Effect of Light and Moisture on Severity of Stemphylium Leaf Spot of Alfalfa

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ABSTRACT

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The effect of various light sequences and moisture periods on severity of Stemphylium leaf spot was tested in a controlled environment chamber on a susceptible alfalfa clone inoculated with the cool-temperature biotype of *Stemphylium botryosum*. Plants inoculated after a 12-hr light period exhibited more disease than those inoculated after a 12-hr dark period, regardless of the postinoculation light sequence. Plants inoculated before a 12-hr light period exhibited more disease than those inoculated before a 12-hr dark period, regardless of the preinoculation light treatment. No leaf spot symptoms appeared if plants were exposed to continuous light after inoculation. Thus, high disease severity was achieved only when plants were exposed to light before and after inoculation, followed by alternating dark/light periods until leaf spot symptoms developed. Disease severity increased when the period of free moisture on the leaves was extended from 1 to 4 days. A 2-day moist period produced symptoms most like those observed in the field in California. Variation in disease severity among treatments was caused by differences in the number, not average size, of lesions. This result illustrates a major distinction in disease expression by the cool-temperature biotype compared with the warm-temperature biotype of *S. botryosum*.

Additional key words: environment, lucerne, *Medicago sativa*, *Pleospora herbarum*

Stemphylium leaf spot of alfalfa (*Medicago sativa* L.) in California, caused by the conidial state *Stemphylium botryosum* Wallr. of *Pleospora herbarum* (Pers. ex Fr.) Rab., is regarded as a "cool-weather disease" (18) and is confined to the cool, moist periods of the year (13,18). In preliminary growth chamber studies using the cool-temperature (California) biotype of *S. botryosum* (8), it was observed that severity of Stemphylium leaf spot was sensitive to temperature, light, and moisture conditions during infection (W. A. Cowling and D. G. Gilchrist, *unpublished*). Temperature was shown to have a differential influence on disease caused by the cool-temperature and the warm-temperature (Eastern North America) biotypes of *S. botryosum*. Disease caused by the cool-temperature biotype was favored at 8–16 C but severely inhibited at 23–27 C, whereas the warm-temperature biotype caused severe disease at the higher temperature range (8).

The effect of light on Stemphylium leaf spot of alfalfa has not been reported in the literature, although light has been observed to affect disease development in many other host-parasite interactions. Shorter day length or darkness (10,12, 16,22) or decreased light intensity (14,23) is reported to increase disease severity. However, a reduction in light intensity decreases severity of Leptosphaerulina leaf spot of alfalfa (17), whereas the severity of anthracnose of alfalfa (24) is reported to be independent of light intensity. The light sequence after inoculation greatly affects total leaf necrosis caused by *Rhynchosporium secalis* (Oud.) J. J. Davis on barley leaves (21).

The objectives of this study were to determine light and moisture conditions favorable for reproducing, under a controlled environment, the severity and symptom appearance of Stemphylium leaf spot (caused by the cool-temperature biotype) as observed in the field in California (8). This information was crucial for the consistent reproduction of the high disease severity levels required for genetic and physiologic studies of this disease under a controlled environment (6–8). The results in this paper are limited to the cool-temperature biotype of *S. botryosum* on alfalfa.

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MATERIALS AND METHODS

Host and pathogen material. The origin and maintenance of the susceptible clone of alfalfa (S2) used in this study were described previously (7). Monoconidial isolates of *S. botryosum* (HV1, HV2, HV3, and HV4) were selected from our collections made throughout central and northern California on the basis of their high relative virulence on alfalfa (7). All isolates produced symptoms typical of the cool-temperature biotype of *S. botryosum* (8). The virulence of inoculum on a susceptible clone was confirmed before or during each experiment by comparison with appropriate control isolates with known high and low virulence.

Disease production and assessment. Methods for the preparation of inoculum; growth and inoculation of plants; measurement and analysis of disease severity recorded as percentage of leaf area necrotic (LAN) on leaves two, three, and four from the stem apex; and measurement and analysis of average leaf and lesion areas on leaf three were the same as used previously (7), except where noted. Temperature during infection was 18–20 C (8), except in light experiments in plant enclosures, which were conducted at 20–22 C.

Diseased leaves were excised from the plant and mounted directly on herbarium paper by fully covering leaves with clear adhesive tape before disease assessment. This method permitted rapid mounting of leaves and preserved the contrast between green, lesion-free areas and bleached, necrotic lesions on leaves for more than 2 yr (D. G. Gilchrist and A. N. Martensen, *unpublished*). Mounted leaves were assessed visually for percent LAN, percentage of leaf area chlorotic (LAC), and number of hypersensitive-like spots (HLS). Leaf area was measured directly on mounted leaves with an area planimeter. Percent LAN and LAC data were arc-sine transformed for analysis of variance as described previously (7).

Light studies. The effect of various light sequences on disease severity was tested in the controlled environment growth chamber described previously (7). Two experimental procedures were used to assess the direct effect of light on the disease severity observed.

In the first procedure, plants were exposed to different light treatments in separate enclosures inside the growth chamber. The frames of the plant enclosures (30 × 48 × 56 cm), constructed from wire and wooden dowels, were designed to cover eight plants (one experimental unit [7]) of the susceptible alfalfa clone. Lamps (cool-white fluorescent) were kept on continuously,

and dark periods were created by covering the frames with black cloth hoods. Three units of plants were equilibrated for 72 hr under each of the following light regimes: continuous light and 12-hr light/12-hr dark sequences ending with either 12-hr light or 12-hr dark immediately before inoculation. Each unit was then inoculated with 25 ml of a conidial suspension of *S. botryosum* isolate HV1, using a standard experimental procedure (7), and the enclosures were covered immediately with plastic bags to maintain a humid environment. Trays beneath the enclosures were flooded with water to aid in maintaining humidity during the infection period. Plant units from each preinoculation light treatment were then exposed to one of three postinoculation light treatments, continuous light or 12-hr light/12-hr dark sequences beginning with either 12-hr light or 12-hr dark immediately after inoculation. Plastic bags were removed at 48 hr, and disease severity was assessed 7 days after inoculation by counting the number of lesions per leaf.

Alternatively, with a second method, certain postinoculation light treatments were repeated without plant enclosures in separate experiments in the growth chamber to assess potential indirect effects of the microenvironment of the enclosures on the observed disease severity. The light/dark treatments were created by using a clock-timer hooked to the chamber lights, and humidity was maintained by a mist system as previously described (7). This system permitted only one treatment to be imposed during each infection period as follows: a) continuous light, b) continuous dark, c) 12-hr light/12-hr dark sequences beginning with 12-hr light, and d) 12-hr light/12-hr dark sequences beginning with 12-hr dark immediately after inoculation. Each of the above treatments was initiated following a 72-hr preinoculation sequence of 12-hr dark/12-hr light. The mist system was kept on for 48 hr, the light treatment for 72 hr, and disease severity was assessed 7 days following inoculation.

Temperature, light, and relative humidity (RH) were measured in both procedures on a time-course basis. RH was determined with a calibrated thermoelectric dew point hygrometer (model 880, EG&G International, Waltham, MA 02154). Light intensity (380–700 nm) was measured at soil level with a filtered pyrheliometer (model 50, Eppley Laboratory, Newport, RI 02840).

Preliminary experiments revealed that 70% of the conidia on the leaf surface in both procedures had germinated by 2 hr after inoculation at 18–20 C and approached a maximum of 98–100% by 24 hr. Thereafter, conidial germination was evaluated at 24 hr by vertical epi-illumination microscopy. For each isolate in each experiment, 200 conidia per leaf were observed on each of the first to fifth leaves from the stem apex. Preliminary experiments also showed that leaf penetration by germ tubes, which began 7–12 hr after inoculation, approached a maximum value by the end of the 48-hr mist period. Thereafter, stomatal penetration was determined on leaflets sampled from leaf position three 48 hr after inoculation in each experiment. Leaflets were cleared in chloral hydrate solution, stained with aniline blue, destained, mounted permanently as reported for Stemphylium leaf spot of birdsfoot-trefoil (9), and examined by phase contrast microscopy. In each experiment, the mean lesion area on leaf three was measured and analyzed by the square-root transformation described previously (7).

Moisture studies. The effect on disease severity of extending the period of free moisture on leaves from 1 to 4 days was tested using the mist system. The most effective light treatment was used, that is, 12-hr light both before and after inoculation followed by 12-hr dark/12-hr light cycles. Following inoculation of four experimental units with isolate HV4, one unit was removed to an equivalent low-humidity growth chamber every 24 hr for 4 days. Seven days after inoculation, three leaves per stem from three stems per plant were excised, taped immediately to herbarium paper, and then assessed for percent LAN, percent LAC, and HLS per leaf.

RESULTS

Relative humidity and light inside plant enclosures. The RH of air inside plant enclosures increased to a maximum of 85–95% in less than 8 min after inoculation of plants, and droplets of moisture remained on leaves for the 48-hr infection period. Cloth hoods, placed over the frames of plant enclosures for 12-hr dark periods, did not affect RH or temperature inside the enclosures compared with the equivalent light period, whether or not the enclosures were covered with airtight plastic bags. Light intensity (380–700 nm) in the growth chamber (410 W/m^2) was 7.6% of full sunlight at noon on 28 July in Davis, CA, and was reduced to 4.8% of full sunlight by plastic covers over the plant enclosures.

Effect of light on disease severity in plant enclosures. Different preinoculation and postinoculation light sequences greatly affected disease severity, despite the relatively low light intensities inside the growth chamber (Table 1). Continuous light or 12-hr light immediately before inoculation resulted in more than twice the number of lesions per leaf than 12-hr dark regardless of the postinoculation light treatment. Similarly, postinoculation light sequences beginning with 12-hr light after inoculation resulted in significantly more lesions than sequences beginning with 12-hr dark. Continuous light after inoculation virtually prevented lesion formation. The experiment was repeated at the same temperature, and similar results were obtained.

It should be noted that the disease severity levels observed (Table 1) were lower than those previously reported on alfalfa clone S2 inoculated with high-virulence isolates of *S. botryosum* (7). However, the results of this experiment are in agreement with those reported in the next set of experiments, where certain postinoculation light treatments were evaluated under free leaf moisture conditions in the growth chamber and disease severity levels were much higher.

Effect of light on disease severity under mist. The percentage of germination of conidia of isolates HV2 and HV3 on the leaf surface 24 hr after inoculation varied from 85 to 95% in four experiments but was not significantly affected by the post-inoculation light regime. As reported previously for this disease (6), leaf penetration by germ tubes from the pathogen occurred exclusively through

Table 1. Effect of selected preinoculation and postinoculation light sequences on disease severity of Stemphylium leaf spot of alfalfa

	Average number of lesions per leaf[q]			
	Postinoculation light sequence[r]			
Preinoculation light sequence	12-hr light/ 12-hr dark	12-hr dark/ 12-hr light	Continuous light	Mean
12-hr dark/ 12-hr light	6.4 v	2.2 xy	0.1 z	2.9 m
12-hr light/ 12-hr dark	2.9 wx	0.8 yz	0.0 z	1.2 n
Continuous light	4.1 w	3.9 wx	0.4 z	2.8 m
Mean	4.5 a	2.3 b	0.2 c	

[q]Lesions on the second, third, and fourth leaves from the stem apex were recorded 6 days after inoculation on 75 leaves per treatment; values followed by a common letter (range v–z for individual treatments, ranges a–c and m–n for respective row and column means) do not differ ($P = 0.01$) according to Duncan's multiple range test.

[r]The 12-hr dark periods were created by covering the experimental units with black cloth in a continuously illuminated growth chamber. The temperature (20–22 C) and humidity (85–95%) were the same in all treatments.

stomata. Stomatal penetration occurred at the same frequency at 48 hr (0.18 penetrations per conidium observed on the leaflet surface after the staining procedure) under the light sequence producing maximum disease severity (12-hr light immediately before and after inoculation followed by 12-hr dark/12-hr light cycles) as under the light treatment producing lowest disease severity (continuous light after inoculation). Light affected disease severity through changes in the number and not the size of lesions on leaves, except for continuous light after inoculation, which decreased both lesion number and size (Table 2).

Effect of duration of mist on disease severity. The duration of the period during which free moisture remained on the leaf surface greatly influenced disease severity and the visual appearance of disease symptoms. Disease severity increased but average lesion area remained constant as the mist period was extended from 1 to 4 days (Fig. 1). Average leaf area (4.3 cm^2) did not vary significantly among mist treatments. Therefore, the increase in disease severity was the result of an increase in the number and not the size of lesions on leaves, as it was for the effect of light. In addition, percent LAC and HLS/cm^2 of living leaf tissue increased exponentially when the mist period was extended longer than 2 days. There were significant correlations between percent LAN, percent LAC, and HLS/cm^2 on plants of clone S2 after 48- and 72-hr mist ($r_{[6]} \geq 0.9$). The high levels of leaf chlorosis and spotting observed after 3 and 4 days of mist in the growth chamber were atypical of symptoms of Stemphylium leaf spot observed in the field in California (6,8).

DISCUSSION

Disease severity, resulting from infection of alfalfa with the cool-temperature biotype of *S. botryosum* at 18–20 C in the growth chamber, was extremely sensitive to light sequences before and after inoculation as well as to the duration of the moist period. A 48-hr mist period was observed to produce high disease severity levels and symptom appearance most like those observed in the field in California. Longer mist periods resulted in a high degree of leaf chlorosis and hypersensitive-like spotting not seen under field conditions. One light sequence consistently resulted in higher disease severity levels than any other: 12-hr light before inoculation followed by 12-hr light/12-hr dark cycles after inoculation. In contrast, continuous light after inoculation almost completely inhibited disease symptom development at an intensity of 5–8% of full sunlight.

These results may explain our inability (6–8,11) to reproduce the results of earlier genetic and physiologic studies of this disease in California (1–5). In the previous work, alfalfa leaves were exposed to continuous light (4,5) or dark (2,3) during 72-hr or longer infection periods. Our studies indicate that such conditions would produce low disease severity levels and symptoms atypical of the disease in the field.

Differences in disease severity resulting from various moisture periods were caused by change in the number, but not the average size, of lesions on leaves. This further illustrates a major difference between the warm-temperature (eastern) and the cool-temperature (California) biotypes of *S. botryosum* (8); average lesion size caused by the former biotype increases with duration of the moist infection period (20), but lesion size caused by the latter did not in these studies. Expansion of lesions caused by the warm-temperature biotype results in the characteristic concentrically ringed pattern observed in the field (8,19). Lesions caused by the cool-temperature biotype do not expand once formed, but the abrupt border does become dark brown with continued exposure to mist (8). These results are consistent with a previous report for the cool-temperature biotype that showed that neither pathogen virulence nor inoculum concentration affected average lesion size (7). Thus, lesion size remains a stable feature of the disease in California.

The possibility that the effect of light on disease produced in plastic bags was confounded with changes in gas (eg, carbon dioxide or oxygen) concentrations was eliminated when similar results were obtained in a ventilated growth chamber using a misting apparatus.

The frequency of stomatal penetration by the fungus was equal in the light treatments that resulted in the highest or lowest disease severity levels, indicating that light affected the infection process after penetration. This contrasts with a report for conidia of *R. secalis* on barley, in which the rate of production and

Table 2. Effect of selected postinoculation light/dark sequences on severity and average lesion size of Stemphylium leaf spot of alfalfa

Postinoculation light sequence[q]	Disease severity (%)[r]		Lesion size[s] (mm^2)
	Isolate HV2	Isolate HV3	Isolate HV2
Experiment 1 12-hr dark/ 12-hr light/...	6.1 c	5.5 c	0.58 y
Experiment 2 12-hr light/ 12-hr dark/...	17.4 b	23.5 a	0.58 y
Experiment 3 Continuous light...	2.4 e	1.4 e	0.34 z
Experiment 4 Continuous dark...	5.0 d	7.6 c	0.49 yz

[q] Plants in each experiment received a 12-hr dark/12-hr light cycle ending with 12-hr light before inoculation, followed after inoculation by the light sequences indicated.

[r] Disease severity was assessed 7 days after inoculation as the percentage of leaf area necrotic. Each value is the mean of 120 leaves; values sharing a common letter do not differ ($P = 0.01$) according to Duncan's multiple range test of data transformed by arc sine.

[s] Each value is the mean area of 150 randomly selected lesions per light treatment; values sharing a common letter do not differ ($P = 0.01$) according to Duncan's multiple range test of square-root transformed data.

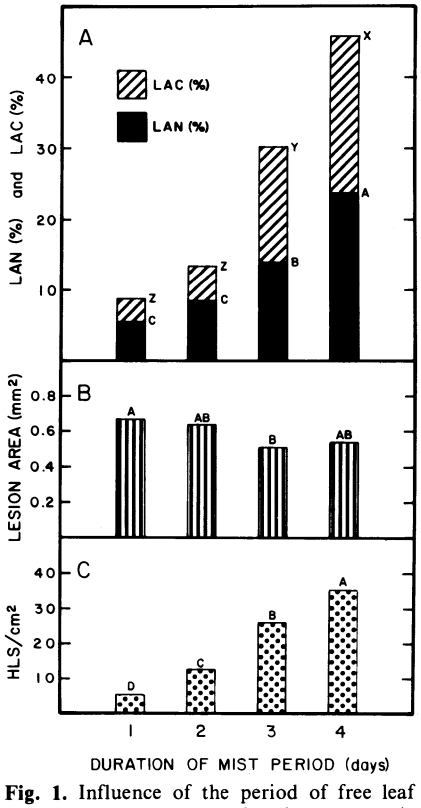

Fig. 1. Influence of the period of free leaf moisture on percentage of leaf area necrotic (LAN) and percentage of leaf area chlorotic (LAC), mean lesion area, and density of hypersensitive-like spots (HLS) on leaves of alfalfa clone S2 inoculated with isolate HV4 of *Stemphylium botryosum*. **(A)** Each percent LAN and percent LAC bar is the average visual rating of 72 leaves. **(B)** Each lesion area bar is the mean of 250 lesions. **(C)** Each HLS/cm^2 bar is the average count on the same leaves rated in (A). Values sharing a common letter do not differ ($P = 0.01$) according to Duncan's multiple range test of data analyzed by arc sine (A) or square-root (B) transformation.

elongation of germ tubes decreased when 12-hr light was substituted for 12-hr dark immediately after inoculation (21).

Inhibition of disease by continuous light after inoculation has been reported for *Alternaria solani* (Ell. & G. Martin) Sor. on potato (12) and *Phytophthora drechsleri* Tucker on safflower (22). In fact, many published reports indicate that longer photoperiods (10,16,22), higher light intensities (14,23), or substitution of light for dark periods after inoculation (21) decreases disease severity. To our knowledge, this is the first report that describes an effect of photoperiod sequences both before and after inoculation on plant disease severity levels, where plants in each treatment were exposed to the same total amount and quality of light. Although these studies were not designed to permit conclusions regarding the optimum periods of light before and after inoculation, it is possible to infer that not only light before but also a dark period within 12–24 hr after inoculation are required for expression of high disease severity levels.

The events responsible for the large differences in disease severity between light treatments appear to be quite light sensitive, because they were influenced at intensities of 5–8% of full sunlight. It would be interesting to speculate that the effect of light on disease may arise from an effect on the phytoalexin response in the host (15). However, extensive fungal growth in leaf tissue does not occur before or during lesion formation by the cool-temperature biotype (6). Therefore, a light-activated host defense system, based on the classical role of phytoalexins as fungistatic agents, may not afford an explanation for our observations.

The consistently high disease severity levels achieved under the environmental conditions used in a previous study (7) and investigated in more detail here have permitted the detection of genetic variability in pathogen virulence (7) and host resistance (6).

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