Morphology and Host Specialization of *Sclerotinia trifoliorum* from Small Hop Clover

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ABSTRACT

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Symptoms of a Sclerotinia disease were observed annually for 8 yr in volunteer stands of small hop clover (*Trifolium dubium*) near Starkville, Mississippi. Apothecia developed from sclerotia in November and December, and patches of dead plants with sclerotia present in and on tissue appeared from February through March. The pathogen was identified as *Sclerotinia trifoliorum* by ascospore morphology. Sclerotia formed by *S. trifoliorum* on small hop clover in the field and apothecia produced from them were smaller than those recovered from berseem and crimson clovers. However, sclerotia and apothecia produced in culture by isolates from the three clover species did not differ significantly in size. Isolates from all three clover species were highly virulent on berseem clover. Isolates from small hop clover were more virulent ($P < 0.05$) on small hop than were isolates from berseem and crimson clovers, whereas isolates from berseem and crimson clovers were more virulent ($P < 0.05$) on crimson clover than were isolates from small hop. Disease severity in field plots of crimson clover infested with sclerotia from berseem and crimson clovers was greater than in plots infested with sclerotia from small hop clover. These results document *S. trifoliorum* as a pathogen of small hop clover for the first time in North America. Isolates of *S. trifoliorum* from small hop clover show significant and strong specialization in pathogenicity to this host.

Small hop clover (*Trifolium dubium* Sibth.) is one of three closely related species, collectively termed "hop" clovers, that were introduced into the United States from Europe approximately 200 yr ago (4). These exotic species have since become naturalized and are now widely distributed over the southern and eastern United States. Small hop clover is the predominant species in much of the Southeast (4). It may be planted there as a pasture crop, but it is most common as a volunteer plant that produces dense stands in pastures and along roadsides and embankments in early spring. Although hop clovers are common in the eastern United States and are documented hosts of several fungal pathogens, they have not been reported as natural hosts of *Sclerotinia* spp. in North America (3,16).

During the past 8 yr, symptoms of a Sclerotinia disease were observed in volunteer stands of small hop clover near Starkville, Mississippi. The disease cycle on small hop clover developed simultaneously with that of *S. trifoliorum* Eriks. on cultivated forage legumes (9,10), but sclerotia produced by the pathogen on small hop clover and apothecia that developed from them were smaller than those typically produced by *S. trifoliorum* on forage legumes (10).

This study evaluates the morphology, identity, and pathogenicity of the causal organism of the Sclerotinia disease on small hop clover.

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MATERIALS AND METHODS

Collection and storage of isolates. Isolates of *Sclerotinia* were collected as sclerotia from parasitized plants in the field in March and April and were air-dried in plastic petri dishes at 23–25 C for 3–4 mo. Sclerotia were hydrated on moist filter paper for 2–4 hr, surface-disinfested in 70% ethanol for 10 sec and in 1% sodium hypochlorite for 2 min, rinsed in sterile distilled water, blotted on sterile filter paper, bisected, and plated on Difco cornmeal agar (CMA) (Difco Laboratories, Detroit, MI). Colonies were transferred after 4–5 days of incubation at 23–25 C. Isolates were stored, and inoculum was produced by transferring cultures to 250-cm^3 flasks that contained an autoclaved mixture of moist wheat and oat grain (6) and incubating for 2 wk at 23–25 C. The infested grain mixture was air-dried in a thin layer for 2 days at 20 C and stored in sealed plastic bags at 10 C (6). Colonies were generated for experiments by removing and plating sclerotia.

Morphology of sclerotia, apothecia, and ascospores. Sclerotia collected in the field from parasitized plants of small hop, berseem (*T. alexandrinum* L.), and crimson (*T. incarnatum* L.) clovers in April were air-dried at 23–25 C for 3–4 mo. The maximal diameters of 100 randomly selected sclerotia from each source were measured. Diameters of apothecia that formed naturally in December from sclerotia from parasitized plants were measured directly in the field.

Seven isolates of *S. trifoliorum* that produced apothecia consistently were each grown on 10 plates of 2% V8 agar for 30 days. Maximal diameters of five adjacent sclerotia from a randomly selected point at the edge of each colony were measured. All sclerotia from each colony then were counted, transferred to an empty petri dish, air-dried for 30 days, and covered to a depth of 2–3 mm with 36 g of silica sand. Sand was saturated with distilled water, and plates were randomized in a growth chamber at 16 C under cool-white fluorescent light (145–290 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity, 12-hr photoperiod). Diameters of the first three mature apothecia that formed from different sclerotia in each plate after 3–8 wk were measured. The experiment was performed twice. Ascospores were observed for size dimorphism as in a previous study (10).

Radial growth rates. Isolates were grown on Difco potato-dextrose agar for

1 day at room temperature. Colony margins were marked, and colonies were then incubated for 24 hr at 28 or 20 C, or for 48 hr at 5 C, in darkness. Three replicate plates of each isolate were randomized at each temperature, and three measurements of radial growth were made for each colony. The experiment was performed twice.

Pathogenicity experiment. Inoculum of infested wheat and grain was prepared in the same manner as for isolate storage (6). After the infested grain was air-dried, it was fragmented by comminuting dry in 22-g portions for 30 sec in a food blender (1.25 L capacity). The mixture was then sieved through four stacked screens (openings 2.36, 1.40, 0.84, and 0.50 mm, respectively) with shaking. Particles retained on the three finest screens were combined for use as inoculum.

Radicles 1–1.5 cm long were formed by germinating seeds of berseem, crimson, and small hop clovers on agar and planting the seeds in clay pots (10.5 cm diameter) (10 seeds per pot) containing sand. Compatible *Rhizobium* inoculum was watered into pots at planting, and seedlings were thinned to eight per pot after 2 wk. A low-nitrogen liquid fertilizer (9-45-15, N-P-K, respectively) was applied to plants weekly, and a complete micronutrient fertilizer (Peters Hydro-Sol 5-11-26, W. R. Grace & Co., Fogelsville, PA) was applied monthly. Plants of berseem and crimson clovers were grown for 9–10 wk and hop clover for 11–14 wk before inoculation in two experiments. Different periods were required to produce plants of comparable size because of the slower growth of small hop clover and seasonal differences in growth rates of all species in the greenhouse.

Fifteen pots, comprising five replicate pots of each clover species, were randomized for each inoculation treatment. Foliage of plants in all pots was sprayed evenly with 50 ml of a sticker solution (Pel-gel, 40 g/L, Nitragin Co., Milwaukee, WI) and dusted evenly with 50 g of inoculum of a single *Sclerotinia* isolate. Controls received autoclaved inoculum. Pots were individually sealed in plastic bags containing water for a saturated atmosphere, and pots of all treatments were arranged in a completely randomized design. Plants were incubated under fluorescent plant-growth lights (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) with a 12-hr photoperiod at 20–22 C. Bags with water were removed, reapplied, and removed at 4, 8, and 12 days after inoculation, respectively. The number of dead plants in each pot was recorded 14 days after inoculation. The experiment was performed twice.

Field experiment. Twenty plots (0.61 × 0.61 m) of crimson clover cultivar Tibbee were each sown with 1.0 g of seed in September of 1988 and 1989, and stands were established with irrigation. All plots were separated by 0.61-m alleys seeded to ryegrass (*Lolium multiflorum* Lam.). One hundred sclerotia from berseem, crimson, and small hop clovers, collected in the field the previous spring and air-dried for 5–6 mo, were spaced evenly in surface soil of each of five randomly selected plots 2–3 wk after clovers were planted. Five control plots received no sclerotia. Disease severity in each plot was estimated the following April as the percentage of stand destroyed by *S. trifoliorum* (9).

Statistical methods. Data for each experiment on number and size of sclerotia, size of apothecia, radial growth rate, and disease severity in field plots (angular-transformed data) were compared by analysis of variance with a completely randomized design, and by use of Duncan's new multiple range test when significant differences occurred. Data for each experiment on pathogenicity of isolates were compared by analysis of variance with a completely randomized design and a factorial arrangement of treatments. Factor A included the three clover species, and factor B included the 13 inoculation treatments (12 isolates and one control). Factor B was further subdivided into orthogonal contrasts based on isolate source hosts (14). Means were separated by use of the LSD test because of the factorial treatment structure.

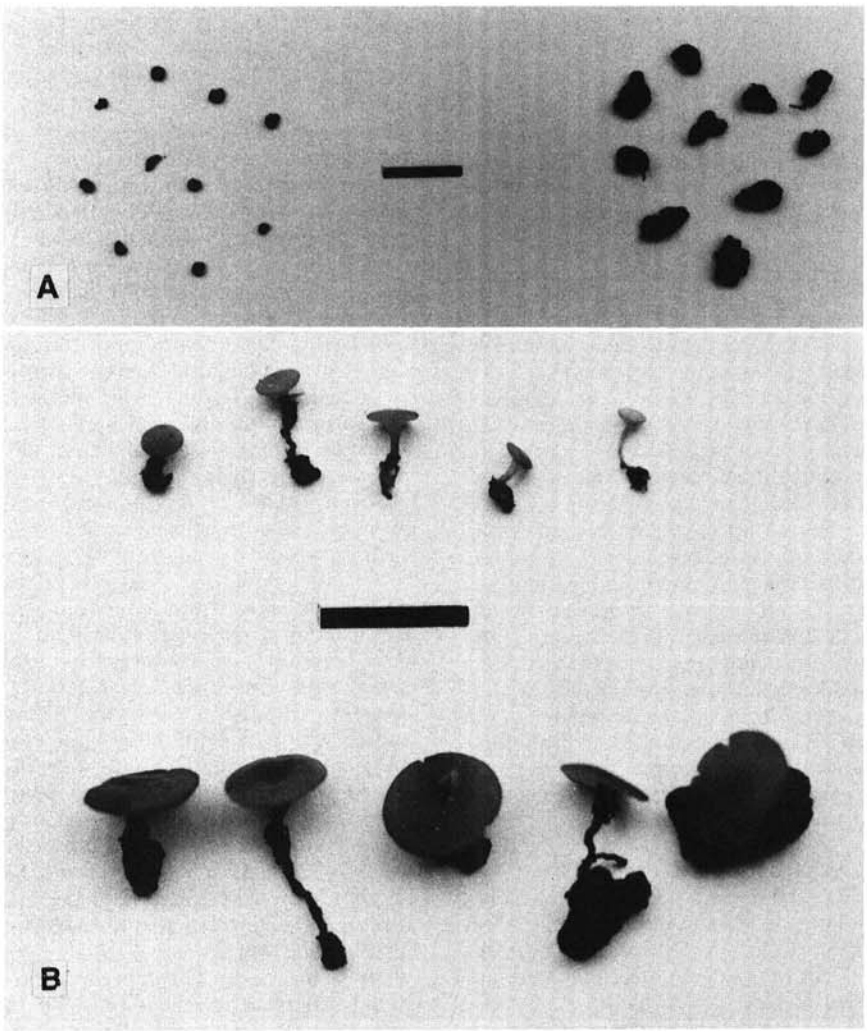

Fig. 1. Comparative sizes of sclerotia and apothecia produced by *Sclerotinia trifoliorum* after parasitism of small hop, crimson, and berseem clovers in the field. **(A)** Sclerotia collected in April from small hop (left) and crimson (right) clovers. **(B)** Apothecia collected in December from sclerotia from small hop (upper) and berseem (lower) clovers. Scale bars are 1 cm.

RESULTS

Symptoms of disease, sclerotia, and apothecia. Patches of symptomatic and dead plants were observed annually in volunteer stands of small hop clover near Starkville from February through April of each year (1984–1991). Individual disease patches were small and irregular, seldom greater than 20 cm in maximal diameter, but these often coalesced to form networks of dead plants within stands. Sclerotia formed in and on dead tissue in March and April, and apothecia developed from sclerotia in the field in November and December.

Sclerotia and apothecia that developed from parasitized small hop clover in the field were atypically small in comparison to those formed by *S. trifoliorum* on berseem and crimson clovers (Fig. 1).

Mean maximal lengths of 100 air-dried, field-collected sclerotia from berseem, crimson, and small hop clovers were 3.3, 2.6, and 1.8 mm, respectively. Mean diameters of 100 apothecia that formed naturally in the field from sclerotia of previously parasitized berseem, crimson, and small hop clovers were 3.8, 3.6, and 1.8 mm, respectively; maximal diameters recorded were 7.5, 6.5, and 3.0 mm, respectively. However, when one isolate from berseem clover, three from crimson clover, and three from small hop clover were grown in laboratory culture, numbers and sizes of sclerotia, and sizes of apothecia did not differ significantly ($P = 0.05$) between any isolate in two experiments. Mean numbers of sclerotia produced by all isolates on 20 plates in two experiments were 6.90–14.05 per plate, mean maximal lengths of sclerotia were 3.75–4.90 mm, and mean diameters of apothecia were 2.45–3.20 mm. Ascospores produced by all isolates in culture and from field-collected sclerotia of the three clover species showed size dimorphism characteristic of *S. trifoliorum* (5).

Radial growth rates. Mean radial growth rates (millimeters per day) of all isolates in two experiments were 3.1–11.7 at 28 C, 6.2–16.3 at 24 C, and 3.7–7.5 at 5 C (data not shown). Significant differences in growth rates occurred between isolates in one or both experiments at each temperature, but these differences were not consistently related to isolate source. At each temperature, growth rates of one or more isolates from small hop clover did not differ significantly from growth rates of one or more isolates from berseem and crimson clovers.

Pathogenicity experiments. Results of two pathogenicity experiments were highly consistent (Tables 1 and 2). On berseem clover, nearly all isolates of *S. trifoliorum* from berseem, crimson, and small hop clovers were highly virulent. Most plants were killed within 2 wk after inoculation, and there were no consistent differences in isolate pathogenicity according to isolate source. In one experiment, crimson isolates as a group were less virulent than berseem and small hop clover isolates due to the weak virulence of one isolate. This difference did not occur in the second experiment (Table 2). On crimson clover, isolates from berseem and crimson clovers were significantly more virulent than isolates from small hop clover in both experiments. However, on small hop clover, isolates from small hop clover were much more virulent than isolates from berseem and crimson clovers in both experiments.

Within groups of isolates, significant differences in virulence were observed for isolates from berseem and crimson clovers in both experiments and for isolates from small hop clover in one experiment (Table 2).

Field experiment. Environmental conditions during 1988–1989 were generally favorable for disease development (9). Disease caused by *S. trifoliorum* was present in all plots including controls, which apparently were infected with sclerotia naturally present in soil (9). Disease severity in plots amended with sclerotia from berseem and crimson clovers, but not small hop clover, was significantly greater than in controls (Table 3). During 1989–1990, the disease cycle was disrupted by a severe freeze in December that killed many leaves after primary ascospore infection. Subsequent disease development was slight, despite favorable environmental conditions.

Table 1. Sources of variation and their significance from analyses of variance of two experiments on the pathogenicity of 12 isolates of *Sclerotinia trifoliorum* on three species of clover[z]

		Experiment 1		Experiment 2	
Source of variation	df	Mean square	*F*	Mean square	*F*
Total	194	12.56	10.38**	12.65	7.76**
Treatment	38	59.11	48.85**	57.89	35.52**
Clover species	2	523.00	432.23**	435.00	266.87**
Isolates	12	44.25	36.57**	41.25	25.31**
Sources	2	79.50	69.70**	32.00	19.63**
Berseem	3	10.33	8.54**	21.00	12.88**
Crimson	3	33.33	27.55**	29.33	17.99**
Small hop	3	4.67	3.86**	3.33	2.04 NS
Control vs isolates	1	132.00	109.09**	269.00	165.03**
Species × isolate	36	18.58	15.36**	23.19	14.23**
Species × sources	4	122.25	101.03**	154.50	94.79**
Species × berseem	6	8.50	7.02**	10.17	6.24**
Species × crimson	6	14.00	11.57**	7.33	4.50**
Species × small hop	6	2.33	1.93 NS	2.33	1.43 NS
Species × control vs isolates	2	15.50	12.81**	49.00	30.06**
Error	156	1.21		1.63	

[z] Each experiment included 12 isolates of *S. trifoliorum* plus one control inoculated onto three species of clover (berseem, crimson, and small hop) with a factorial arrangement of treatments. Isolates were further subdivided into three groups according to source, with four isolates each from berseem, crimson, and small hop clovers. Each treatment combination was represented by five replicate plots arranged in a completely randomized design. ** = Significant at $P = 0.01$; NS = not significant at $P = 0.05$.

Table 2. Pathogenicity of 12 isolates of *Sclerotinia trifoliorum* on three species of clover

		Mean numbers of plants killed by isolates based on source[z]											
		Experiment 1						Experiment 2					
		Berseem clover		Crimson clover		Small hop clover		Berseem clover		Crimson clover		Small hop clover	
Isolate source	Isolate	Single isolate	Isolate group	Single isolate	Isolate group	Single isolate	Isolate group	Single isolate	Isolate group	Single isolate	Isolate group	Single isolate	Isolate group
Control	0	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
Berseem	1	7.8	7.95	0.6	3.95	0.0	0.25	8.0	8.00	0.2	4.00	0.0	1.25
	2	8.0		4.8		0.2		8.0		4.6		2.0	
	3	8.0		5.8		0.8		8.0		5.4		0.4	
	4	8.0		4.6		0.0		8.0		5.8		2.6	
Crimson	1	7.6	6.00	2.2	1.55	0.2	0.10	8.0	8.00	6.0	3.50	2.0	0.70
	2	7.4		2.2		0.2		8.0		2.4		0.6	
	3	8.0		1.8		0.2		8.0		5.6		0.2	
	4	1.0		0.0		0.0		8.0		0.0		0.0	
Small hop	1	7.0	7.55	0.0	0.30	5.0	6.67	6.8	7.65	0.8	1.15	6.8	7.60
	2	7.4		0.0		8.0		8.0		0.8		8.0	
	3	8.0		0.8		6.8		8.0		1.6		8.0	
	4	7.8		0.4		6.8		7.6		1.4		7.6	

[z] Eight plants in each of five replicate pots. LSD ($P = 0.05$) for isolate means: experiment 1 = 1.35, experiment 2 = 1.59, LSD ($P = 0.05$) for isolate group means: experiment 1 = 0.68, experiment 2 = 0.79.

Table 3. Severity of disease induced by sclerotia of *Sclerotinia trifoliorum* from three source hosts after application to field plots of crimson clover[z]

Source of sclerotia	Mean percentage of stand killed by disease	
	1988–1989	1989–1990
0 (control)	29.8 a	17.2 a
Berseem clover	76.0 b	39.0 b
Crimson clover	74.0 b	21.4 a
Small hop clover	30.0 a	22.6 a

[z] Field-collected sclerotia from each source host were added to soil in each of five replicate plots of crimson clover cultivar Tibbee in September of each year. Disease severity was estimated the following April. Means within each column not followed by the same letter differ significantly ($P = 0.05$) according to Duncan's new multiple range test. Statistical analyses were performed on angular-transformed data.

Disease severity was greater than in controls only in plots amended with sclerotia from berseem clover (Table 3).

DISCUSSION

Results of this study demonstrate that small hop clover is a natural host of *S. trifoliorum* in Mississippi. The pathogen caused disease in volunteer stands each spring for up to 8 yr, despite varied environmental conditions that both favored and inhibited disease development. Although *S. trifoliorum* is not yet reported on small hop clover from other areas, both the host and pathogen are common and widely distributed in the southeastern United States (2,4,10,17). This common distribution suggests that small hop clover might serve as a propagative host that could account for the presence of *S. trifoliorum* on sites seldom or never planted to forage legumes. In instances in which disease developed in first-year plantings or after very long rotations (9,17), inoculum may have been present as a result of parasitism of volunteer small hop clover in previous years.

Sclerotia and apothecia produced by *S. trifoliorum* on small hop clover in the field were atypically small in comparison with those found on most other forage legumes (Fig. 1) (9,17). These small sizes initially suggested an identity of the pathogen as *S. minor* Jagger rather than *S. trifoliorum* (5,7). In laboratory culture, however, isolates from small hop, berseem, and crimson clovers produced sclerotia and apothecia that were similar in size. Thus, the small sizes of sclerotia and apothecia produced by *S. trifoliorum* on small hop clover in the field represent environmental- or host-induced effects rather than genotypic features of the pathogen.

Inoculation experiments under controlled conditions revealed highly significant host species × isolate source interactions. These interactions indicate that host specialization in pathogenicity occurred among the isolates studied. All isolates were highly virulent on berseem clover in one or both experiments, and this species is, therefore, a universal suscept for all three isolate-source groups. Isolates from berseem and crimson clovers were significantly more virulent on crimson clover than were isolates from small hop clover. However, on small hop clover, the pattern was reversed in that all hop clover isolates were highly virulent, whereas all berseem and crimson isolates were only weakly virulent (Table 2). Isolates from small hop clover, therefore, demonstrate significant specialization in pathogenicity to that host.

Several previous studies have suggested evidence for possible host specialization in pathogenicity of *S. trifoliorum* on forage legumes (12). Nilsson-Leissner and Sylven (8) observed that isolates from alsike clover (*T. hybridum* L.) caused greater infection on alsike than on red and white clovers, whereas isolates from red and white clovers did not cause greater infection on alsike. Cappellini (1) observed significant isolate × host interactions during one season in field plots of alfalfa and red and white clovers that were inoculated with individual isolates from different sources. He suggested that the results indicated the possible existence of natural pathogenic strains within *S. trifoliorum*. Scott and Fielding (13) observed different patterns of pectolytic enzyme production by isolates of *S. trifoliorum* after their inoculation onto different legume species. They suggested that pectolytic enzymes may be involved in adaptation of *S. trifoliorum* to a particular legume host. Results of the present study support these earlier suggestions for the occurrence of host specialization in *S. trifoliorum*. The similar and significant isolate source × host species interactions, which were demonstrated repeatedly, appear to provide the strongest evidence obtained to date for that phenomenon. Knowledge of the potential for host specialization in pathogenicity of *S. trifoliorum* is essential for future development of cultivars with stable and broadly effective resistance. Similar studies with *S. sclerotiorum* (Lib.) de Bary, in contrast, have not revealed significant host specialization in pathogenicity (11,15).

Results from 2 yr of field experiments on crimson clover generally support results of controlled inoculation experiments. Sclerotia from berseem clover caused significantly greater disease than in controls in both years, and sclerotia from crimson clover caused greater disease in one year. Sclerotia from small hop clover, however, did not cause significantly greater disease than in controls in either year. This evidence from field experiments, therefore, suggests that the host species × isolate source interactions revealed in controlled inoculation experiments are representative of interactions that occur in the field. It is not known if possible differences in apothecium size, ascospore production, and primary infection contributed to final differences in disease severity.

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