The Host-Pathogen Interaction of Alfalfa and Stemphylium botryosum

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ABSTRACT

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The nature of the host-pathogen interaction of alfalfa and *Stemphylium botryosum* was investigated by histological means. In a resistant plant of checkered alfalfa *(Medicago cancellata),* growth of the pathogen was inhibited and restricted to the cells surrounding the infection site. The host response was characterized as hypersensitive. On a susceptible host *(M. sativa)* the pathogen grew luxuriantly and produced abundant typical Stemphylium leafspot symptoms. Toxins produced by *S. botryosum* play an important role in disease development and their relationship to resistance and susceptibility was established. On susceptible plants, a crude filtrate from a culture medium inoculated with *S. botryosum* produced symptoms identical to those incited by the fungus. The resistant host was unaffected by the filtrate. A positive correlation between toxin test scores and those obtained by inoculation with the pathogen permitted the use of toxin tests as an improved rapid screening technique for resistance.

Additional key words: toxins, hypersensitive reaction.

Stemphylium botryosum Wallroth, the conidial stage of *Pleospora herbarum* (Fries) Rabenhorst, has been reported causing disease in alfalfa *(Medicago sativa* L.) mostly in the eastern United States (3, 4, 8). The fungus attacks leaflets, petioles, stems, peduncles, flowers, pods, and seeds, causing defoliation and wilting. On leaflets the symptoms appear as punctate lesions, with light colored centers and brown margins. The lesions become larger and irregular in shape, and may coalesce to form extensive necrotic areas. The fungus is pathogenic to several other forage legumes.

Histological studies of the *S. botryosum*-alfalfa relationship were conducted by Smith (9). He reported that penetration of the germ tube was mainly through stomata, but may be directly between epidermal cells. Nelson (6), reported that a dense cluster of hyphae develops in the substomatal cavity following penetration, and dead areas free of mycelium occur in advance of and on both sides of the hyphae. He suggested that a toxin produced by the pathogen may be involved in the disease. A detailed study of the interaction (7), demonstrated that in nonsusceptible species the pathogen did not develop beyond the primary hyphae stage and damage to the cells was restricted to the area surrounding the primary hyphae. The toxins were believed to play a minor role in the development of the disease in this study.

The production of toxins by *S. botryosum* and their role in the development of disease has been studied recently (1) and at least three different toxins have been separated. One of these, 'Stemphylin' (2) produces brown necrotic spots on lettuce *(Lactuca sativa* L.) and some other species including *M. sativa.*

The present studies were undertaken to learn more about the nature of the resistance to *S. botryosum* in alfalfa. This was accomplished by histological studies of the interaction in susceptible and resistant alfalfa. These studies revealed the involvement of fungal toxins in the disease and their host-specificity was investigated in detail.

MATERIALS AND METHODS

Histological studies.—A resistant plant from the species *M. cancellata* (P.I. 315457) and a susceptible plant from *M. sativa* cultivars (SW 44) were selected for histological studies of the host response to the pathogen. A single-spore isolate of *S. botryosum,* strain WS-1, supplied by the Department of Plant Pathology, U.C. Davis, was used to produce inoculum. The fungus was cultured on V-8 juice agar and incubated for 3 weeks at 20 C under alternate periods of 12 hours of darkness and fluorescent light. The inoculum contained spores in suspension (7×10^4 spores/ml).

Detached leaves of the susceptible and resistant plants were floated on water containing 5% sucrose. The inoculum was sprayed with an atomizer to form small droplets on the upper surface of the leaves. The inoculated material was incubated at 20 C and 100% relative humidity (RH). Leaves were collected at 12, 24, 36, 48, 72, 96, 120, 148, 172, and 196 hours after inoculation for microscopic examination. Whole mounts were prepared following the procedure described by Pierre and Millar (7).

Fungal toxins.—A crude culture filtrate of *S. botryosum* was used to study the role of toxins in the disease and their relation to resistance. A single spore

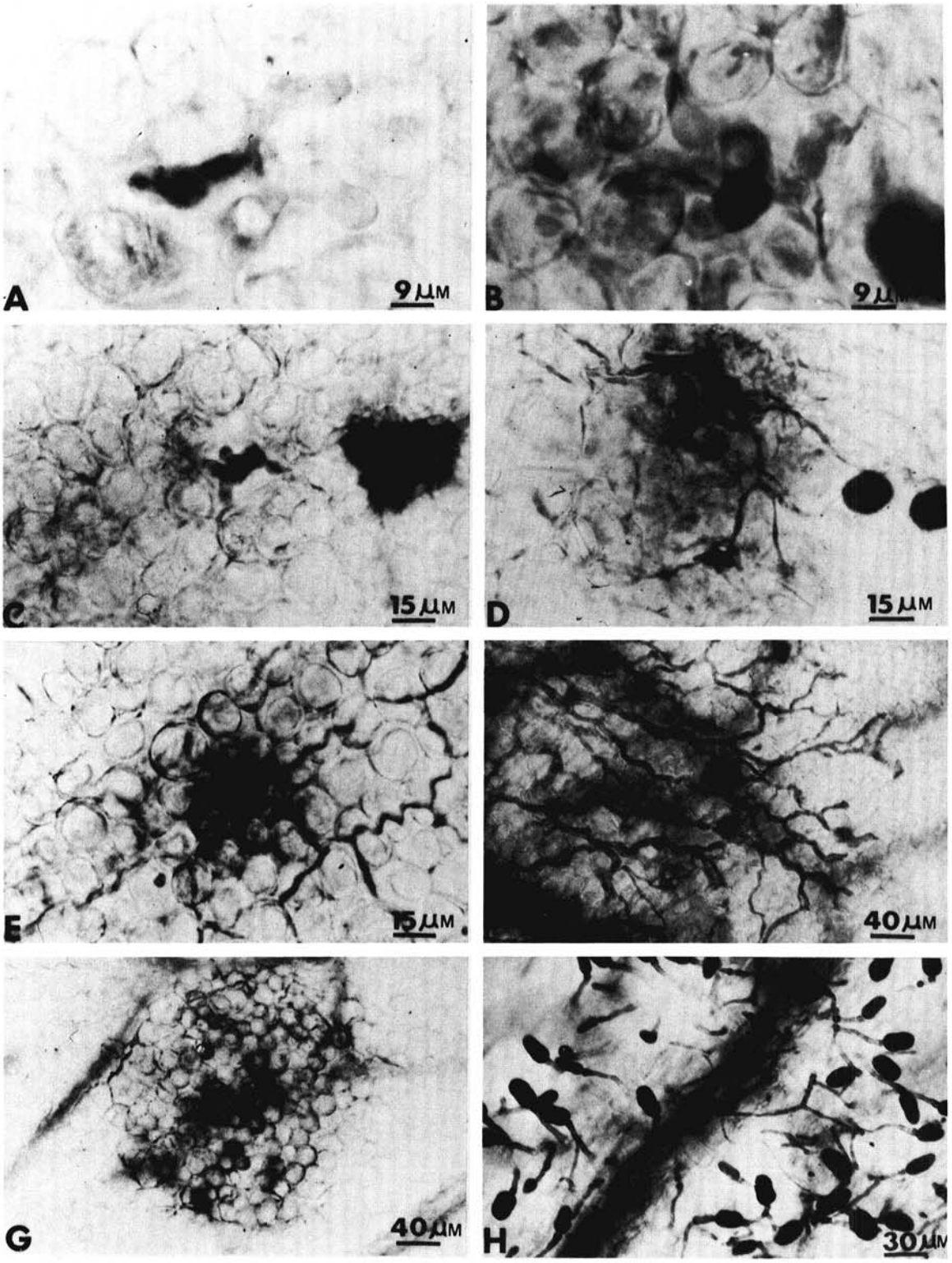

Fig. 1-(A to H). Postpenetration development of *Stemphylium botryosum* **A, C, E, and G)** in resistant host *(Medicago cancellata),* 24, 48, 96, and 196 hours after inoculation, respectively, and **B, D, F, and H)** in the susceptible host *(M. sativa,* 24, 48, 96, and 196 hours after inoculation, respectively.

isolate of *S. botryosum* was grown in 1-liter Roux bottles containing 200 ml of a liquid medium (2). The cultures were incubated without shaking for 24 days at 20 C.

After incubation the contents of 10 bottles were passed through four layers of cheesecloth and filtered using a Whatman No. 1 filter paper. The light-brown filtrate (750 ml) was concentrated to 45 ml by flash evaporation under reduced pressure at 45 C and sterilized by filtration through a Millipore filter (0.45 μm pore size). The same procedure was followed for the control which consisted of 750 ml of liquid medium not inoculated.

Excised leaves from alfalfa plants growing in pots in the greenhouse were used for toxin bioassays. The leaflets were placed in petri dishes with a moist filter paper in the bottom. A small needle puncture was made in the center of each leaflet and a drop of the test solutions was applied. The concentrated filtrate was diluted 1:1, 1:3, 1:15, 1:31, 1:63, 1:127, 1:255, 1:511, and 1:1023 (v/v) with sterile distilled water before application to susceptible and resistant hosts. The petri dishes were covered to prevent evaporation and were incubated at 20 C for 3 days. The effects of components of the culture medium were determined by testing these solutions on the susceptible and resistant hosts.

Clones from a number of *Medicago* introductions of different origins and genetic constitutions were selected for studies of correlation between sensitivity to the toxin and susceptibility to the pathogen. The toxin bioassay was conducted as described above but the filtrate was diluted 1:3 (v/v) with sterile distilled water. Simultaneously, a corresponding set of plants was inoculated using single drops of a spore suspension (5×10^4 spores/ml) and kept in a growth chamber at 20 C and 100% RH for 3 days. Readings were made 3 days later for the toxin tests and 8 days after inoculation for reaction to the pathogen. In both tests the plants were scored on a scale from 1 to 5 according to the lesion size. Plants with score 1 or 2 were considered resistant (R), and plants with score 3, 4, or 5 were considered to be susceptible (S).

RESULTS

Germination of conidia.—In leaf samples taken from the susceptible and resistant plants 12 hours after inoculation, almost 100% germination of conidia was observed. Germination usually occurred simultaneously from different cells of the same conidium. Germ tubes grew straight and along the junctures of the epidermal cells on the *M. cancellata* leaves. Conidiophore-like structures were observed growing directly from conidia.

Penetration.—On the resistant host, there was a 20% penetration by germ tubes 12 hours after inoculation. In most cases ingress was through stomata. At this stage, no cell injury (browning of the cells) was observed in the area surrounding the infection site. Apparently, penetration directly between cells of the epidermis did not occur.

TABLE 1. Response of alfalfa clones to inoculation with a spore suspension of *Stemphylium botryosum* compared with their reactions to a toxic filtrate from a culture medium inoculated with the same strain of the pathogen

Species	Alfalfa clone	Toxin test score	Disease score
Medicago cancellata	57-17	1 (R)	2 (R)
	15-3	3 (S)	4 (S)
	15-4	2 (R)	2 (R)
	15-16	3 (S)	3 (S)
	57-10	2 (R)	2 (R)
	58-2	2 (R)	3 (S)
	58-4	1 (R)	1 (R)
	43-29	2 (R)	1 (R)
	43-28	3 (S)	1 (R)
M. sativa × *M. cancellata*	S-17 × 14-9	2 (R)	3 (S)
	8-1	1 (R)	2 (R)
	21-24	2 (R)	4 (S)
	21-26	2 (R)	2 (R)
M. falcata	17-9	3 (S)	3 (S)
	17-12	3 (S)	4 (S)
	17-17	2 (R)	4 (S)
	17-19	3 (S)	4 (S)
	17-21	2 (R)	2 (R)
M. hemicycla	81-8	4 (S)	4 (S)
	81-18	2 (R)	1 (R)
	24-11	3 (S)	2 (R)
	24-12	2 (R)	2 (R)
	24-16	4 (S)	4 (S)
	25-19	4 (S)	4 (S)
	81-818	4 (S)	5 (S)
	81-1813	3 (S)	5 (S)
	81-1815	3 (S)	4 (S)
	81-18129	5 (S)	4 (S)
M. sativa	I44	5 (S)	5 (S)
	S-17	5 (S)	5 (S)

Twenty-nine percent penetration of germ tubes occurred after 24 hours. In a few cases hyphae grew out of the stoma and continued growing on the leaf surface. After 36 hours, the germ tube penetrations increased to 80.0%, but no cellular injury appeared.

On the susceptible plant, there was 66.7% of germ tube penetrations 12 hours after inoculation. Penetration was through stomata and directly between cells of the epidermis in about the same frequency. After 24 hours 73.9% of the germ tubes had penetrated, and at 36 hours the percentage had increased to 85.7%.

Postpenetration development.—In the resistant *M. cancellata,* small primary hyphae were observed in the substomatal cavity after 24 hours (Fig. 1-A). They did not grow beyond the cells immediately surrounding the cavity. This situation was similar in subsequent samples, even 8 days after inoculation (Fig. 1-A, C, E, F). In a few cases primary hyphae were observed between the first two layers of cells after 5 days. Complete inhibition of fungal growth was apparent when the hyphae interacted with the cells of the resistant host. In this host, adverse cellular effects were noticed only after 48 hours and they were restricted to a small area. The injured area increased gradually and after 7 days it included up to 30 cells from the infection site. The cells were brown, but did not show evidence of collapsing.

In the susceptible *M. sativa,* the primary hyphae were detected 12 hours after inoculation in the second and third layers of cells from the infection site. After 24 hours the primary hyphae were larger (Fig. 1-B) and a slight browning of the cells was observed. After 36 hours, secondary hyphae developed between the first five to six layers of cells. A few hyphae emerged from different stomata. After 48 hours, secondary hyphae grew and branched intercellularly and the host cells were injured over larger areas (Fig. 1-D).

Discoloration in the leaf veins and browning of the cells in advance of the fungal hyphae confirmed the participation of toxins in the interaction. However, after 96 hours the hyphae grew between apparently healthy cells (Fig. 1-F). This suggests that toxins are not of primary importance in the later stages of development, even though they may be essential for the establishment and initial growth of the pathogen.

Hyphae were observed emerging through the cuticle of the leaves and in some areas the fungus began sporulating (Fig. 1-H). In later stages the cells collapsed and were dark brown.

Toxin bioassays.—The application of the culture filtrate of the pathogen to leaves of the susceptible *M. sativa* plant produced symptoms identical to those observed in the diseased plants. Sequential changes occurred in the development of these symptoms. Initially, the cells surrounding the toxin drop became water-soaked, probably due to the action of the toxin on cell permeability resulting in loss of water and cell turgor. Within 24 hours, the host cells became discolored and finally a brown necrotic spot developed. The histological studies demonstrated that the changes induced by the fungus after penetration were similar to those produced by the filtrate alone. The control solution failed to induce symptoms, demonstrating the absence of effects by components of the culture medium.

On susceptible host leaves, the culture filtrate was toxic up to a dilution of 1:63 (v/v). The cells in the resistant host were unaffected by the filtrate, even at the highest concentration.

In the toxin tests and inoculation with spores of the fungus the host plants showed a wide range of variation in responses (Table 1). The correlation analysis between scores obtained for both methods yielded a positive correlation coefficient of $r = 0.73$. When the plants are classified as resistant or susceptible there are only six plants in which the toxin and spore inoculation tests did not correspond.

DISCUSSION

On the susceptible alfalfa host, *S. botryosum* showed a typical pattern of disease development. Our observations suggest that the fungus affects the host cell permeability. However, other effects directly on the protoplast as indicated by Pierre and Millar (7) are not excluded. As expected, the host cells did not develop any physical barrier to prevent colonization and reproduction by the fungus.

On the resistant host the growth of the pathogen was inhibited soon after penetration and it did not grow beyond the primary hyphae stage. The host reaction to the fungus was evidenced by the browning of the cells surrounding the infection site. The reaction was localized and can be considered as a hypersensitive reaction. Hypersensitivity (tissue necrosis) was apparent after 72 hours. This suggests, according to Király's hypothesis (5), that a preformed mechanism of defense inhibits the growth of the pathogen and hypersensitivity is the effect of that inhibition.

Our histological studies revealed that fungal toxins were involved in the early stages of the disease development. The toxin bioassays with a crude filtrate reaffirmed other reports of the production of toxic substances by *S. botryosum.* We found that the filtrate produced identical symptoms to those produced by the fungus on susceptible plants. However, on resistant plants the filtrate did not produce symptoms. For this reason the toxin or toxins present in the filtrate are considered host-specific toxins.

A correlation coefficient of $r = 0.73$ showed that there was a positive relationship between toxin tests and inoculations with spores. The differences found could be attributed to the fact that different genotypes, even carrying different genes for resistance or susceptibility, may not have the same sensitivity to a given concentration of the toxins.

As a screening technique, the use of toxins would have the advantages of eliminating susceptible plants and would permit the screening of larger populations in a reduced space. Likewise, there should be fewer escapes when compared with inoculation by spores.

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