Fig. 3) the ψ value of the soil-tissue mix from the driest soil treatment was about -65 bars. It seems probable that, even though the soil was initially at $\psi = -100$ bars, MS were actually formed in tissues at $\psi = -65$ bars or higher. Similarly in soil with an initial ψ value of -32 bars (Fig. 3), MS were actually formed in tissues at $\psi = -23$ bars, or higher.

In tomato fields, MS of *V. dahliae* are produced at the end of the growing season when the infected vines are incorporated into the soil (N. Ioannou, *unpublished*). In California, irrigations of mechanically harvested tomatoes usually are discontinued several weeks before harvest. Thus, soil moisture at time of harvest probably is always within the range favorable for MS production (Fig. 3). After harvest, the vines usually are left on the soil surface for several days before they are plowed under. Thus, unless the vines desiccate to values less than -80 bars, loss of tissue moisture during this period may enhance the production of MS (Fig. 4). The results of this study suggest that alteration of cultural practices which affect the water status of soil and plant debris, both before and after the vines are plowed under, could provide a means for reduction of inoculum density in soils. Conceivably, flooding soil immediately after the infested residues are incorporated would inhibit the formation of MS.

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