Infection of Milk Thistle (*Silybum marianum*) Leaves by *Septoria silybi*

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ABSTRACT

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Septoria silybi infected milk thistle (*Silybum marianum*) frequently when daylight was provided during high humidity inoculation periods but rarely when light was excluded, because of the pathogen's requirement for open stomata to penetrate thistle leaves. When stomata were artificially closed with abscissic acid, *S. silybi* failed to penetrate even during daylight. Conversely, the application of fusicoccin opened stomata on thistle leaves kept in the dark and permitted penetration. Hyphae of *S. silybi* encountered stomata as frequently on milk thistle leaves held in the dark as on those exposed to daylight. Hydrotropism, the directed growth of germ tubes and branches toward stomata, was not exhibited by *S. silybi*. Superficial hyphae reached or crossed stomatal pores with similar frequency, irrespective of whether humidity gradients might have existed at such sites or not. *S. silybi* infected milk thistle after periodic (8 hr/day for 6 days) or prolonged (continuously for 2.5 days) postinoculation drying. The amount of disease occurring on plants exposed to periodic drying was comparable to those incubated continuously at high humidity. Severity of disease resulting from inoculation with a given number of spores of *S. silybi* increased proportionally with leaf age. Whereas *S. silybi* infected young and old leaves of milk thistle with similar efficiency, the rate of lesion expansion was 2.5 times higher on old leaves than on young leaves.

Additional keywords: biological control, mycoherbicide.

Milk thistle (*Silybum marianum* (L.) Gaertner) is widespread in California, where it invades land disturbed by grazing, machinery, or natural erosion. While most troublesome in pastures and rangeland, it also grows in fallow croplands and in wasteland such as ditchbanks, field margins, railroad right-of-ways, and roadsides. This species not only limits productivity of infected fields but also poses a major hazard to animal health by injuring grazers with its long thorns. Milk thistle can be controlled by cultural practices that prevent seedling establishment, by mechanically killing thistles before bolting and seed set, and with broad-spectrum herbicides. Three aspects of this weed make it a good candidate for biocontrol by plant pathogens. First, this weed grows as a winter annual in California, when conditions for infection of the plant are good. Second, milk thistle reproduces only by seed. If a pathogen can prevent the production of viable seed, then the weed cannot persist from one year to the next. Third, chemical control is not usually economical in pastures and rangeland. In addition, there is no herbicide selective for milk thistle. Available chemicals also kill beneficial broad-leafed pasture species such as clover. There is much current interest in the use of plant pathogens as weed management agents. Considerable attention has focused on the use of endemic plant pathogens applied infrequently but at inundative doses to hosts at a susceptible stage and under appropriate environmental conditions (bioherbicide approach) (21,22). Successful and consistent management of weeds with plant pathogens will require a thorough understanding of the biological and epidemiological features of the pathogen being used.

We have observed a disease of milk thistle caused by *Septoria silybi* in numerous locations in central California over a period of several years, indicating that this fungus is a highly successful pathogen of its host. Saccardo (17) and Oudemans (11) list *S. silybi* as the only *Septoria* pathogen of milk thistle and give *S. marianum* as the sole host known for *S. silybi*. Except for Saccardo's and Oudemans's descriptions, no other publication has dealt with a *Septoria* of milk thistle. We, therefore, have studied the infection of milk thistle by *S. silybi* to determine its suitability as a biological control agent. During initial studies on the potential of *S. silybi* as a biological control of milk thistle in California pasture, infection was found to be much greater on thistles that received sunlight during high humidity inoculation periods than on those that did not. Particular attention was placed on the interaction of light with other environmental factors on this pathogen and its host because of this important epidemiological trait.

MATERIALS AND METHODS

Conidia of a strain of *S. silybi* originally collected from a diseased milk thistle leaf near Jackson, CA, were produced on V8-Elliott agar (0.36 g of KH_2PO_4, 1.06 g of Na_2CO_3, 0.5 g of $MgSO_4$, 5 g of glucose, 1 g of asparagine, 16 g of agar, 200 ml of V-8 juice, 800 ml of H_2O) cultures incubated alternatively for 12 hr at 18 C in the light and 11 C in the dark. Plates were inoculated by spreading mycelial fragments over the surface. Once the petri plate cultures formed abundant cirri (after about 10 days), the plates were frozen at −8 C until needed for inoculum harvest. Spores were removed from plates by swabbing with a sterile cotton applicator in a small volume of sterile water.

In vitro spore germination and extension. Spore concentrations of suspensions were determined with a hemacytometer and diluted to a concentration of 10^6 spores/ml in 1:1 potato-dextrose broth-sterile distilled water containing 30 μg/ml each of streptomycin and chloramphenicol. Drops (0.02 ml) of the suspension were incubated in hanging drop slides in the dark, and the slides were placed in seven different incubation chambers maintained at temperatures between 5 and 37 C. All temperature treatments were accomplished simultaneously and with drops of spores from the same initial suspension. Germination of 300 spores was recorded after 18 and 24 hr for each treatment. Spores with germ tubes extending 2.5 μm or longer were considered germinated. The cover slips were removed from hanging drop slides and placed on a hemacytometer so that germ tube extension could be estimated with the hemacytometer's 50-μm grid. The germ tube length for a given spore was defined as the sum of the lengths of all the germ tubes growing from that spore.

Plant inoculations. Milk thistle seedlings were grown to a height of about 15 cm in a sand-peat mixture from seeds collected near Berkeley, CA. Plants normally contained three to five sets of

leaves at the time of inoculation. Spores were suspended in distilled water and concentrations adjusted with a hemacytometer. With the exception of microscopic studies, plants having leaves of different ages were inoculated by spraying spore suspensions with an air-pressurized hand sprayer until runoff. Cotyledons were distinguished from the oldest true leaves (old leaves) and from the youngest set of true leaves (young leaves). Inoculum concentration in all experiments was 2×10^4 spores/ml except where noted. Spores were applied precisely to adaxial leaf surfaces in experiments involving microscopic examination of postinoculation events. Four inoculation loci were outlined with water-resistant ink on each leaf of the first true leaf pair (old leaves), and a 15-μl drop of a 10^6 spores/ml suspension was applied by pipette to each locus. Plants inoculated either by spraying or with a pipette were placed immediately in premoistened clear or opaque black plastic bags and closed tightly. The bagged thistles were then placed in a greenhouse maintained at 16 C for the duration of the treatment period. Bags were opened and closed twice daily to permit thorough gas exchange. In treatments where high humidity was interrupted daily, plants were removed from bags at 8:00 a.m., placed in a greenhouse maintained at 21 C, and watered carefully so that leaves were not wetted. Plants were remoistened with a mist of sterile distilled water and returned to premoistened clear or black plastic bags at 4:00 p.m. After treatment periods that ranged from 2 to 6 days, sets of thistles were maintained uncovered in a greenhouse at an average temperature from 21–24 C. Relative humidities were measured with a wet-bulb psychrometer. Nighttime temperatures in both clear and dark plastic bags were 16.5–17 C. Midday temperature was 25–26 C in dark bags and 26–27 C in clear bags. The relative humidity was 100% during both day and night in both clear and dark bags.

The percentage of individual leaves that comprised necrotic lesions typical of *Septoria* infection were estimated using visual assessment keys depicting 0, 3, 6, 12, 25, 35, 50, 65, 75, 88, 94, 96, and 100% disease. The keys for cotyledons and old leaves were diagrammatic while the key for young leaves consisted of photographs of actual diseased leaves. Both sets were verified as to true disease severity with a microcomputer-controlled video image analysis system (10).

Lesion development. Lesion expansion on young and old leaves of individual plants was compared. The two lesions of a pair were located at similar positions on their respective leaves, e.g., very top, top, middle, or bottom portion of a leaf, and both lesions were selected to be the same initial size. To ensure that selected lesions were discrete and would not coalesce with others, plants were spray inoculated with a spore suspension containing only 50 spores/ml. Lesion diameters were remeasured with a precision of 0.5 mm 4 days after lesion pairs were established.

Modification of stomatal aperture. Fusicoccin from Italchemia S.A. and ± cistrans abscissic acid (ABA) were used to artificially open and close stomata, respectively. Fusicoccin (10^{-2} M), ABA (10^{-4} M), and distilled water used as a control were all applied to leaves in two ways. In a leaf injection method, the mesophyll of leaves left intact on thistle plants was infiltrated through abaxial surfaces with a syringe and needle until the undersides of leaves at and around inoculation sites became watersoaked. With a cut leaf method, the petioles of leaves cut from thistles were immediately placed in vials containing the test solution. Fusicoccin and distilled water were applied at 2.5 days after inoculation to leaves of thistles maintained in dark bags. One hour after application, thistles with treated leaves and vials containing cut leaves were returned to dark bags and incubated at 16 C until samples were taken 36 hr later. ABA and distilled water were applied 3 days after inoculation to leaves of thistles that had been in clear bags the last day but in dark bags the first 2 days after inoculation. Thistles with infiltrated leaves and vials containing cut leaves were returned to clear bags after ABA application, maintained at 16 C, and subjected to 10 hr of daylight, 10 hr of darkness, and then 10 hr of daylight.

The condition of stomata (whether wide open, partially open, or closed) was determined by microscopic examination of epidermal strips. Strips excised with a razor blade and removed with fine forceps were placed immediately in a drop of microscope immersion oil on a glass slide and then covered with a coverslip, as described by Hsiao and Fisher (9).

In vitro spore germination and germ tube extension was measured for conidia of *S. silybi* suspended in potato-dextrose broth to which either ABA (to concentrations of up to 10^{-3} M) or fusicoccin (to concentrations of up to 10^{-2} M) was added. Germination and germ tube growth were unaffected by 10^{-3} M ABA or 10^{-2} M fusicoccin. The extent and pattern of spore germination and superficial hyphal growth also appeared normal on all leaves treated with ABA or fusicoccin.

In vivo spore germination. Spore germination on intact leaves was measured with parlodion films. A drop of parlodion (parlodion 4%, w/v, in ether-absolute alcohol 1:1, v/v) was applied with a glass rod to an inoculation locus 3 days after inoculation. Once the film dried (after about 20 sec) it was peeled from the leaf and mounted in lactophenol containing 0.15% cotton blue. When a repeated impression was made of an inoculated leaf surface, no additional spores were recovered. Spores with germ tubes greater than or equal to 2.5 μm in length were considered germinated.

Leaf surface hyphal growth and stomatal penetration. Inoculated leaf tissue was cleared and stained for observation of superficial hyphal growth and host penetration. Inoculation loci were excised with a cork borer 2–4.25 days after inoculation. The 1-cm-diameter leaf disks were cleared for 2 hr in Carnoy's solution (1:2 acetic acid-absolute alcohol), rinsed in water, and mounted in lactophenol with 0.15% cotton blue. Three or four leaves (replications) were used per treatment in the experiments, and four disks were taken from each. The trials relating light and high humidity to superficial hyphal growth and host penetration involved three measurements for each of several adjacent fields of view (×400) observed for each leaf disk: the number of stomatal pore crossings, the number of those crossings with penetration, and the total hyphal growth. The total length of all the hyphae in a field was quantified as the number of field of view diameters to which each hypha could be estimated to extend. Individual hyphae, which were usually very difficult to distinguish, were not enumerated. Enough fields of view were examined on the four leaf disks of each replication so that all replications included a total estimated hyphal growth of between 50 and 60 fields. In the experiments where stomatal aperture was modified by ABA or fusicoccin, only stomatal pore crossings and the number of crossings with penetration were counted. In these cases, enough fields of view were examined for all replications to include a total of between 45 and 80 observed stomatal crossings. A hypha was considered to cross a stomatal pore only if it reached or crossed the aperture and not if it traversed only guard cells.

Statistical methods. Statistical computations were made with software provided by Statistical Analysis Systems (SAS Institute Inc., Cary, NC). The SAS general linear models procedure (GLM) was used to perform analysis of variance on untransformed observations of disease severity, stomatal crossings by hyphae, and penetration. With the exception of differences in lesion expansion, which was assessed with a paired *t*-test, main effect differences were assessed with *F*-tests.

RESULTS

Spore germination and hyphal growth. Spores of *S. silybi* germinated and hyphae grew over a wide temperature range in culture (Fig. 1). Both spore germination and germ tube elongation occurred at temperatures between 5 and 33 C. Maximum frequencies of spore germination occurred between 12 and 33 C. However, germ tube elongation was most rapid at between 18 and 25 C in culture (Fig. 1). Germ tubes were observed to fragment at temperatures above 30 C, and this disruption of hyphae resulted in a lower total hyphal length for a given spore.

Effect of sunlight and stomatal aperture on infection. Disease severity was much greater on thistles exposed to light than on

thistles kept in continuous darkness during incubation at a high humidity (Table 1). The severity of disease at 12 days after inoculation of plants held in the dark and inoculated with 2×10^4 spores/ml was even significantly less than the disease severity of plants exposed to light that were inoculated with only 2×10^2 spores/ml for both old leaves ($F = 5.25$, $P = 0.03$) and young leaves ($F = 29.35$, $P = 0.0001$). Increased disease severity in the light was not due to increased spore germination or more frequent stomatal pore crossings. Germination of spores of *S. silybi* within 3 days after inoculation averaged 68.5% on leaves kept in continuous darkness and 64.8% on leaves exposed to daylight during moist treatments and did not differ significantly ($F = 1.20$, $P >> 0.10$). Similarly, the number of hyphae of *S. silybi* that reached or crossed stomatal pores on leaves kept in the dark and on leaves exposed to light did not differ ($F = 0.44$, $P > 0.1$) (Table 2). The oil immersion technique used to examine stomatal apertures gave consistent results, and the size of apertures were constant for at least 30 min after tissue preparation. In addition, it was possible to observe superficial hyphal growth and host penetration by taking strips of inoculated epidermis and mounting them in oil. Differential interference contrast microscopy of these specimens yielded good images of fungal and plant structures unaffected by any fixing, clearing, or staining processes.

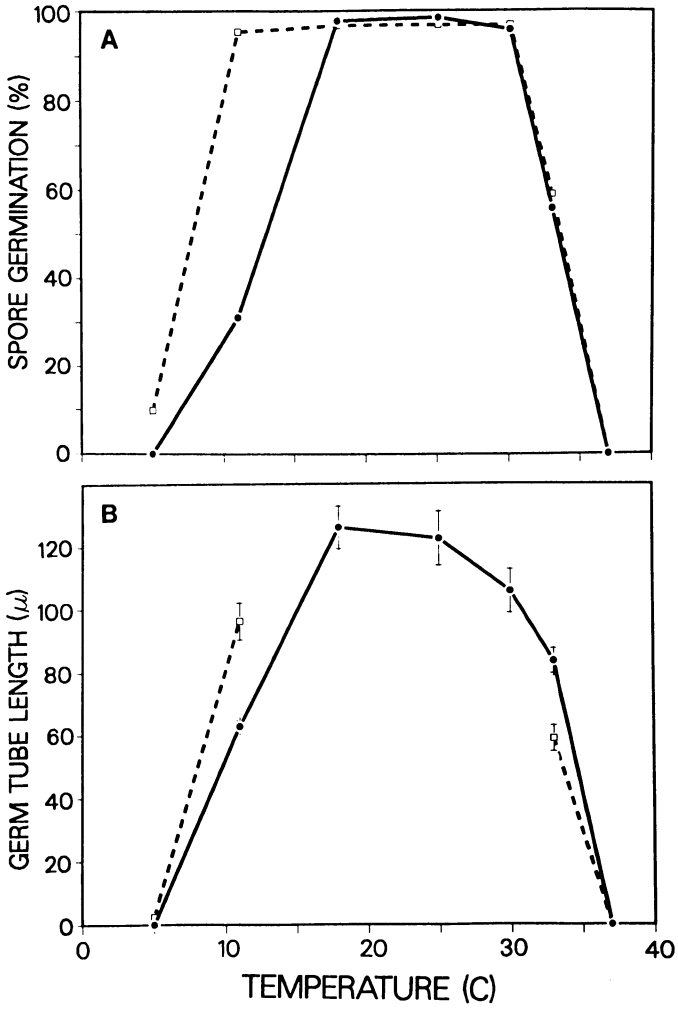

Fig. 1. A, Germination and germ tube elongation of *Septoria silybi* at different incubation temperatures (5, 11, 18, 25, 30, 33, and 37 C). Values for spore germination after 18 hr (●) and 24 hr (□) are means for three replicate hanging drop samples in which 300 spores were examined for each sample. **B,** The mean total length (μm) of germ tubes from 28 different spores assayed in each of three replicate samples was measured after incubation at the different temperatures shown on the abscissa for 18 hr (●) and 24 hr (□). Germ tube length at 24 hr for spores incubated between 18 and 30 C was not quantified exactly, but was greater than 140 μm.

Penetration of milk thistle stomata by *S. silybi* was frequently observed on plants exposed to light (41.6% of pore crossings) but was rare for plants incubated in continual darkness (Table 2). Entry of *S. silybi* into plants was observed only at stomata. Hyphae penetrated stomata without appressoria. The hyphae appeared somewhat constricted as they passed through the stomatal aperture, returning to normal diameter once through. Superficial hyphae did not terminate at such stomata but extended beyond. Substomatal vesicles were not observed. Penetration of stomata occurred as early as 40 hr after inoculation on plants incubated continuously at high humidities.

Treatment of milk thistle leaves with ABA prevented penetration of hyphae into plants exposed to daylight. The treatment of milk thistle leaves with ABA (10^{-4} M) caused stomata to close within 1 hr of application even though they were exposed to light. All stomata remained tightly closed for the duration of the 30-hr treatment. The stomata of intact and excised water-treated control leaves that were also exposed to daylight were always wide open by 8:00 a.m. and remained wide open until at least approaching darkness at 6:30 p.m. By 8:30 p.m., the stomata were all closed. All stomata remained closed on plants incubated in continual darkness. While hyphae penetrated stomata of water-injected leaf tissue on the majority of pore crossings (57.9%), they did so very rarely on ABA-treated tissue (0.4%). The difference was highly significant ($F = 23.88$, $P < 0.01$). The results for treatments of excised leaves were similar; ABA reduced the percentage of stomata penetrated by *S. silybi* to 0.5% compared with 35.5% for leaves treated with water alone.

Application of fusicoccin to milk thistle leaves permitted their

TABLE 2. Stomatal pore crossings and penetration by hyphae of *Septoria silybi* on leaves of milk thistle incubated in the light or kept dark under moist incubation conditions[a]

Light	Stomatal crossings/field[b] (no.)	Stomatal crossings with penetration (%)
+	0.570	41.6
−	0.614	0

[a]Samples were taken at 4 days after inoculation. The means reported are for three leaf disks per treatment, with each disk coming from a different leaf.
[b]Microscope field of view diameters were used to estimate hyphal growth.

TABLE 1. Disease severity[a] of leaves of different ages on milk thistle plants inoculated with *Septoria silybi* and incubated in a moist chamber in the presence and absence of light

Days after inoculation[b]	Old leaves			Young leaves		
	2×10^4 spores/ml		2×10^2 spores/ml	2×10^4 spores/ml		2×10^2 spores/ml
	+ Light	− Light	+ Light	+ Light	− Light	+ Light
10	21.6 ± 3.6	3.0 ± 0.6	3.1 ± 0.9	18.0 ± 3.0	0.6 ± 0.3	3.3 ± 1.0
12	79.6 ± 5.8	12.8 ± 3.2	26.6 ± 5.3	40.2 ± 5.8	2.1 ± 0.6	17.3 ± 4.1

[a]The mean percentage of necrotic tissue on milk thistle ± the standard error are reported for the 28 separate leaves of each treatment.
[b]Disease severity was measured 10 and 12 days after inoculation. Inoculated plants were kept moist in clear and opaque plastic bags for 4 days following inoculation.

penetration by *S. silybi* even in the dark. Fusicoccin (10^{-2} M) altered stomatal apertures within 3 hr of treatment and continued to do so for the 36-hr treatment period in this study. It was necessary to use a relatively high concentration of fusicoccin since the stomata of epidermal peels placed in solutions of this compound opened only at concentrations of 10^{-3} M or greater. The degree of stomatal opening with fusicoccin was variable among stomata of a single leaf. Most pores were either wide or partially open, but some were closed. In contrast, the water-treated controls which were kept in darkness always had only closed stomata. Hyphae penetrated stomata of excised leaves treated with water and held in the dark on only 0.9% of the observed stomatal crossings. An increase in the frequency of stomatal penetration to 7.2% occurred after treatment of excised leaves with fusicoccin ($F = 4.53$, $P = 0.08$).

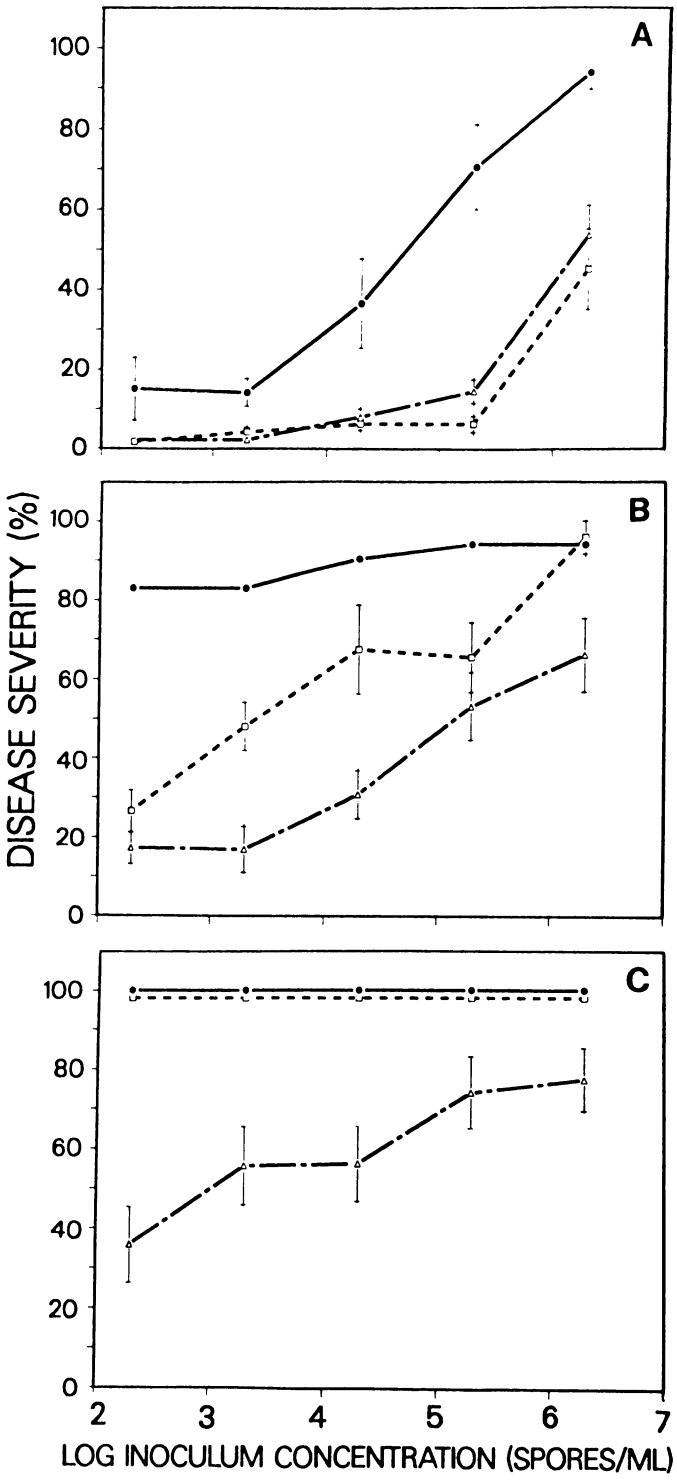

Fig. 2. Severity of disease caused by different numbers of spores of *Septoria silybi* on milk thistle leaves of different ages at different times after inoculation. Milk thistle plants were inoculated with the spore concentrations of *S. silybi* shown on the abscissa and incubated in high humidity chambers with alternating light and darkness for 4 days. The severity of disease on cotyledons (●), old true leaves (□), and young leaves (△) was assessed **A**, 9 days, **B**, 12 days, and, **C**, 18 days after inoculation. The vertical bars indicate the standard error of the mean percent of each of 12 replicate leaves for each treatment that was necrotic after the indicated incubation time.

Disease severity and inoculum dosage. Disease severity on milk thistles incubated in the light increased with spore dosage applied to leaves of three age classes (cotyledons, young leaves, and old leaves) and with time after inoculation (Fig. 2). Disease developed more rapidly on older leaves than on younger leaves. By 12 days after inoculation most cotyledons exhibited more than 80% disease severity even at inoculum concentrations as low as 200 spores/ml. On old and young true leaves at 12 days after inoculation, the severity of disease was much less than this amount for plants inoculated with as many as 2×10^5 *S. silybi* spores/ml (Fig. 2). Disease severity of these true leaves after 12 days was proportional to leaf age and inoculum concentrations between 2×10^2 and 2×10^6 spores/ml (Fig. 2). Nearly all cotyledons and old true milk thistle leaves were killed by 18 days after inoculation with 200 or more spores per milliliter. Even young leaves had considerable disease 18 days after inoculation with as little as 200 spores/ml (Fig. 2). Few plants were killed even by 18 days after inoculation with *S. silybi*. While cotyledons and at least

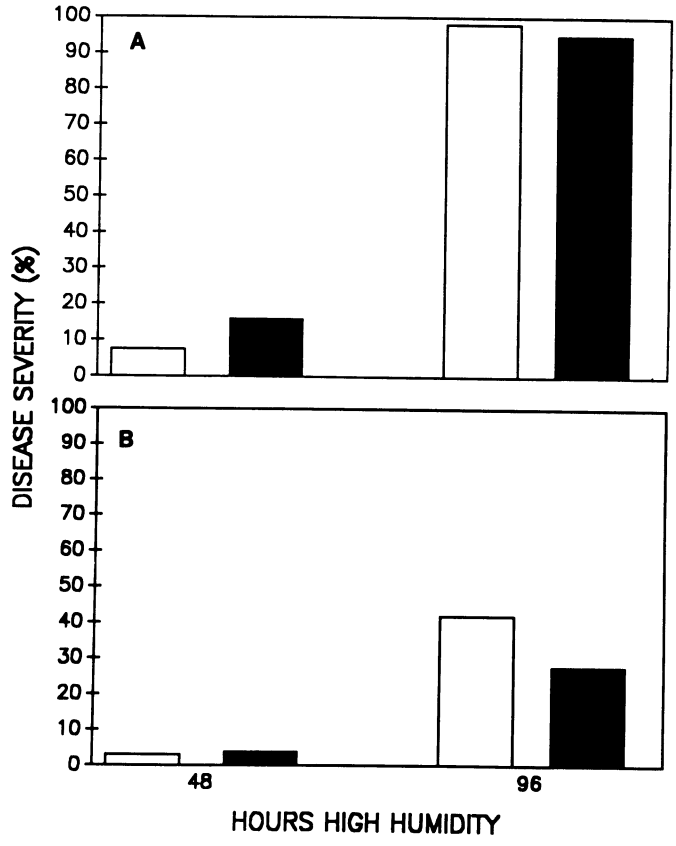

Fig. 3. Effect of duration and interruption of high humidity incubation of milk thistle leaves of different ages on severity of disease caused by *Septoria silybi*. Milk thistle plants with both **A**, old leaves and, **B**, young leaves were incubated in high relative humidity conditions continuously in clear plastic bags (open bars) or wetted discontinuously (solid bars) for 48 or 96 hr. Discontinuously wetted plants were alternately wetted for 16 hr in clear plastic bags and then allowed to dry in the light on a greenhouse bench for 8 hr. High humidity treatment periods totaling 48 and 96 hr were achieved in interrupted incubations after 3 and 6 days, respectively. Disease severity was assessed 10 days after inoculation. The mean percent of each of 22 leaves per treatment that was necrotic is reported.

the distal portions of all treated true leaves 1 cm or longer at the time of inoculation were killed, leaves smaller than this remained alive and permitted the survival of seedling plants.

Increased disease severity caused by *S. silybi* on older milk thistle leaves compared to younger leaves was associated with a more rapid rate of lesion expansion and not a higher number of lesions. A similar number of discrete lesions were observed on old and young leaves of milk thistle after inoculation with a given concentration of spores of *S. silybi* (data not shown). Lesions remained very small on young leaves, while they increased more rapidly in size with time on older leaves. Lesions of similar sizes (from 1 to 3 mm diameter) were paired on leaves of different ages on the same milk thistle plant and lesion diameter measured 4 days later. The rate of lesion expansion on old leaves (0.86 mm/day) was significantly higher than on young leaves (0.35 mm/day) when compared with a paired *t*-test ($t = 5.41$, $P < 0.001$).

Infection during interrupted moist periods. Milk thistle plants exposed to nightly wetting and daily drying were efficiently infected by *S. silybi* (Fig. 3). Significantly more disease developed on old leaves subjected to 48 hr of interrupted high humidity conditions than to those exposed to continuous moisture of the same duration ($t = 2.30$, $P < 0.05$). Greenhouse relative humidity fell to 37–48% and midday temperatures reached 29–33 C during the dry periods interrupting moist incubation. *S. silybi* infected milk thistle even when inoculum was allowed to dry for 60 hr after inoculation (data not shown). The severity of disease occurring on plants in which the moist incubation period was delayed up to 60 hr after inoculation did not differ appreciably from that of plants that received a moist incubation period immediately after inoculation (data not shown).

Stomatal pore crossing and penetration by hyphae of *S. silybi* occurred with comparable efficiency irrespective of whether leaves remained moist or were dried during exposure to daylight (Table 3). The frequency of stomatal crossings did not differ significantly between milk thistle plants removed to the dry greenhouse for 8 hr a day and those kept in the high humidity of clear plastic bags ($F = 3.37$, $P > 0.10$; Table 3). The percentages of pore crossings in which hyphae entered stomata were also similar on leaves kept continuously in high humidity to that on leaves receiving interrupted moisture treatments (Table 3).

DISCUSSION

Some fungal pathogens appear to require open stomata to penetrate their hosts. Light affects penetration of wheat by *Puccinia graminis* apparently by opening stomata (4). Infection was linearly related to the duration of light following a dark and moist period of at least 3 hr. Significantly less disease developed if stomata were kept closed by subjecting plants to water stress. Appressoria of *Mycosphaerella citri* form over stomata of citrus leaves just as often in continuous darkness as in a daily light/dark regime, but penetration of those stomata occurs much more frequently when the inoculated plants receive daylight (23). While this may have been due to light-induced stomatal opening, it is possible that slight temperature fluctuations within incubation chambers affected relative humidity and leaf wetness differently between the two treatments (23). Light intensity greatly affects infection of wheat by *Septoria tritici* or subsequent symptom development (3,7). Disease severity to plants incubated with *S. tritici* in the dark is greatly reduced (7). No studies of the role of light in the penetration of wheat by *S. tritici* have appeared, however. *Cercospora beticola* was prevented from penetrating sugar beet by spraying leaves with ABA, which kept stomata closed (6). These authors suggest that *C. beticola* penetrates principally open stomata. They note, however, that although ABA did not directly affect the growth and development of the fungus in culture and in planta, the possibility that it affected fungal development in some way could not be entirely discounted. Penetration of milk thistle by *S. silybi* is also prevented by application of ABA to host leaves. As with Feindt et al, we cannot entirely exclude the possibility that ABA influenced fungal morphogenesis in some way, even though in vitro conidium germination and germ tube growth were unaffected and fungal development appeared normal in planta. It is also possible that ABA had an effect on host physiology additional to closing stomata that may have influenced penetration by *S. silybi*. Normally, *S. silybi* rarely penetrates thistles during continuous darkness (Table 1). But when stomata were opened with fusicoccin, penetration occurred. Our results strongly suggest that *S. silybi* penetrates only stomata that are open.

TABLE 3. Stomatal pore crossing and penetration by hyphae of *Septoria silybi* on leaves of milk thistle that remained moist or were dried while exposed to daylight[a]

High humidity[b]	Stomatal crossings/field[c] (no.)	Stomatal crossings with penetration (%)
Continuous	0.748	38.5
Interrupted	0.654	40.0

[a]Values are means for three leaves.
[b]Milk thistle plants of both treatments were incubated in darkness and high humidity for the first 2 days after inoculation. Plants then either received daylight for 8 hr a day under conditions of high humidity in clear plastic bags or were incubated in a dry greenhouse. All plants were returned to darkness and high humidity each evening. Leaf disk samples were taken from leaves 4 days after inoculation when kept continuously moist or after 6 days from leaves whose surface moisture was interrupted by dryness.
[c]The mean number of microscope field of view diameters was used to estimate hyphal growth.

The response to open stomata by *S. silybi* may have been tactile, chemical, hydrotropic, or by another mechanism. A tactile infection response to stomata has been found for various rust fungi (18,19,24) and for hop downy mildew (16). If hyphae of *S. silybi* responded to milk thistle hydrotropically, then penetration might have been infrequent in fusicoccin treatments since there may not have been a humidity differential between stomatal pores and the air at the leaf surface. Although ambient RH in incubation chambers was 100% at all times and temperature differences between clear and dark bags were only 1 C or less, moisture conditions may have varied subtly at the leaf surface. We noticed that there was often more condensation on leaves in dark bags than in clear bags during daylight hours. It is possible that on the surface of light absorbing leaves in clear bags the RH was less than 100% and a slight humidity gradient between leaf surface and stomatal pores existed. The low frequency of penetration in the fusicoccin treatments might also be explained by incomplete stomatal opening.

Hydrotropism has been postulated for various fungal pathogens (2,5,8,13,14). The penetration of *Cercospora musae*, *Cercospora beticola*, and *Ramularia areola* is much greater under nighttime wetting/daytime drying than under continuous moisture (8,12,13). The investigators have attributed this phenomenon to hydrotropism, the directed growth of germ tubes and branches toward the moisture of stomata (8,13,14). Germ tubes and branches of *Cercosporidium personatum* and *Cercospora zeae-maydis* also show a positive tropism toward stomata on leaf surfaces without free water (where the relative humidity at the leaf surface might be less than 100%), but grow seemingly at random on leaves in the presence of free water (2,5). In our study, branching and growth of germ tubes of *S. silybi* did not appear directed in any way, and hyphae reached or crossed stomatal pores with similar frequency irrespective of whether leaves were exposed to continuous or discontinuous moisture in the dark where hydrotropism could not have occurred (Tables 2 and 3). Although hydrotropism (in the sense of growth toward open stomata) was not exhibited by *S. silybi*, it is still possible that the growth of hyphal tips into pores from hyphae already positioned above apertures was a hydrotropic or chemotropic response.

S. silybi regularly penetrated stomata of milk thistle when daylight exposure was restricted to periods of leaf dryness (Table 3). This result suggests that hyphae positioned over stomatal openings are able to branch and extend into open stomata when free moisture is lacking. The very high humidity at stomatal

openings may allow for sufficient extension of hyphae, which had grown in free moisture overnight, into the pores. Hyphae of *S. silybi* are capable of growth without free moisture in vitro. A small percentage (4.3%) of hydrated then briefly air-dried conidia of *S. silybi* germinated on coverslips kept in 100% humidity chambers (data not shown). The germ tubes of these conidia extended and branched to lengths of between 30 and 300 μm under the condition of 100% RH but no free moisture (data not shown). Hyphal extension on leaf surfaces lacking free moisture can also be inferred for those pathogens to which hydrotropism has been ascribed (2,5,8,13,14).

S. silybi exhibits many traits desirable in a pathogen to be used as a biological control agent of a weed such as milk thistle. The temperature range over which spores of *S. silybi* can germinate and germ tubes elongate is rather large. The ability of such spores to germinate and grow at temperatures as low as 5 C would be of utility in the infection of a winter annual plant like milk thistle in Mediterranean climates such as California, where moisture is present primarily during the cold winter months. *S. silybi* tolerates daily or prolonged drying, and nightly high humidity periods can apparently provide the moisture required for infection. Various other fungi have been shown to germinate and penetrate after a series of short leaf-wetness periods (1,15,20). The ability to survive drying would allow *S. silybi* to infect milk thistle when individual periods of rain or dew are not of adequate duration to complete the infection process. Very low concentrations of spores of *S. silybi* as inoculum are sufficient to cause considerable portions of infected leaves to become necrotic. Inundative applications of spores of this pathogen would likely require sufficiently low numbers of spores to be economically feasible. Lesions incited by *S. silybi* on milk thistle are not determinate in size and generally expand with an increasing rate on leaves as they age. Thus, after a sufficiently long time following inoculation, most leaves, even those with a relatively low initial disease severity, will become heavily necrotic and many will die. *S. silybi* is not well adapted to kill inoculated plants, however. Young leaves with incomplete lesion expansion will likely occur, thus permitting the survival of infected plants. Infected plants would, however, probably be seriously compromised in their ability to compete with other weed and crop species due to the removal of infected leaves as they matured. *S. silybi* may therefore have promise in the management of milk thistle where it occurs in competition with other crop or pasture plant species.

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