Use of a Host-Pathogen Interaction System to Test Whether Oxalic Acid is the Sole Pathogenic Determinant in the Exudate of *Sclerotinia trifoliorum*

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ABSTRACT

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A host-pathogen interaction system was used to test the hypothesis that oxalic acid is the sole pathogenic determinant in the exudate of *Sclerotinia trifoliorum*. The system allowed exposure of germinating alfalfa seedlings (*Medicago sativa*) to continuously produced fungal exudate without physical contact with the fungus. Blockage of diffusion of macromolecular components (>3,500 mol wt) of the exudate without alteration of oxalic acid levels reduced the observed inhibition of alfalfa radicle length by 40–50%. Such results indicate that in this system oxalic acid is not the sole inhibitory factor and that other, yet unidentified, macromolecular components share a codeterminant role in the observed inhibitory effect. The techniques described should be generally applicable to characterization of exudates of other pathogenic fungi or bacteria that are cultured on solid media, while allowing flexibility in design of pertinent bioassays.

Additional keywords: electroelution of proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Sclerotinia trifoliorum Eriks. is a major fungal pathogen of forage legume crops such as alfalfa (*Medicago sativa*) and clover (*Trifolium* sp.) (7). The general inability to develop germ plasm of either alfalfa or clover with resistance to this pathogen and related *Sclerotinia* species has focused attention on the need for more detailed characterization of the pathogenic determinants involved in disease development. Extensive work (4) has documented the importance of oxalic acid production by *Sclerotinia* sp. as a factor in pathogenesis; however, the role of other factors, such as fungal extracellular enzymes, remains controversial. In contrast to earlier studies (6), a codeterminant role for oxalic acid and several extracellular enzymes has been suggested (2,5,11,12).

The relative importance in pathogenesis of oxalic acid has recently been reassessed by production of mutants of *Sclerotinia* specifically lacking the ability to synthesize oxalic acid (3). These oxalic acid minus mutants were nonpathogenic in bioassays with *Phaseolus vulgaris*, yet produced equivalent, if not elevated, levels of pectin methyl esterase, polygalacturonase, and cellulase as deduced from enzyme activity measurements. Although the results of this approach strongly suggest a primary role for oxalic acid in *Sclerotinia* pathogenesis, the possibility that production of other

unidentified extracellular enzymes (or other factors) was altered in the mutants was not excluded. Moreover, germ plasm selection techniques using oxalic acid alone as a screening agent have failed to yield alfalfa germ plasm with resistance to *S. trifoliorum* (9). Thus, the basic question of whether oxalic acid produced by *Sclerotinia* species is the sole determinant in pathogenesis has not been conclusively resolved.

In this report, we describe a novel approach designed to test whether factors other than oxalic acid in the exudate of *S. trifoliorum* contribute to pathogenesis. This host-pathogen interaction system (14), with modifications as described, allowed analysis of effects of fungal exudate components on alfalfa seedlings without physical contact of the seedlings with the continuously growing fungus. Our results argue against oxalic acid as the sole active determinant in this system. The techniques described should be generally applicable to characterization of exudates of other pathogenic fungi or even bacteria that are cultured on solid media, while allowing flexibility in design of pertinent bioassays.

MATERIALS AND METHODS

Fungal isolate and plant material. A highly virulent (8) isolate of *S. trifoliorum* (collected from alfalfa and kindly provided by Dr. R. G. Pratt, USDA-ARS, Mississippi State, MS) was routinely maintained on 1.7% cornmeal agar plates at 15 C with transfer to new plates at 2-wk intervals. Seeds of alfalfa (*M. sativa* 'Moapa-69') were used in bioassays described below after surface sterilization by orbital shaking in 90% ethanol for 10 min, in 1% sodium hypochlorite containing 0.05% Tween-20 for 10 min, and in sterile water (four rinses). Maintenance of cultures and bioassays were done in nonilluminated, controlled temperature chambers.

Host-pathogen interaction system (HPIS). The concept and physical description of the two-sided petri plate system (Lutriplate Inc., Starkville, MS) used to expose a host to a fungal pathogen exudate without physical contact to the growing fungus were as previously published (14). However, details of assembly of the system as applicable to work with *Sclerotinia* are provided because they vary significantly from the original report (14). Various media used in this study for growth of *S. trifoliorum* on one side of the HPIS were as follows: cornmeal agar (CMA) at 1.7%; V8 agar (20% V8 juice in 2.0% Difco agar); Schenk and Hildlebrandt agar (SH) as previously described (10) but without growth regulators; and potato-dextrose agar (PDA) at 3.9%. The fungal growth media were autoclaved (20 min at 121 C and 100 kPa), then poured into one side of the double-sided petri plate to a depth of 5 mm (31 ml). The temporary center support on which the media solidified was carefully removed, a thin layer of nontoxic cement (Testor Corp., Rockford IL) was applied to the inner retaining ring, and a precut autoclaved polycarbonate membrane filter (0.2-μm porosity, Nucleopore, Pleasanton, CA) was then affixed to the retaining ring. Plates were left overnight to ensure solid bonding of the filter with the retaining ring as required for complete restriction of fungal mycelia to the fungal compartment. The opposite side of the plate was then filled with 31 ml of autoclaved 1.2% agarose (Ultrapure, Bethesda Research Laboratory, Gaithersburg, MD).

The fungal side of the plate was inoculated with a 7-mm plug of *S. trifoliorum* from cultures maintained as described above. The lid was added and sealed from the underside with weather strip caulking cord (Mortell Company, Kankakee, IL). The edge of both lids were then wrapped in Parafilm. Plates were incubated for 72 h at 15 C to initiate growth of the fungus before bioassays (described below) and are referred to as preinoculated.

As a modification to the system so far described, we used an additional membrane filter (3,500 mol wt cutoff, SpectraPor, Los Angeles, CA) to restrict not only the fungus to one side of the plate but also certain macromolecular components (>3,500 mol wt) of the fungal exudate from diffusion to the bioassay side. This dialysis-type membrane was sterilized by autoclaving, rinsing in 70% ethanol, and washing in sterile water (5 min). It was then simply placed over the bonded polycarbonate filter (0.2-μm porosity), forming a reservoir to hold the subsequently added agarose solution.

Bioassay of alfalfa seedlings. To quantify effects of the diffusible fungal exudate, a bioassay was devised using germinating alfalfa seedlings. Fifty surface-sterilized seeds were placed on the agarose side of preinoculated and noninoculated control plates. After 7 days of incubation at 25 C, radicle length of germinated seedlings was measured for each plate. Each analysis was replicated four times.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extracellular fungal proteins. Extracellular proteins that were produced by *S. trifoliorum* and diffused to the agarose side of the HPIS were electroeluted from 13-mm-diameter agarose plugs using a standard device (Model 1750, Isco Inc., Lincoln, NB). The buffer systems used were 12.5, 4, and 1 mM Tris-glycine at pH 8.0 containing 0.02% SDS in the tank's outer, inner, and sample compartments, respectively. Typically, four plugs per sample cup were electroeluted and concentrated at 50 V for 20 min followed by 100 V for 3 h. The voltage polarity was reversed for 3 s before the collection of a concentrated sample. Agarose plugs from equivalent but noninoculated plates were carried through the same electroelution protocol. Collected samples were mixed with SDS sample buffer and heated for 3 min at 95 C before analysis by SDS-PAGE on 10–20% polyacrylamide gradient gels. Gels were stained with either Coomassie Blue R-250 by standard procedures or silver (15). Where indicated, stained gels were scanned with a video densitometer (BioMed Instruments, Fullerton, CA).

Other techniques. Changes in pH of the host side of the HPIS resulting from diffusion of the fungal exudate were made directly on the agarose surface with an Orion Model 91-36 flat surface electrode. To prevent contamination of the plates, the electrode was washed very briefly in 70% ethanol and quickly rinsed with sterile water just before each measurement. Each determination was the average of four readings taken at equidistant locations on the surface. Equivalent results were obtainable by remelting the agarose medium and measuring pH of the solution at a controlled temperature.

Determination of oxalic acid concentration in the agarose side of the HPIS after growth of the fungus on CMA was carried out by an enzymatic (oxalate oxidase) method (Sigma kit 591). The agarose medium of noninoculated and inoculated plates was removed from the HPIS and melted in the microwave. To obtain assay values within the linear range of standards, aliquots were diluted 20-fold in water at 50 C. Fifty microliters of such diluted samples were then used in the enzymatic assay (37 C, 5 min) along with standards of known concentrations. Negative controls included were malate and citrate.

RESULTS AND DISCUSSION

Characterization of the HPIS. The validity of our approach depended on the following criteria: complete restriction of the growing fungus to the pathogen side of the HPIS; unrestricted diffusion of fungal-produced exudate from the pathogen side to the host side of the HPIS; fractionation of the fungal-produced exudate within the HPIS to prevent diffusion of chosen size classes of exudate components (e.g., >3,500 mol wt) without affecting the diffusion of oxalic acid; a reliable, objective bioassay with sufficient sensitivity to test the relative effects of the unfractionated vs. fractionated exudate.

The results of Figure 1 represent an artificial test of the HPIS designed to determine whether known components are freely diffusible through the HPIS and whether they can be fractionated as described. Ovalbumin (43,000 mol wt) and bromophenol blue (400 mol wt) were added to one side of the HPIS as an agarose plug. After several days incubation, analysis of agarose medium taken from the opposite side of the HPIS revealed that both the dye and ovalbumin protein diffused through the system with the polycarbonate filter (0.2-μm porosity) as the center membrane barrier (I, Fig. 1A,B). Equivalent amounts of the low molecular weight dye diffused through the HPIS containing both the poly-

carbonate and dialysis (3,500 mol wt cutoff) filters (II, Fig. 1A), yet diffusion of the protein was completely blocked under this condition (II, Fig. 1B). These results show that the center membrane barriers are contiguous with the media of each side of the HPIS and can be employed to control the diffusion of substances of defined size class within the system.

The production of extracellular exudate components by *S. trifoliorum* and the diffusion of these components within the HPIS were characterized in the next set of experiments. The acidification of the host side of the HPIS, which was expected due to the well documented (4) capacity of the fungus to excrete oxalic acid, was a convenient and pertinent parameter for estimation of exudate production and movement in the system. We first compared several different growth media for their effects on growth rate and production of diffusible exudate (Fig. 2). In all cases we found that the polycarbonate filter was completely effective in restricting the rather invasive fungus to the pathogen side of the HPIS. The data revealed that PDA and V8 agar supported more rapid growth rates (half-time ~2.5 days) as compared to CMA and SH agar (half-time ~3.7 days). Growth of the fungus on all media was correlated with decreases in pH in the opposing agarose side of the HPIS (Fig. 2) indicating rapid diffusion of fungal exudate through the polycarbonate filter barrier. No changes in pH were observed for noninoculated control plates beyond initial equilibration with the pH of the individual growth media (Fig. 2, inset).

Based on known pK_a values for oxalic acid, we estimated that oxalic acid concentrations of at least 1–2 mM would be necessary if the maximum pH changes observed (Fig. 2) were primarily due to oxalic acid. Specific determination of oxalic acid in the host side medium (agarose) by an enzymatic method (Materials and Methods) revealed that oxalic acid concentration reached 6.0 mM (SE = 0.9 mM, $n = 4$) after 10 days growth of the fungus on CMA. Considering the minimal buffering capacities of either CMA or agarose (data not shown), such levels of oxalic acid suggest that it was the primary acidic metabolite responsible for the observed pH changes.

Further characterization of the fungal exudate involved analysis for the presence of extracellular proteins. After growth of *S. trifoliorum* on the various media tested in Figure 2, polypeptides of both fungal and medium origin were electroeluted from agarose plugs of the host side of the HPIS and resolved by SDS-PAGE. Typical results of such analyses are shown in the silver-stained gel of Figure 3. By strict comparison to noninoculated plates, it is clear that several polypeptides were associated with the fungal exudate. We noted both qualitative (bands 2 and 5, Fig. 3) and quantitative (bands 1, 3 and 4, Fig. 3) differences in the exudate proteins depending on the growth medium used. Although the silver-staining technique revealed the total spectrum of exudate proteins, the most prominent bands were the 23-kDa protein (band 4, CMA), and the 34- and 74-kDa proteins (bands 3 and 1, both V8 and SH). Their higher relative abundance in the exudate was evident on duplicate gels (not shown) that were stained with Coomassie blue instead of silver. These same patterns of extracellular protein components were observed for other independent field isolates of *S. trifoliorum* including two from the American Type Culture Collection (46758 and 52584) (data not shown).

The ability to specifically block diffusion of such macromolecular components in the exudate without affecting diffusion of oxalic acid is shown in Figure 4. After growth on CMA, both polypeptide profiles (A) and pH (B) of the opposing agarose medium were determined for plates that contained the polycarbonate filter either with (II) or without (III) the dialysis filter.

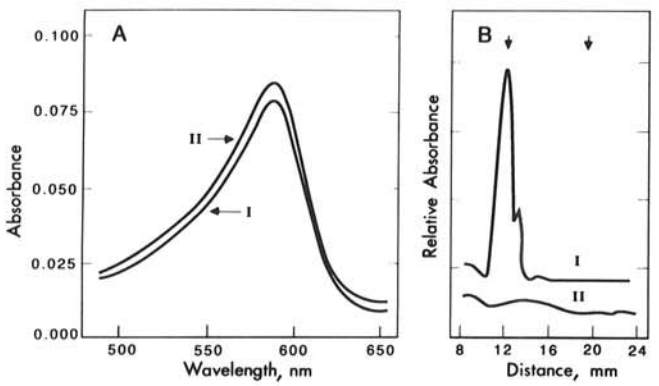

Fig. 1. Control of diffusibility of added components through the host-pathogen interaction system (HPIS). A noninoculated HPIS was constructed with agarose (1.2%) on both sides of the center membrane barrier. The membrane consisted of the polycarbonate (0.2-μm porosity) filter either alone (I) or in conjunction with the dialysis (3,500 mol wt cutoff) filter (II) as described in Materials and Methods. A 10-ml plug of agarose (1.2%) containing 0.3% ovalbumin (Sigma, grade V) and 0.03% bromophenol blue dye was placed on the surface of one side of the system. After 7 days of incubation as described for inoculated plates (Materials and Methods), plugs of agarose from the opposite side of the membrane barrier were analyzed for relative levels of bromophenol blue by direct scanning in a Perkin-Elmer lambda 6B spectrophotometer (Perkin-Elmer, Norwalk, CT) (**A**) and for ovalbumin protein by electroelution and SDS-PAGE (Materials and Methods) as shown by densitometric scans of the pertinent region of the minigel (**B**). Verticle arrows denote the positions of standards (BioRad, Richmond, CA) of 42.7 (ovalbumin) and 31.0 (carbonic anhydrase) kDa resolved in the displayed region of the gel (B).

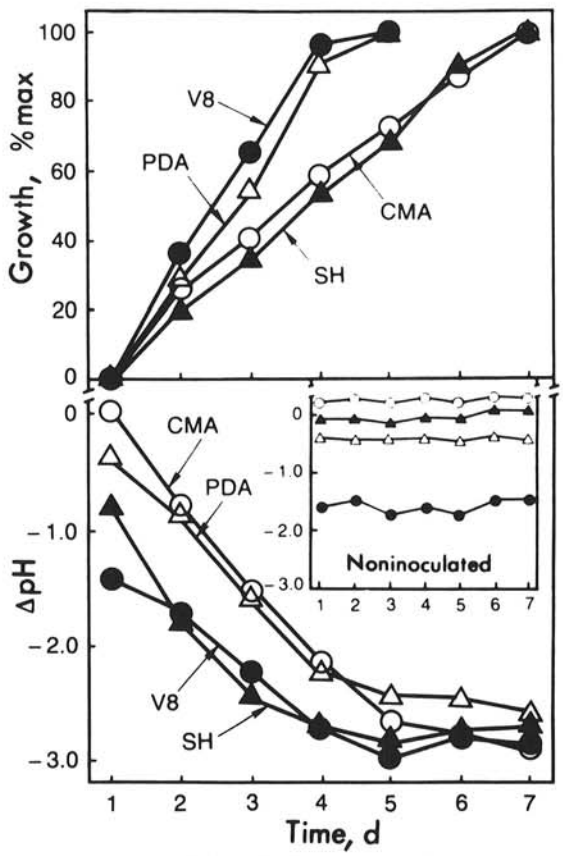

Fig. 2. Growth characteristics of *Sclerotinia trifoliorum* and associated changes in pH of the host side of the host-pathogen interaction system (HPIS). Plates containing the indicated growth media were prepared as described in Materials and Methods using the polycarbonate (0.2-μm porosity) filter as the center barrier between growth medium and agarose sides. After inoculation of plates, measurements of mycelial radius (maximum 4.1 cm) and changes in pH of the opposing (agarose) side of the HPIS were made at the indicated times. Values represent the average of three replicates. The standard errors for growth and pH measurements were in the range of 2.4–7.4% and 0.01–0.17 ΔpH, respectively, and were not shown for clarity. The inset represents equivalent pH measurements made on noninoculated control plates. The initial pH readings (day 0) of the individual growth media were as follows: 6.10, CMA; 4.40, V8; 5.80, SH; 5.60, PDA. The initial pH (day 0) of the opposing agarose medium was 6.0.

The dialysis filter completely blocked the diffusion of protein components to the agarose medium (II vs. III, Fig. 4A) but did not alter diffusion of oxalic acid as evidenced by similar changes in pH (II vs. III, Fig. 4B). Thus, the HPIS with the modifications as described represents a unique approach toward manipulation of a fungal exudate produced during continuous growth of the fungus on solid media.

Bioassay of fungal exudate within the HPIS. The relative effects of the diffusible, whole exudate vs. the fractionated exudate were tested in bioassays that were based on measurements of radicle length of germinating alfalfa seedlings (Materials and Methods). The relevance of such bioassays toward characterizing the toxicity of fungal exudate components has recently been demonstrated with red clover seedlings that were exposed to liquid culture filtrates of *Fusarium roseum* (1). In the following experiments, CMA served as the fungal growth medium. Presterilized seeds were placed on the host side of preinoculated and noninoculated plates. After 7 days of incubation, radicle lengths of the seedlings were measured and analyzed as shown in Figure 5. First, the uniformity of radicle lengths (~4.5 cm) obtained for the various noninoculated control plates (treatments 1,2,3A,4A, and 5A, Fig. 5) rules out the possibility of nonfungal effects from either the media (CMA vs. agarose) or the use of the various filters in the HPIS. The maximum possible inhibition of radicle length was gauged by comparison of noninoculated and inoculated plates, which lacked any membrane barrier for restriction of the fungus (3A vs. 3B, Fig. 5). Radicle lengths were reduced from 4.9 to 2.0 cm under this condition of direct seedling invasion by the fungus. Significant inhibitory effect on radicle growth was also observed when the seedlings were exposed to the whole exudate without contact to the fungus through the use of the 0.2-μm filter (4A vs. 4B, Fig. 5). If oxalic acid was the sole determinant involved in the inhibitory effects measured in this bioassay, then equivalent effects on radicle length would be observed for seedlings exposed to the fractionated exudate lacking larger macromolecular components. However, the results indicated that the extent of inhibition was reduced for seedlings exposed to the fractionated exudate (5A vs. 5B, Fig. 5).

The inhibitory effect of the whole fungal exudate was confirmed in several other cultivars of alfalfa (e.g., Apollo, Thor, Lahonton) using CMA as the growth medium (data not shown). Although the overall extent of inhibition varied with cultivar within a range of 40–70%, the decreased inhibition within any particular cultivar due to exclusion of the larger macromolecular components of the exudate was similar to that shown in Figure 5 (40–50% less inhibition). Alternately, inclusion of oxalic acid in the media of noninoculated plates in amounts sufficient to mimic the pH reached in inoculated plates inhibited radicle length significantly less than the whole exudate (D. E. Rowe, *unpublished*). Such results and those reported here lead us to conclude that oxalic acid is not the sole pathogenic determinant responsible for the inhibitory effects measured in the bioassay. Rather, the results suggest that oxalic acid and other macromolecular components of the exudate share a codeterminant role in the observed inhibitory effects on alfalfa seedlings. Future work on identification of these other active exudate components and demonstration of their role will further clarify the molecular basis of disease development induced by this fungal pathogen.

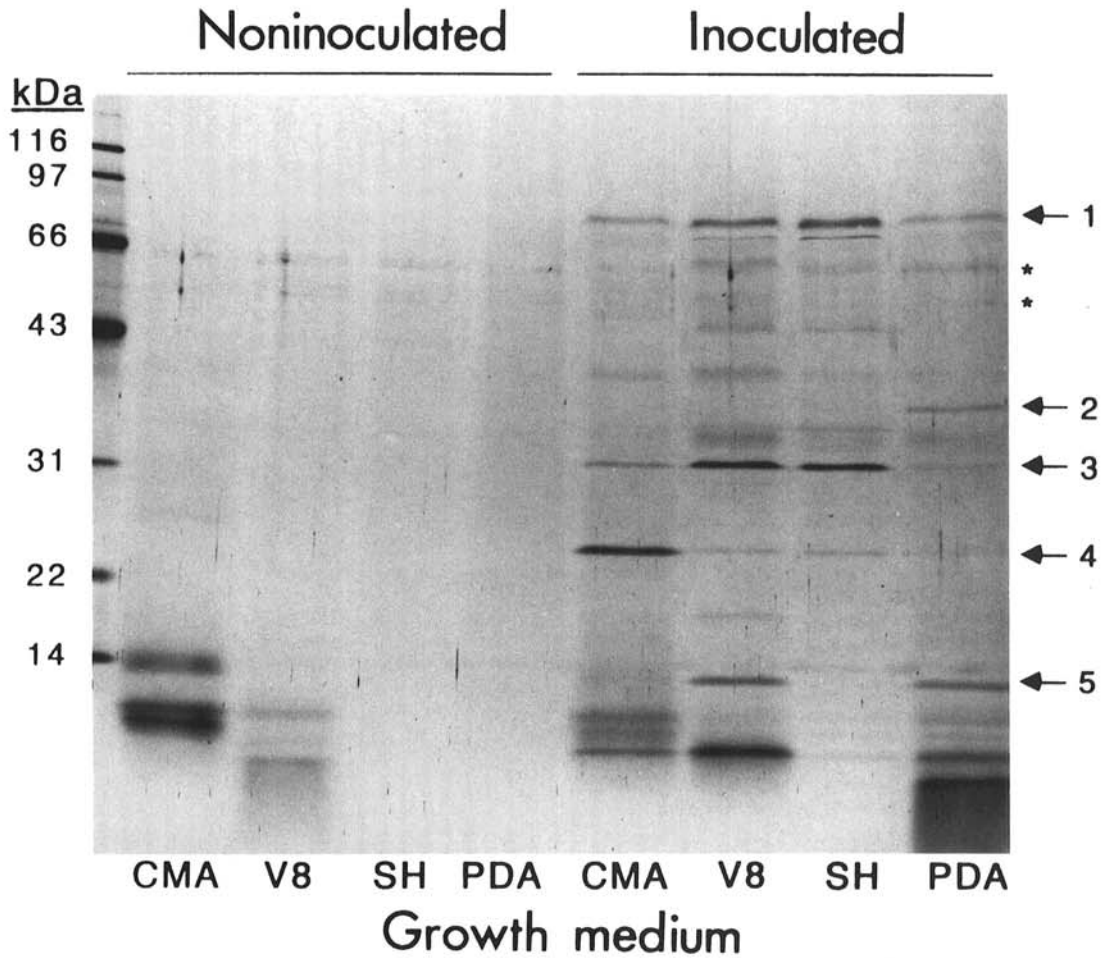

Fig. 3. Effect of growth medium on the spectrum of extracellular polypeptides produced by *Sclerotinia trifoliorum* in the host-pathogen interaction system. Noninoculated and inoculated plates as described in Figure 2 were incubated for 7 days. Agarose plugs from the opposing side of the polycarbonate (0.2-μm porosity) filter were analyzed for protein components by electroelution and SDS-PAGE (Materials and Methods). Protein standards (BioRad, Richmond, CA) of known molecular weight are shown on the left. Several fungal polypeptides are denoted by arrows on the right for reference in the text. Asterisks denote artifactual bands unrelated to the experiment but often observed in silver-stained SDS-PAGE gels (13).

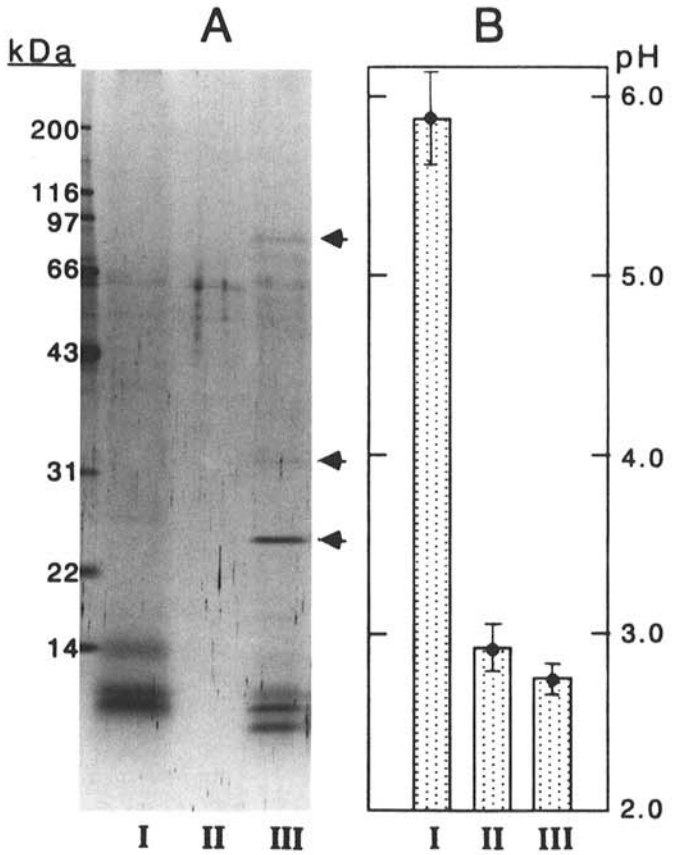

Fig. 4. Manipulation of diffusibility of fungal exudate components within the host-pathogen interaction system (HPIS). The HPIS plates contained cornmeal agar as growth medium. The center membrane barrier was the 0.2-μm filter either alone (I and III) or in conjunction with the dialysis (3,500 mol wt cutoff) filter (II). The noninoculated control (I) was carried through the same regime. Measurements were made after 10 days of incubation. **A,** SDS-PAGE analysis of polypeptides electroeluted from the agarose side of HPIS; **B,** pH measured on the agarose side of the HPIS. The arrows in A correspond to bands 1, 3, and 4 as denoted in Figure 3.

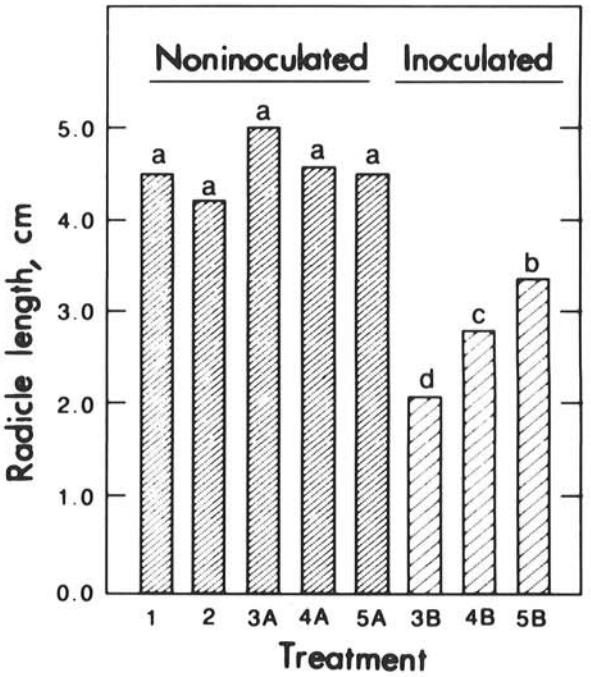

Fig. 5. Inhibitory effect of whole vs. fractionated fungal exudate on alfalfa seedling radicle growth. Bioassays of the effects of the diffusible fungal exudate on alfalfa seedling radicle length were as described in Materials and Methods. Cornmeal agar (CMA) was used as growth medium in the host-pathogen interaction system (HPIS). The experimental design was a balanced completely random design with eight treatments (fixed effects) and four replications. The analysis of variance indicated that variation among treatments had F values with $P < 0.001$ for measured radicle lengths. A separation of treatment means was performed using Duncan's multiple range test at $P = 0.05$ as indicated by letters above the bars. Bioassay treatments were as follows: 1 and 2, noninoculated regular petri plates containing CMA and agarose, respectively; 3A and 3B, noninoculated and inoculated HPIS plates but without any membrane barrier between pathogen and host sides (i.e., fungus unrestricted); 4A and 4B, noninoculated and inoculated HPIS plates with the 0.2-μm porosity filter as membrane barrier between pathogen and host sides (i.e., fungus restricted, whole exudate diffusible); 5A and 5B, noninoculated and inoculated HPIS plates with both the 0.2-μm filter and dialysis (3,500 mol wt cutoff) filter as membrane barriers between pathogen and host sides (i.e., fungus restricted, only exudate components less than 3,500 mol wt diffusible).

LITERATURE CITED

1. Blain, F., Bernstein, M., Khanizadeh, S., and Sparace, S. A. 1991. Phytotoxicity and pathogenicity of *Fusarium roseum* to red clover. Phytopathology 81:105-108.
2. Favaron, F., Alghisi, P., Marciano, P., Magro, P. 1988. Polygalacturonase isoenzymes and oxalic acid produced by *Sclerotinia sclerotiorum* in hypocotyls as elicitors of glyceollin. Physiol. Mol. Plant Pathol. 33:385-395.
3. Godoy, G., Steadman, J. R., Dickman, M. B., Dam, R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 37:179-191.
4. Lumsden, R. D. 1979. Histology and physiology of pathogenesis in plant diseases caused by *Sclerotinia* species. Phytopathology 69:890-896.
5. Marciano, P., Di Lenna, P., Magro, P. 1983. Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. Physiol. Plant Pathol. 22:339-345.
6. Maxwell, D. P., and Lumsden, R. D. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60:1395-1398.
7. Pratt, R. G., Dabney, S. M., Mays, D. A. 1988. New forage legume hosts of *Sclerotinia trifoliorum* and *S. sclerotiorum* in the Southeastern United States. Plant Dis. 72:593-596.
8. Pratt, R. G., and Rowe, D. E. 1991. Differential responses of alfalfa genotypes to stem inoculations with *Sclerotinia sclerotiorum* and *S. trifoliorum*. Plant Dis. 75:188-191.
9. Rowe, D. E., and Welty, R. E. 1984. Indirect selection for resistance to Sclerotinia crown and stem rot on alfalfa. Can. J. Plant Sci. 64:145-150.
10. Schenk, R. U., and Hildebrandt, A. C. 1972. Medium and techniques for induction and growth of monocotyledonous plant cell cultures. Can. J. Bot. 50:199-204.
11. Scott, S. W., and Fielding, A. H. 1982. Polysaccharide degrading enzymes of *Sclerotinia trifoliorum*. Trans. Br. Mycol. Soc. 78:166-170.
12. Scott, S. W., and Fielding, A. H. 1985. Differences in pectolytic patterns induced in *Sclerotinia trifoliorum* by different legume host species. Trans. Br. Mycol. Soc. 84:317-324.
13. Tasheva, B., and Dessev, G. 1983. Artifacts in sodium dodecyl sulfate-polyacrylamide gel electrophoresis due to 2-mercaptoethanol. Anal. Biochem. 129:98-102.
14. Tomaso-Peterson, M., and Krans, J. V. 1990. Evaluation of a new in vitro cell selection technique. Crop Sci. 30:226-229.
15. Wray, W., Boulikas, T., Wray, V. P., Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118:197-203.