Expression of Pathogen Virulence and Host Resistance During Infection of Alfalfa with *Stemphylium botryosum*

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ABSTRACT

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Infection of *Medicago sativa* by *Stemphylium botryosum* (cool-temperature biotype) was examined histologically to assess the sequence and form in which pathogen virulence and host resistance were expressed. Pathogen growth during the infection process and host reaction to infection were followed simultaneously in a set of nine host clone-pathogen isolate (h-p) combinations. The h-p set consisted of the factorial combination of three alfalfa clones and three pathogen isolates chosen to encompass the range of relative host resistance and pathogen virulence observed in the field in California. In all h-p combinations, leaf penetration by germ tubes occurred exclusively through stomata, while subsequent fungal growth (revealed by aniline blue dye) was restricted to the development of a bulbous mycelium in the substomatal cavity. The formation of the bulbous mycelium coincided with an effect on adjacent palisade mesophyll cells that resulted in retention of dye by walls of the cells, in contrast to unstained walls of cells remote from the site of penetration. Penetrations in which dye was retained by host cell walls located more than 0.1 mm from the bulbous mycelium (the radius of the smallest lesion formed on susceptible leaves) were recorded as "effective" penetrations. Differences in relative virulence of isolates (expressed as the relative disease severity produced on a susceptible alfalfa clone at a fixed inoculum concentration) were correlated with the frequency of stomatal penetration. In addition, isolates with high relative virulence produced a higher proportion of effective penetrations than isolates with low virulence. The frequency of stomatal penetration was not affected by host resistance. Differences in relative host resistance were expressed as the relative frequency with which host cells collapsed and formed necrotic lesions in response to effective penetrations. Therefore, relative virulence is expressed independently of and prior to the expression of relative resistance in this interaction.

Additional key words: *Pleospora herbarum*, lucerne, histology.

Genetic and physiological studies of pathogen virulence and host resistance often focus on, or arise from, observations of the sequence of histological events which appear to determine the relative compatibility of a given host-parasite interaction (17,18). The interpretation of data from such studies would be facilitated by, if not dependent upon, the development of a model presenting both the form and sequence of determinative events at the level of cellular interaction of host and pathogen.

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Previous histological studies on Stemphylium leaf spot of alfalfa (*Medicago sativa* L.), caused by *Stemphylium botryosum* Wallr. (= *Pleospora herbarum* (Br.) Rab.), have been used as the basis of proposals concerning the determinative role of phytotoxins and phytoalexins in the disease (4,19,20). However, all previous studies have been based on either the interaction of susceptible *M. sativa* plants with pathogens and nonpathogens or virulent *S. botryosum* isolates on susceptible *M. sativa* and nonhost plants. Correlative relationships in plant disease, such as relative pathogen virulence with tolerance to host-produced phytoalexins, can provide evidence for determinative mechanisms of disease if accompanied by appropriate genetic analysis (23). Unfortunately, interactions of hosts with nonpathogens or nonhosts with pathogens are less useful

in this regard. In no case have simultaneous histological comparisons been made of compatible and incompatible combinations of *S. botryosum* and *M. sativa*.

The original histological work of Smith (21) in Wisconsin, although frequently quoted in connection with the disease on alfalfa (4,13,20), was conducted on Stemphylium leaf spot of red clover (*Trifolium pratense* L.), which is caused by a physiological race of *S. botryosum* not pathogenic on alfalfa (21). No evidence has been provided that the observations and conclusions from red clover could be extrapolated to the disease on alfalfa. Later histological studies of Stemphylium leaf spot of alfalfa in eastern North America (19,20) may not be comparable directly to the results of similar studies in California (4) since different biotypes of the pathogen occur in the two regions of the continent (8).

The present study was initiated to complement recent (5,6,12) and ongoing research directed towards elucidating the genetic and molecular mechanisms of pathogen virulence and host resistance in the *M. sativa-S. botryosum* host pathogen (h-p) relationship. Specifically, the experiments were designed to determine where, when, and in what manner pathogen virulence and host resistance were expressed during the infection process. The conclusions of this study are restricted to the form of the disease caused by the cool-temperature biotype of *S. botryosum* found on alfalfa in California (8). A preliminary report on this work has been published (7).

MATERIALS AND METHODS

Host and pathogen material. Monoconidial isolates of *S. botryosum* expressing high virulence (HV), moderate virulence (MV), and low virulence (LV) were obtained from alfalfa production fields near Salinas, CA, and maintained in pure culture as described previously (6). Alfalfa clones originated from single plants that had been selected for apparent resistance (clone M9), moderate susceptibility (clone M3), and susceptibility (clone S2) to Stemphylium leaf spot from severely diseased cultivars (*M. sativa* 'Lew,' 'WL512,' and 'WL450,' respectively) in the field in California. Growth and maintenance of alfalfa clones in the greenhouse was described previously (6). The three pathogen isolates represented the widest range of relative virulence in our collections of *S. botryosum* from alfalfa in California (6), while the three alfalfa clones comprised the widest range of relative host reaction to *S. botryosum* observed in alfalfa cultivars in the field.

Disease production and assessment. Methods for the preparation of inoculum, inoculation of plants, assessment and analysis of disease severity, and measurement of average leaf and lesion areas were the same as described previously (6). The light, moisture, and temperature conditions in the growth chamber during infection, also the same as used previously (6), were favorable for the production of disease severity levels and symptoms similar to those observed in the field in California (5,6,8).

Experiments were designed to permit assessment of the infection process in each h-p combination from inoculation to lesion formation. Eight replicate plants of each host clone were inoculated as a group by spraying with 25 ml of conidial suspensions containing 10^5 conidia per milliliter. The plants were then misted for 48 hr in the growth chamber as previously described (6).

Observation of the fungus before leaf penetration. Preliminary experiments revealed that 70% of the conidia on the leaf surface had germinated by 2 hr after inoculation at 18–20 C, and percentage germination approached a maximum of 98–100% by 24 hr. Therefore, leaves were sampled from each h-p combination 24 hr after inoculation to examine conidial germination on the leaf surface by a stereo microscope with reflected epi-illumination. Percentage germination of 200 conidia was assessed on a leaflet sampled from leaf position three from the stem apex in each h-p combination. The number of germ tubes per conidium and length of the longest germ tube on each conidium was assessed on 50 conidia per h-p combination.

Observation of the fungus during leaf penetration. Preliminary experiments showed that leaf penetration by germ tubes at 18–20 C began 7–12 hr after inoculation, the frequency of penetration reached a maximum near the end of the 48-hr mist period, and leaf lesions appeared on susceptible clones 48–72 hr after inoculation. Therefore, penetration of the host by germ tubes, development of the fungus in the host, and host reaction to presence of the fungus was observed at 24, 48, 72, and 120 hr after inoculation. Two leaflets were sampled from each h-p combination at leaf position three at each time point. Leaflets were immediately fixed and cleared by immersion in a saturated chloral hydrate solution, stained with aniline blue, and destained by a method similar to that used for Stemphylium leafspot of birdsfoot trefoil (11). However, the destained leaves were not counterstained in bismarck brown (11), but were viewed directly by phase contrast microscopy following permanent mounting in synthetic resin on glass slides.

The total number of leaf penetrations on each leaflet was recorded at 24 and 48 hr after inoculation. The efficiency of germ tube penetration of leaves was computed so that a comparison could be made to similar measurements reported in an earlier study (4). The efficiency of germ tube penetration was estimated from the total number of penetrations per leaflet observed after staining divided by the average number of germ tubes on the leaflet surface before staining.

Observation of host reaction following penetration by the fungus. Initial observations of stained tissue revealed that a certain frequency of penetrations was distinguished by an apparent effect on the cell walls surrounding the penetration site. Thereafter, penetrations observed 48 hr following inoculation were defined as "effective" if they were surrounded by an area of leaf tissue in which palisade mesophyll cells were outlined by retention of aniline blue dye in the cell wall. Specifically, and of relevance to later lesion development, only those penetrations in which the affected distance was greater than the radius of the smallest necrotic lesion measured in this study were recorded as effective.

A frequency distribution histogram of the areas of 1,000 lesions on leaves of the susceptible clone caused by the MV isolate was constructed to compare the size of lesions with the area of tissue affected by penetrations at 48 hr. Lesion area was measured with an area planimeter as previously described (6). The square-root transformation converted lesion area to lesion radius, which was plotted also in a frequency distribution histogram. The radius of the smallest lesion size category, thus obtained, was used as the threshold distance for defining "effective" penetrations.

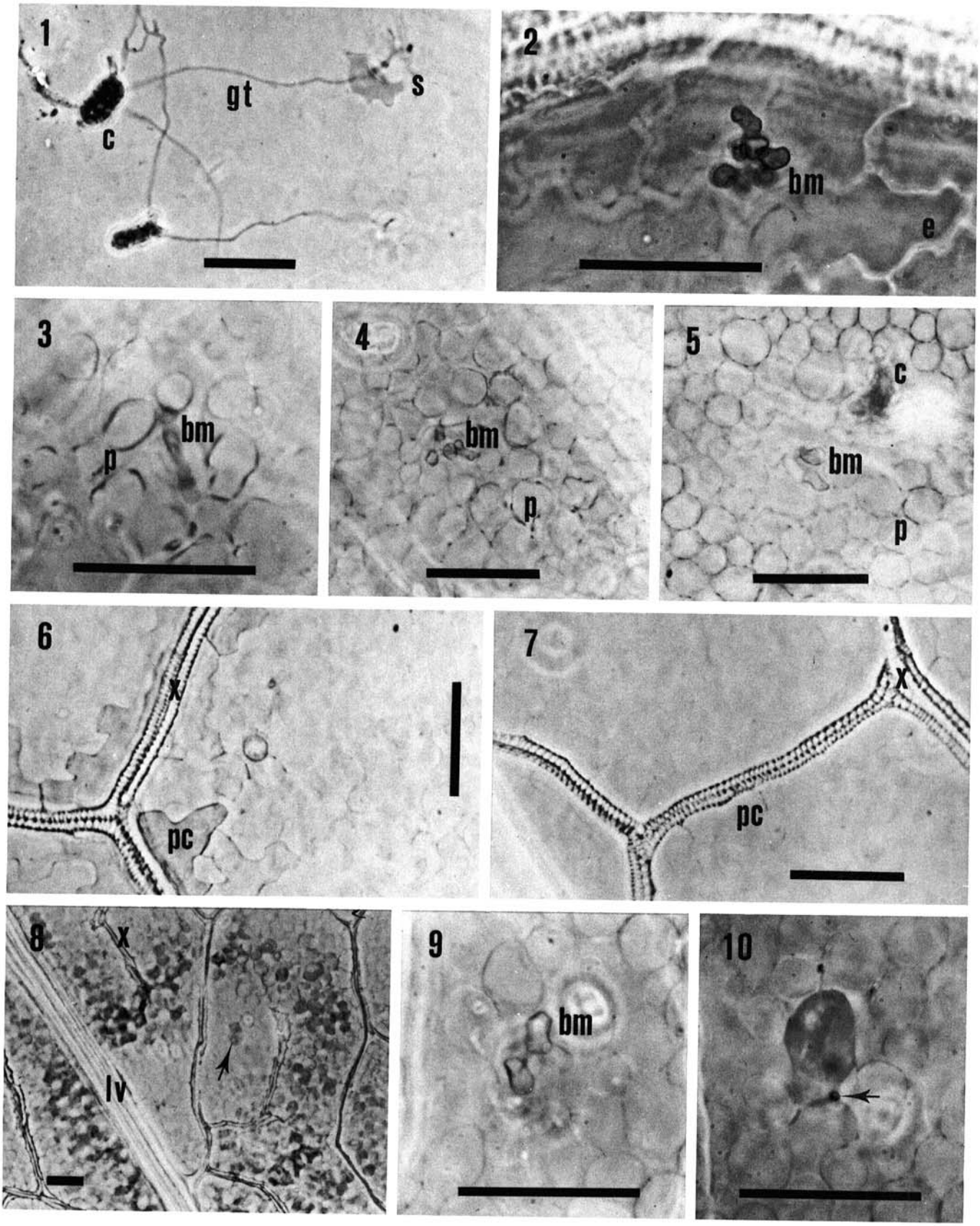
Photomicrographs were taken of stained leaflets to record growth of the fungus during and after penetration of leaflets by germ tubes, host reaction to presence of the fungus, and appearance of the host and pathogen during the development of disease symptoms.

RESULTS

Prepenetration growth and development of the fungus. The average number of conidia present on individual leaflets 24 hr after inoculation was 344 (range 104–921). The high variability may have been due to variation in leaflet area within and among alfalfa clones, the uneven distribution of conidia on the leaf surface due to the inoculation technique, or the removal of conidia from leaves by mist droplets moving over the leaf surface. However, groups of eight plants of each clone received the same volume and concentration of inoculum, and there were no significant differences in the mean numbers of conidia remaining on leaves among clones after 24-hr mist.

There were no consistent differences or trends in prepenetration growth of the fungus on alfalfa leaves 24 hr after inoculation (percent germination of conidia, number of germ tubes per conidium, and length of the longest germ tube of each conidium) that could account for the differences in disease severity recorded among the nine h-p combinations (Table 1).

Leaf penetration by the fungus. Germ tubes penetrated leaves exclusively through stomata. Only one of 2,129 leaf penetrations observed in this study occurred directly between epidermal cells. Furthermore, germ tube growth did not appear to be influenced by the proximity of conidia to stomata; for example, germ tubes were observed to pass over stomata without penetration (Fig. 1). After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stomatal penetration, germ tubes formed a bulbous structure in the substomatal cavity (Fig. 2) and very little, if any, further growth of the mycelium was observed throughout the period required for symptom development.

The frequency of stomatal penetration at 24 and 48 hr after inoculation appeared to be associated with pathogen relative virulence (V), and increased greatly in this time period (Table 2). In addition, the analysis of variance (Table 2) revealed that the ability of the three pathogen isolates to penetrate the host was not affected by relative host resistance (R). Similarly, the level of host resistance did not affect differentially the relative frequency of total leaf penetrations by isolates with different virulence, as indicated by the lack of significance for the R×V interaction (Table 2).

The efficiency of leaf penetration by germ tubes 48 hr after inoculation was estimated from the average total number of penetrations of leaflets (Table 2) divided by the average number of germ tubes observed on leaflets prior to clearing and staining. This calculation was necessary because the number of conidia remaining on leaves after the staining procedure was closely related to the total number of penetrations (or relative virulence) of an isolate; the average number of conidia remaining on leaflets inoculated with the MV and LV isolates was 28.6 and 19.5%, respectively, of those inoculated with the HV isolate. The estimated efficiency of leaflet penetration by germ tubes of the HV, MV, and LV isolates (averaged over all alfalfa clones) was 19.4, 3.1, and 1.6%, respectively.

Effect of the fungus on host tissue after penetration. The walls of palisade mesophyll cells surrounding the bulbous mycelium in the substomatal cavity appeared to be affected by the presence of the fungus as observed by the retention of aniline blue dye in the cell walls. At 24 hr, dye retention was restricted to cells in the immediate vicinity of the bulbous mycelium (Fig. 3). At 48 hr, the effect had spread to cells a considerable distance away from the nearest mycelium (Fig. 4). The same phenomenon was observed in cell walls surrounding stomatal penetrations in both susceptible (Fig. 4) and resistant (Fig. 5) host tissue. In addition to dye retention in palisade mesophyll cell walls, the dye was commonly retained in parenchymatous cells surrounding the vascular bundles beneath the sites of penetration (Fig. 6), and along leaf veins leading away from the site of penetration. This effect was not visible in unpenetrated regions of the same leaflet (Fig. 7).

The possible relationship between dye retention by cells at 48 hr and the subsequent formation of lesions on leaves 48–72 hr after inoculation of susceptible clones was investigated by comparing the area of tissue affected by penetrations at 48 hr with the size of lesions subsequently formed on leaves of susceptible clones. The frequency distribution histogram of lesion area on the susceptible clone is plotted in Fig. 11A. The radius of lesions was estimated from the square-root transformation of lesion area data and is plotted in Fig. 11B. The assumption that lesions were circular appeared to be correct for small lesions but not for large lesions, which tended to be elongated and restricted between leaf veins. The radius of the smallest lesion measured at 7 days was 0.1 mm (Fig. 11B). Therefore, this distance was chosen as a threshold value for defining "effective" penetrations at 48 hr; that is, those penetrations that were accompanied by dye retention in host cell walls more than 0.1 mm (five to seven palisade mesophyll cells) from the nearest mycelium.

The frequency of effective penetrations on leaflets 48 hr after inoculation was associated with pathogen virulence (Table 3). Also, comparison of Tables 2 and 3 indicates that the proportion of effective to total penetrations on leaflets was much higher for the HV isolate (55.2%) than for the LV isolate (5.9%). Analysis of variance of the number of total and effective penetrations at 48 hr indicated that there was a significant relationship of type of penetration to the number of penetrations per leaflet. This is interpreted to mean that the number of effective penetrations per leaflet was more closely associated with pathogen virulence than was total number of penetrations per leaflet ($F = 12.79$, $P \leq 0.01$). The level of host resistance did not affect the frequency of effective penetrations, and did not affect differentially the relative frequency of total or effective leaf penetrations by isolates with different virulence, as the R×V interaction was not significant.

The number of effective penetrations per leaflet at 48 hr was correlated very closely with the "equivalent number of lesions per leaflet" ($r^2 = 0.988$) ultimately formed on the susceptible and moderately susceptible alfalfa clones (Table 3). The high correlation suggested that effective penetration and cell collapse occurred in a closely related sequence of events between 48 and 72 hr after inoculation of leaves of the susceptible clones. However, on the resistant clone similar numbers of effective penetrations did

TABLE 1. Effect of host resistance and pathogen virulence on the germination of conidia and length of germ tubes of *Stemphylium botryosum* (cool-temperature biotype) on alfalfa leaves 24 hr after inoculation

Pathogen relative virulence[c]	Conidial germination, number and length of germ tubes[a]			
	Alfalfa clone relative resistance[b]			
	Susceptible	Moderately susceptible	Resistant	Mean
High	96.3 (3.5; 360)	98.0 (3.3; 280)	98.0 (2.7; 320)	97.4 (3.2; 320)
Moderate	86.5 (2.8; 280)	94.3 (2.9; 360)	94.2 (2.9; 360)	91.7 (2.9; 330)
Low	92.7 (2.8; 250)	90.6 (3.0; 310)	91.3 (2.8; 340)	91.5 (2.9; 290)
Mean	91.8 (3.1; 300)	94.3 (3.1; 310)	94.5 (2.8; 340)	

[a] The parameters measured were percent germination (outside parentheses), number of germ tubes per conidium (first value inside parentheses), and length of the longest germ tube growing from each conidium (second value inside parentheses μm). Each percent germination value is the result of observation of 200 conidia per host clone-pathogen isolate combination; the other two parameters were measured on 50 conidia per combination.

[b] Host resistance is defined as the relative percent leaf area necrotic (LAN) on clones of alfalfa after inoculation with a high virulence isolate of *S. botryosum*.

[c] Pathogen relative virulence is defined as the relative percent LAN induced by isolates of *S. botryosum* on a susceptible clone of alfalfa at a fixed inoculum concentration.

Figs. 1–10. Photomicrographs of the sequence of events during infection of alfalfa by *Stemphylium botryosum* (cool-temperature biotype). Alfalfa clones susceptible, moderately susceptible, or resistant to Stemphylium leaf spot were inoculated with conidial suspensions of isolates of *S. botryosum* with high (HV), moderate (MV), or low (LV), relative virulence. Leaflets were fixed and cleared 24, 48, 72, and 120 hr after inoculation, stained with aniline blue, and mounted for observation by phase contrast microscopy. The bar in each photomicrograph represents 40 μm. **1,** Two conidia (c) and germ tubes (gt) of the MV isolate on the leaf surface of the susceptible alfalfa clone (24 hr). One germ tube has penetrated a stoma (s). **2,** A bulbous mycelium (bm) of the HV isolate immediately below a stoma of the moderately susceptible clone (24 hr). Leaf epidermal cells (e) are visible in the same focal plane. **3,** Cell walls of palisade mesophyll cells (p) of the moderately susceptible clone outlined with dye near a bulbous mycelium of the HV isolate in the substomatal cavity (24 hr). **4,** An "effective" penetration of the HV isolate at 48 hr on the susceptible host; the bulbous mycelium is surrounded by affected palisade mesophyll cells (distinguished by the uptake of aniline blue dye) further than 0.1 mm from the nearest mycelium. **5,** An effective penetration of the HV isolate at 48 hr on the resistant clone. **6,** Parenchymatous cells (pc) surrounding the xylem (x) elements on leaves of the susceptible clone appear stained below an effective penetration of the HV isolate (48 hr). **7,** A "healthy" (nonpenetrated) region of same leaflet photographed in Fig. 6; parenchymatous cells are not stained by aniline blue dye. **8,** A small lesion on the susceptible alfalfa clone is limited by a lateral vein (lv) and xylem elements. Only one site of penetration by the MV isolate was observed in the lesion (arrow). **9,** Close-up of region below arrow in Fig. 8 showing bulbous mycelium in substomatal cavity. **10,** Extent of growth of mycelium in Fig. 9 (arrow), one host cell level below the bulbous mycelium.

not lead to necrotic lesion formation. In the latter case, only faint chlorotic spots, if anything, were observed on leaves at the time of disease assessment.

Lesion formation. Lesions appeared on the susceptible and moderately susceptible clones as grey collapsed areas on the leaf after the mist was turned off at 48 hr. The grey collapsed areas acquired the bleached color and abrupt border typical of the California form of the disease (5,8,13) 4–5 days after inoculation. Microscopic examination of lesions revealed a ring of aniline blue-stained mesophyll cells surrounding a thin unstained region where the epidermis was no longer obvious (Fig. 8). Necrotic palisade mesophyll cells inside the lesion appeared to be of regular size and shape, but the stained cells around the border had lost their structure and integrity (Fig. 8). Mycelial growth inside lesions remained restricted to the bulbous structure (Fig. 9) or very close to this structure (Fig. 10). Mycelia were never observed to ramify profusely through the tissue during lesion formation. In fact, ramification of mycelia was observed only when necrotic tissue was rehydrated, for example, by misting the plants again after lesion formation. Microscopic examination of 300 leaf lesions following misting indicated that mycelial ramification and subsequent sporulation were restricted to the necrotic area within the sharply delimited lesions.

DISCUSSION

The host-pathogen interaction of *M. sativa* and the cool-temperature biotype of *S. botryosum* appears to be influenced by pathogen relative virulence and host resistance at distinctly different sites and times in the infection process (Fig. 12). Pathogen relative virulence, defined as the relative disease severity caused by an isolate on a susceptible alfalfa clone (6), is expressed before symptoms become visible as the ability of the fungus to penetrate host stomata and to affect cells surrounding the site of penetration. In contrast, host resistance is expressed as the ability of host cells to avoid cell collapse in leaf tissue affected earlier by a penetration, and is manifested as a reduction in number, rather than size, of lesions on leaves (Table 3) (5). Thus, the observed effect of fungal penetration on host cell walls appears to be a necessary step in the expression of virulence, but is not the step which determines resistance or susceptibility in the host.

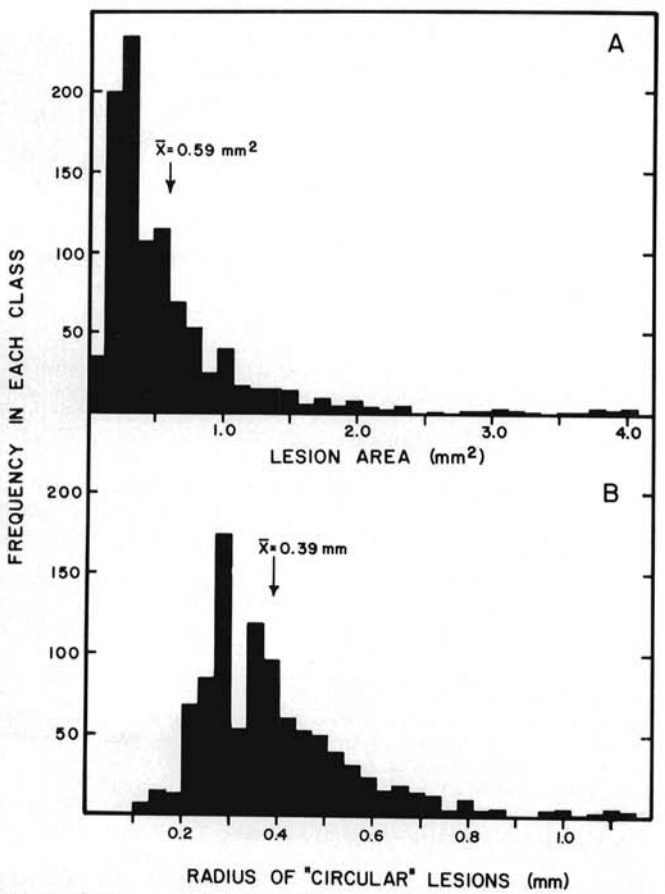

Fig. 11. Frequency distribution histograms of lesion area and radius of lesions produced by the moderate virulence isolate of *Stemphylium botryosum* (cool-temperature biotype) on the susceptible alfalfa clone. **A,** Lesion area (square millimeters) of 1,000 lesions measured on leaves 7 days after inoculation. **B,** Radius (millimeters) of assumed "circular" lesions derived from the square-root transformation of data in Fig. 11A.

The results and conclusions of this study differ in several ways from those of a previous report in which the interaction of the cool-temperature (California) biotype of *S. botryosum* and alfalfa was studied (4). The differences may be explained in part by differences in methodology used in the respective studies. In the earlier reports (3,4), alfalfa leaves were detached, floated on sucrose solutions, inoculated with conidia of *S. botryosum*, and placed under continuous light at approximately 100% relative humidity for 196 hr (2). Germ tube penetration through stomata or directly between epidermal cells was reported to occur with equal frequency, and efficiency of germ tube penetration was as high as 85.7% by 36 hr after inoculation (4). The latter figure may be exaggerated because, as found in the present studies, the staining procedure removes many conidia from the leaf surface if they have not already penetrated stomata.

Continuous light after inoculation was not used here because it caused large reductions in disease severity in experiments preliminary to these studies (5). Also, continuous high relative humidity and sucrose in the floating medium of the detached leaf study (3,4) may have encouraged growth of the mycelium in host tissue beyond that which would have occurred in leaves of an intact plant. In any case, no evidence was provided to confirm that the infection process observed in the detached leaf system (3,4) occurred in the same manner in the intact plant.

Plants in the present study were kept intact throughout the

TABLE 2. Effect of host resistance and pathogen virulence on the total number of stomatal penetrations per alfalfa leaflet by germ tubes of *Stemphylium botryosum* (cool-temperature biotype) 24 and 48 hr after inoculation

Pathogen relative virulence[c]	Stomatal penetrations per leaflet at (24 hr) 48 hr[a]			
	Alfalfa clone relative resistance[b]			
	Susceptible	Moderately susceptible	Resistant	Mean
High	236 (78)	182 (93)	184 (67)	210 A (79) Y
Moderate	53 (22)	35 (3)	9 (5)	32 B (10) Z
Low	18 (28)	32 (23)	1 (0)	17 B (17) Z
Mean	102 B (42) Z	83 B (40) Z	65 B (24) Z	

[a] Alfalfa leaflets were sampled at two time intervals (T) after inoculation, 24 and 48 hr, and then cleared, stained, and examined by microscope to count the total number of penetrations per leaflet (leaf three from the stem apex) by germ tubes. Values inside parentheses are counts at 24 hr; those outside parentheses are counts at 48 hr. Each value within the table is the average of counts on two leaflets per host clone-pathogen isolate combination. The data were square-root-transformed for analysis of variance. F-values from analysis of variance: $F(R)_{[2,\ 18]} = 3.26$ (N.S.); $F(V)_{[2,\ 18]} = 30.27$ ($P \leq 0.0001$); $F(T)_{[1,\ 18]} = 8.89$ ($P \leq 0.01$); $F(R\times V)_{[4,\ 18]} = 0.99$ (N.S.); $F(V\times T)_{[2,\ 18]} = 2.73$ (N.S.); $F(R\times V\times T)_{[6,\ 18]} = 0.26$ (N.S.). Means in the table accompanied by the same letter (range Y–Z, 24 hr; A–B, 48 hr) do not differ ($P = 0.05$) according to Duncan's multiple range test. N.S. = not significant.

[b] Host resistance (R) is defined and the alfalfa clones used are the same as in Table 1.

[c] Pathogen virulence (V) is defined and the isolates of *S. botryosum* used are the same as in Table 1.

infection process. The environmental conditions used here were favorable for the production of both the form and magnitude of disease severity levels observed in the field in California (5,6,8), and were the same conditions used to detect genetic variability of pathogen virulence (6) and host resistance (5) in this disease. Under these conditions, leaf penetration occurred exclusively through the stomata and efficiency of penetration was much lower than reported previously (4). In addition, mycelial ramification in leaf tissue was not observed following penetration or during lesion formation. In all cases, visible growth of the fungus in this study was restricted close to the bulbous mycelium formed in the substomatal cavity.

The substomatal bulbous mycelium has been reported in many previous histological studies, but, in contrast to our observations, growth of the fungus away from the bulbous mycelium was reported to occur before tissue necrosis became visible (4,19,20). Conclusions differed about the position of the mycelium during tissue necrosis—either ahead of (20) or behind (4,19) the area of necrotic cells. For Stemphylium leaf spot of *T. pratense,* Smith stated that "this [substomatal] bulbous mycelium apparently makes very little growth until several host cells immediately around the stoma have been killed" (21). The aforementioned conclusions have followed from the common assumption that stained cells were dead, an assumption that does not seem to be valid for the current study. The cells surrounding effective penetrations, although they retained aniline blue in their walls 48 hr after inoculation, apparently were not killed at this point. The evidence for this conclusion is: affected cells retained stain before lesions became visible on leaves of the susceptible clone; and the uptake of dye was similar in tissue of the susceptible and resistant clones at 48 hr, even though the latter never developed necrotic symptoms.

Since the exact cause of the microscopically visible host symptoms is not known at present, several possible explanations cannot be excluded. These include host cell reaction to pathogen- or host-produced molecules which diffuse through the water layer surrounding the cells, or a direct pathogen-induced communication between adjacent host cells. It has been established that *S. botryosum* produces compounds in liquid media that are toxic to alfalfa tissue (4,5,12), although our evidence indicates that the compounds produced in culture are not host specific (5,12). If phytotoxins are involved nonspecifically in the cell wall staining reaction and subsequent symptom development, the resistant reaction could be due to host-regulated synthesis of sublethal concentrations of toxin by the bulbous mycelium, or an induced self-repair process of host cells similar to that proposed by Hanchey and Wheeler (15). Their hypothesis states that "susceptible plants are unable to repair damage caused by toxin" (15). The latter hypothesis is consistent with the observations in this disease (Table 3) (5), barley leaf scald or blotch (14), and corn northern leaf blight (16), that resistance is expressed as a reduction in the number, rather than size, of lesions on leaves. In these diseases, a moderately resistant plant is able to prevent some, but not all, penetrations from forming lesions. Tissue contiguous to the site of a penetration may be stimulated to a "preinduced state" by that penetration, such that it can respond to any further penetrations by repairing damage caused by the phytotoxin, thereby preventing lesion formation. Accordingly, resistance would be expected to be correlated with the relative frequency of penetrations which are unable to cause lesions.

Further evidence for the role of a diffusible product of pathogen metabolism in lesion formation is the elongated and vein-limited

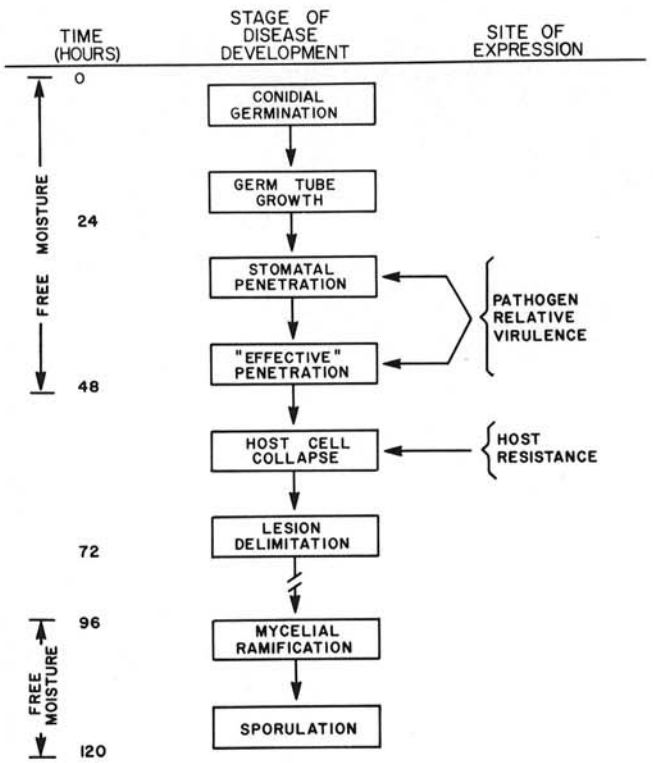

Fig. 12. Summary flowchart of the sites and times at which pathogen virulence and host resistance are expressed during the infection of alfalfa by *Stemphylium botryosum* (cool-temperature biotype).

TABLE 3. Relationship between the number of effective penetrations of alfalfa leaflets 48 hr after inoculation and equivalent number of lesions per leaflet subsequently produced on alfalfa clones with a range of relative resistance inoculated with *Stemphylium botryosum* isolates (cool-temperature biotype) with a range of relative virulence

Pathogen relative virulence[d]	Effective penetrations[a] and equivalent lesions per leaflet[b]			
	Alfalfa clone relative resistance[c]			
	Susceptible	Moderately susceptible	Resistant	Mean
High	161 (95)	82 (50)	91 (0)	111 A (48) X
Moderate	12 (8)	10 (9)	1 (0)	8 B (6) Y
Low	2 (0.3)	2 (0.3)	0 (0)	1 B (0.2) Z
Mean	58 B (34) X	31 B (20) Y	31 B (0) Z	

[a]The number of "effective" penetrations per leaflet by germ tubes of *S. botryosum* (outside parentheses) is the number of stomatal penetrations associated with the retention of aniline blue dye in the walls of cells further than 0.1 mm from the penetrated stomata. Each value is the average count of all penetrations on two leaflets per host clone-pathogen isolate (h-p) combination. Means accompanied by the same letter (range A–B) do not differ ($P = 0.05$) according to Duncan's multiple range test of square-root-transformed data.

[b]Equivalent number of lesions per leaflet (inside parentheses) was calculated as: average percent leaf area necrotic (LAN) × average leaflet area per average lesion area for each h-p combination. Disease severity caused by the high virulence, moderate virulence, and low virulence isolates on the susceptible alfalfa clone was 44.5, 3.9, and 0.2% LAN, respectively, and on the moderately susceptible clone was 32.7, 5.9, and 0.2% LAN, respectively (averages of 81 leaves); no lesions were formed by any isolate on the resistant clone. The average area of all lesions on 10 leaves for the same isolates on the susceptible clone was 0.69, 0.65, and 0.72 mm^2, and on the moderately susceptible clone was 0.61, 0.79, 0.70 mm^2, respectively. There were no significant differences in lesion area among h-p combinations. Average leaf area (leaf three) of the susceptible clone (5.01 cm^2) was significantly larger than the moderately susceptible clone (3.45 cm^2), and average leaflet area was assumed to be one-third of these values. Mean values for equivalent number of lesions per leaflet accompanied by the same letter (range X–Z) do not differ ($P = 0.05$) according to Duncan's multiple range test.

[c]Host resistance is defined and the alfalfa clones used are the same as in Table 1.

[d]Pathogen virulence is defined and the isolates of *S. botryosum* used are the same as in Table 1.

pattern of dye uptake by tissue surrounding the site of effective penetrations (Figs. 6 and 8); this pattern is similar in size and shape to the lesions formed subsequently on a susceptible clone. The time required for simple diffusion of a compound released from the bulbous mycelium through the region of an effective penetration can be calculated theoretically (1). Such calculation for a phytotoxin similar to that produced by *S. botryosum* (molecular weight ~17,000) (5) show that 30 min would be adequate to disperse that phytotoxin through a volume of water equivalent in size to an effective penetration. This is not inconsistent with the time required for the postulated effects of phytotoxin on host cells 24–48 hr after inoculation. Physical barriers to diffusion, such as leaf vascular bundles, could limit the region of affected tissue to between veins. In contrast, the xylem elements could distribute the compound along veinlets, thereby accounting for the effects on leaf tissue observed in Fig. 6, and the subsequent elongated shape of some necrotic lesions on susceptible plants.

It has been postulated that variation in lesion area in this disease arises from the coalescence of two or more "successful infections" by the fungus (6). Such a postulate would require that there were always more effective penetrations (as defined in this report) than lesions ultimately formed on leaves of susceptible clones. The data in Table 3 therefore support such a hypothesis.

Definition of the term "relative virulence," used originally to describe the disease-producing capacity of an isolate of *S. botryosum* (6), may need refining in the light of the observations of the h-p interaction in the present study. An isolate of *S. botryosum* with high relative virulence may be more appropriately described as having high "potential relative virulence," because it exhibits the potential for producing high disease severity before host resistance is expressed. This distinguishes our use of the term "virulence" from its use in h-p interactions where differential interactions of host and pathogen occur. In the latter sense, qualitative expression of virulence of a pathogen isolate depends on the genotype of the host it is infecting (9,22). Our observations indicate that the expression of pathogen phenotype is quantitative (6) and nonspecific at each step as defined by Day (10), that is, independent of host resistance. Similarly, the expression of host phenotype (whether or not host cells collapse or remain viable in the region of an effective penetration) is independent of the potential relative virulence of the pathogen isolate.

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