Differential Responses of Alfalfa Genotypes to Stem Inoculations with *Sclerotinia sclerotiorum* and *S. trifoliorum*

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ABSTRACT

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Differences in susceptibility of alfalfa genotypes to *Sclerotinia sclerotiorum* and *S. trifoliorum* were evaluated by comparing the extent of pathogenesis in individually inoculated stems of replicated clones. Stems were inoculated by applying cotton pads, saturated with 20% V-8 juice and infested with each pathogen, around tips and enclosing with tape. Plants were incubated in a saturated atmosphere for 3 days to establish infection and then in ambient air for 11 days. Pathogenesis proceeded basipetally within stems, and the length of necrosis from the inoculation point to the margin of healthy tissue was measured on each stem at 2 wk after inoculation. In replicated experiments with each *Sclerotinia* sp., significant differences among 19 genotypes in the extent of pathogenesis of stems were observed, but no significant differences between experiments or interactions between genotypes and experiments. Mean responses of genotypes to the two pathogens were not significantly correlated. These results indicate that the stem-inoculation technique may be used to identify consistent differences among alfalfa genotypes in susceptibility or tolerance of stem tissues to *S. sclerotiorum* and *S. trifoliorum*. The differences in susceptibility to the two pathogens appeared to occur independently among the 19 genotypes in this study.

Sclerotinia crown and stem rot, caused by *Sclerotinia trifoliorum* Eriks., is a major disease of alfalfa (*Medicago sativa* L.), *Trifolium* spp., and other forage legumes in temperate climates (9,14,19). On alfalfa in the United States, the disease is most severe in fall plantings, which are common in the southeastern states (8,24). Primary infection of alfalfa and other forage legumes occurs in the fall and early winter when sclerotia in soil germinate to form apothecia. These apothecia discharge ascospores that cause local lesions in host leaves (15,19). From midwinter through early spring, growth of mycelia commences within and between plants, and leaves, stems, and crowns are rotted. This disease development results in patches of dead plants that may enlarge and coalesce through early spring to cause major losses in young stands (8,24). Disease also occurs in spring-seeded or older alfalfa stands, but damage there is usually less severe (8,24).

Sclerotinia sclerotiorum (Lib.) de Bary also has been documented as a pathogen of alfalfa in North America. Apothecia of this species were observed in the spring, and parasitism occurred during spring and summer months (1,5,7).

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No cultivars or germ plasms of alfalfa have been developed with resistance to either *S. trifoliorum* or *S. sclerotiorum*, and there are currently no confirmed reports of how existing cultivars differ in susceptibility to these pathogens. Progress in developing resistant germ plasm has been hampered by the erratic occurrence and intensity of natural disease in field plots within and between years (23) and by a tendency for artificial inoculation techniques either to overwhelm plants during screening (13,23) or to give inadequate selection pressure (6). Scott (19) noted that resistance ratings of plants or cultivars may change within a few days during artificial screening experiments.

Welty and Busbice (23) found significant correlations between disease ratings of alfalfa entries during two field screening experiments with *S. trifoliorum*. They suggested that some entries evidenced a field tolerance to the disease based on their general degree of adaptivity to the location or to subtle differences in susceptibility. No tolerance was evidenced when the same entries were subjected to the disease under controlled conditions. Similar results were noted by Pierson et al (13) in comparisons of cultivars in the field and as seedlings under controlled conditions. Elgin and Beyer (6) evaluated clones, believed to differ in field resistance, and their polycross progenies under controlled conditions. They observed significant correlations between disease scores of some clones and progenies, but progenies of even the most resistant-appearing parents were still considered susceptible.

The rate or extent of lesion development in individually inoculated or infected stems has been used to evaluate host resistance to *S. sclerotiorum* in soybean (*Glycine max* (L.) Merr.) (2,4), sunflower (*Helianthus annuus* L.) (20), and *Brassica* spp. (21). Similar observations have been used to compare the virulence of isolates of *S. sclerotiorum* on vegetables and ornamentals (17) and sunflower (12) and of *S. minor* Jagger on peanut (*Arachis hypogaea* L.) (3,10). We are not aware of previous reports on the use of stem inoculation techniques, or measurements of the extent of necrosis in individual stems, to evaluate resistance of forage legumes to *S. trifoliorum* or *S. sclerotiorum*.

Pratt et al (14) inoculated individual stems of four species of peas and vetches with *S. sclerotiorum* and *S. trifoliorum* to demonstrate pathogenicity of isolates. Infection progressed basipetally from inoculation points at stem tips, and the extent of pathogenesis differed among host species. These observations suggested that a stem inoculation technique, which evaluates the progression of pathogenesis within host stems, might also be used to identify differences in susceptibility among genotypes. Alfalfa was chosen as the model to test this hypothesis because it is a natural host of the two *Sclerotinia* spp., it is a perennial plant that forms stems promptly, and clones are easily replicated by rooting stem cuttings.

The purpose of this study was to determine whether differential responses to stem infections with *S. sclerotiorum* and *S. trifoliorum* occur among select alfalfa genotypes. A preliminary report of portions of the study has been presented (16).

MATERIALS AND METHODS

Nineteen alfalfa genotypes were evaluated for responses to the two *Sclerotinia* spp.; 10 derived from cultivar Moapa '69, 8 from Narragansett, and 1 from PI 143369 (Table 1). None of these genotypes had been evaluated previously for responses to *Sclerotinia* infection. Stem cuttings of each genotype were rooted for 2–3 wk in sand, and roots were dusted with commercial *Rhizobium* inoculum. Plants were established individually in clay pots (570 cm^3 capacity) with sand or plastic pots (870 cm^3 capacity) with a mixture of soil and peat (1:1, v/v). Plants were clipped repeatedly for several

months to induce development of multiple stems. An aqueous solution of a 5-11-26 commercial fertilizer with micronutrients was applied weekly during maintenance growth and a 20-20-20 fertilizer was applied twice weekly for 2–4 wk before inoculations to promote growth of numerous stems that were similar in age and development.

An isolate of *S. sclerotiorum* from hairy vetch (*Vicia villosa* Roth) in Louisiana (14) and an isolate of *S. trifoliorum* from alfalfa at Starkville, Mississippi, were used in all experiments. Preliminary inoculations established that both were highly virulent on alfalfa.

To prepare inoculum, pieces of absorbent cotton, each about 0.04–0.05 g, were rolled into loose balls by hand, autoclaved dry in glass petri dishes, flooded with sterile 20% V-8 juice, and placed on margins of 1- to 3-day colonies of each *Sclerotinia* sp. on cornmeal agar. After 3 days, when cotton pieces were completely infested with mycelium (Fig. 1A), they were individually transferred to pieces of masking tape (2.7 × 3.8 cm) on plates of water agar and spread with forceps to cover an area about 2.0 × 2.25 cm in the center of each piece of tape (Fig. 1B).

Before inoculation, three stems in each pot were measured from base to tip and numbered. Each pot was placed in a plastic cup (580 cm^3 capacity) that was set in a transluscent plastic bag (13 cm wide, 78 cm long). The pot was watered to saturation and the cup was filled to the rim with water. One piece of tape with infested cotton was firmly sealed around the three stems, with the bottom edge of the tape 3.2 cm from the tips (Fig. 1C). The plastic bag was extended over the plant and sealed to create a humidity chamber (Fig. 1D). All pots were randomized on a bench under fluorescent plant growth lights (2,100 lx) on a 12-hr photoperiod at ambient temperatures of 17–20 C for *S. trifoliorum* and 24–27 C for *S. sclerotiorum*. After 3 days, bags and cups were removed. Plants were maintained under the same temperature and light conditions and watered daily or as required to prevent wilting. Two weeks after inoculation, stems were cut at the base of the tape, and the length of necrosis was determined by subtracting the final length of healthy stem from the initial length.

Two experiments were performed at different times with each pathogen. Data from each experiment were compared by analysis of variance with a completely random design and four replicates per treatment. The mean length of necrosis in three stems in each pot (pot mean) was used as a replicate in statistical analyses. For analysis of combined experiments, data were analyzed in a randomized block design with four replicates per block. Means significantly different at $P \leq 0.05$ were separated by use of Duncan's new multiple range test. Relationships of initial stem lengths to necrosis were determined by correlational analysis.

RESULTS

After 3 days of incubation in a saturated atmosphere, symptoms of initial infection by either *Sclerotinia* sp. on stems were manifested by wilting and gray-brown discoloration of terminal leaves distal to the inoculation point, incipient brown necrosis that extended as much as 0.5 cm down the proximal stem, and white mycelial growth on stems at the tip or bottom of the tape that enclosed inoculum (Fig. 2A). Within a few hours after plants were removed to ambient air, infected leaf and stem tissue dried, shriveled, and became light brown or gray. During the next 11 days, infection progressed basipetally within stems from the inoculation point. Margins of healthy and infected tissue were usually distinct, with healthy tissue green or yellow at the edge of advancing necrosis. Infected or dead tissue was constricted, necrotic, dried, and gray-brown. Some margins were characterized by a narrow blackened zone (about 2 mm wide) between healthy and dead tissue (Fig. 2B). Occasionally, necrosis progressed erratically down stems on one or more sides. Final lengths of necrosis usually ranged from 1–2 to 15–20 cm from inoculation points at 2 wk (Fig. 2C). In preliminary experiments, both *Sclerotinia* spp. were reisolated consistently from margins of necrotic stem tissue as described for peas and vetches (14).

Mean lengths of necrosis induced by *S. sclerotiorum* and *S. trifoliorum* in the alfalfa genotypes, in individual and combined experiments, are given in Table 1. Highly significant ($P \leq 0.01$) differences among genotypes were noted in combined analyses of data for the two experiments with each pathogen. Differences between experiments and interactions be-

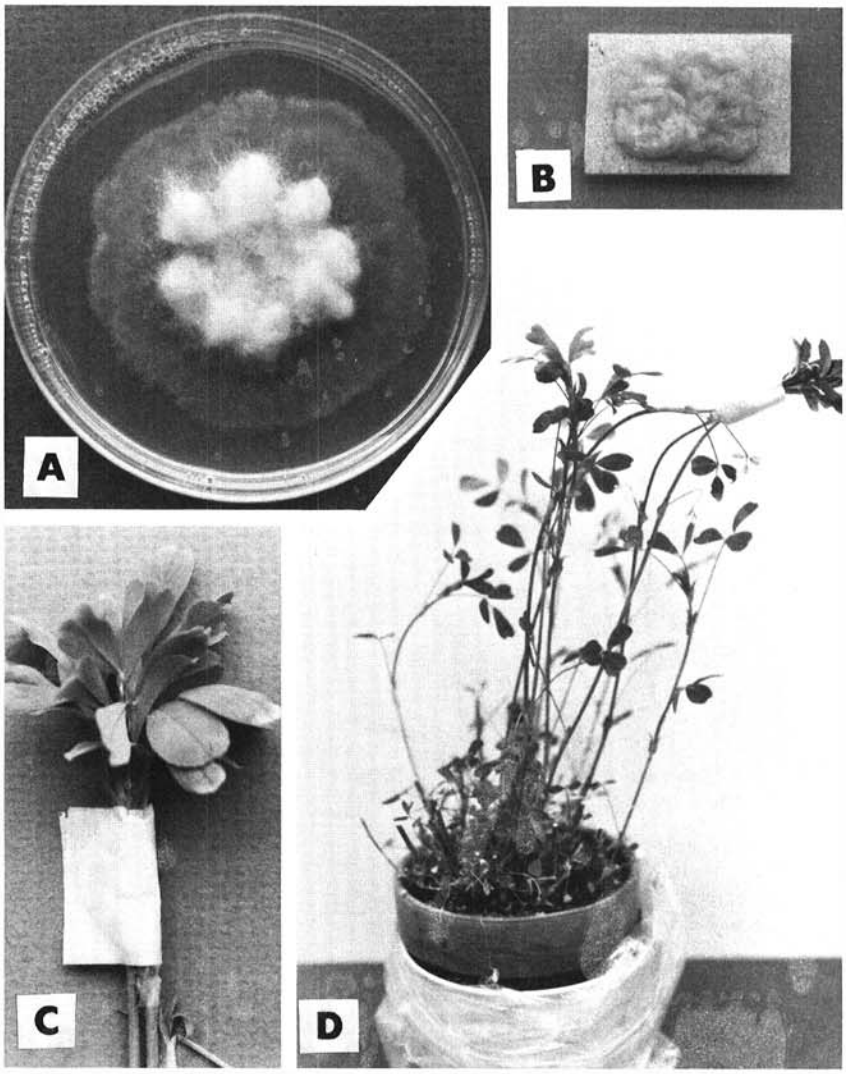

Fig. 1. Procedure for inoculation of alfalfa stems with *Sclerotinia sclerotiorum* or *S. trifoliorum*: **(A)** Pieces of absorbent cotton saturated with 20% V-8 juice are incubated on a *Sclerotinia* colony for 3 days, then **(B)** spread on the sticky surface of masking tape. **(C)** Tape and cotton are sealed around three alfalfa stems, with the bottom edge of the tape 3.2 cm from the tips, and **(D)** the plant with stems inoculated is set in a water-filled cup inside a plastic bag, which is drawn over the stems and sealed to create a humidity chamber.

tween genotypes and experiments (differences in rank of genotypes between experiments) were not significant for either pathogen. Length of necrosis was not correlated with initial stem lengths in any experiment. Genotype means for combined experiments with the two pathogens (Table 1) also were not significantly correlated ($r = 0.08$).

DISCUSSION

Significant differences in pathogenesis occur among alfalfa genotypes after stem-tip inoculations with *S. sclerotiorum* and *S. trifoliorum*. The overall consistency of results obtained in replicated experiments, as indicated by significant differences among genotypes in combined analyses of experiments with each pathogen, suggests that the observed stem responses reflect genotypic differences in host susceptibility. Because no significant correlation occurred for results obtained with the two pathogens, it also appears that factors that condition host susceptibility to them are independent or not closely related.

Although the progression of infection by *S. sclerotiorum* and *S. trifoliorum* differed in inoculated stems, none of the 19 genotypes investigated in these studies showed clearly resistant responses. Means of host responses to both pathogens appeared to represent a continuous gradient rather than distinct categories of disease severity (Table 1). These results are consistent with conclusions and suggestions of previous studies with *S. trifoliorum* on alfalfa: All genotypes studied were basically susceptible (6,13,23), but differences in susceptibility were sometimes manifested with controlled inoculations (6,22), and these might have accounted in part for differences in the field performance of germ plasms in the presence of the disease (13,23).

Some artificial inoculation techniques with *S. trifoliorum* on alfalfa have distinguished differences in susceptibility of germ plasms (6,22), but these have mainly separated the extremes of host reaction types. Other similar techniques have failed to reproduce differences between germ plasms that were evident in the field (13,23). The stem-inoculation technique utilized in these experiments, in contrast, appears to separate narrower differences in host susceptibility or tolerance to each *Sclerotinia* sp. Variability in the extent of necrosis on stems of a genotype was often high within and among pots (Fig. 2), as evidenced by the lack of significant differences between genotypes within individual experiments with each *Sclerotinia* sp. (Table 1). When data from individual experiments were combined in analysis, however, the additional replications were sufficient to reveal significant differences between means of genotypes (Table 1). These genotypes had not been subjected to prior selection and were not known or suspected to differ in susceptibility to either *Sclerotinia* sp. Therefore, the overall magnitude of differences observed between them may represent a relatively narrow range of intermediate host responses rather than extremes (6).

Results of this study on pathogenesis of alfalfa stems by *S. sclerotiorum* and *S. trifoliorum* are generally similar to results obtained in two previous studies with *S. sclerotiorum* on excised (4) and intact (2) stems of soybean. Chu et al (4) observed overall differences among cultivars in the extent of lesion development on excised soybean stems, and these corresponded broadly to cultivar differences observed in the field. However, high variability in stem reactions often gave different rankings of cultivars between experiments and caused a low reproducibility of results. Causes for this variability were not apparent (4). Similar unexplained variability was often observed in this study within and between experiments (Fig. 2C). Boland and Hall (2) also encountered unexplained variability between tests in the extent of lesion development in intact soybean stems, and repeated tests were required to reveal significant differences between cultivars. A similar situation prevailed for both *Sclerotinia* spp. in this study (Table 1). In contrast, Sedun and Brown (20) obtained very consistent differences between sunflower lines in the rate of stem lesion expansion from infection that was initiated in roots, and responses of lines to both *S. sclerotiorum* and *S. minor* were similar. Price and Colhoun (17) also obtained consistent results with stems of *Brassica* spp. inoculated with *S. sclerotiorum*, and they considered the rate of stem lesion expansion to be a reliable indicator of resistance.

Several mechanisms of pathogenesis have been proposed to account for the parasitism of *S. sclerotiorum* and *S. trifoliorum* on host plants (11). In par-

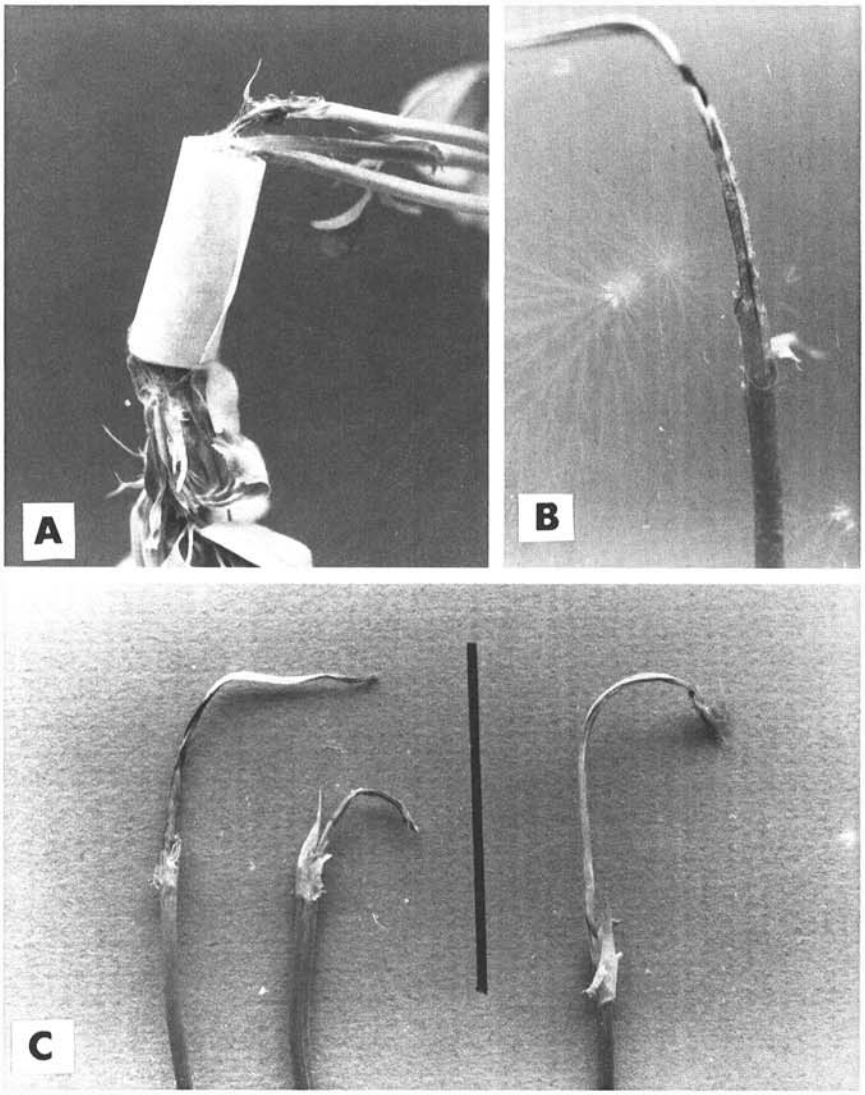

Fig. 2. (A) Initial symptoms of infection of alfalfa stem tips by *Sclerotinia* after 3 days of incubation in saturated atmosphere. Leaves are partly collapsed and rotted at the distal (lower) end of the tape, and stems are rotted and constricted below the proximal (upper) end. **(B)** Close view of margin of necrotic and healthy tissue in a single infected stem. **(C)** Final extent of necrosis in three stems of one alfalfa genotype 2 wk after inoculation with *S. sclerotiorum*. Scale bar = 5 cm.

Table 1. Lengths (cm) of necrosis induced by *Sclerotinia sclerotiorum* and *S. trifoliorum* in stems of 19 alfalfa genotypes and significant differences in combined experiments[x]

Genotype and *F* value[y]	*S. sclerotiorum*			*S. trifoliorum*		
	Expt. 1	Expt. 2	Combined expts.	Expt. 1	Expt. 2	Combined expts.
Mo22	8.83	5.35	7.08 a	7.03	7.30	7.15 abcd
Mo8	3.98	7.03	5.49 ab	8.78	8.20	8.49 abcd
Mo53	5.00	5.25	5.12 abc	6.28	6.18	6.21 bcd
Na23	5.25	4.90	5.07 abc	6.33	8.23	7.28 abcd
Mo38	5.28	4.85	5.04 abc	5.48	4.83	5.16 d
Na32	3.85	5.33	4.59 abc	6.30	4.68	5.48 cd
Mo21	5.22	2.99	4.10 bc	6.85	3.50	5.18 d
Na43	4.38	3.45	3.91 bc	5.68	6.10	5.88 cd
Mo34	3.55	4.08	3.82 bc	6.60	11.28	8.95 abc
Na38	3.83	3.58	3.69 bcd	7.23	6.00	6.60 abcd
Mo32	4.15	3.00	3.58 bcd	6.13	3.83	4.99 d
I1313	3.95	3.20	3.58 bcd	5.50	7.03	6.26 bcd
Na39	3.70	3.20	3.46 bcd	10.25	9.05	9.65 ab
Mo6	4.23	2.40	3.31 bcd	8.83	8.00	8.42 abcd
Na35	3.80	2.63	3.21 bcd	7.53	8.88	8.21 abcd
Mo10	3.45	2.63	3.03 bcd	8.24	11.26	9.75 a
Mo54	2.50	3.30	2.90 bcd	8.03	4.18	6.09 cd
Na34	2.33	3.00	2.65 cd	8.45	7.35	7.89 abcd
Na44	1.20	0.88	1.04 d	6.33	5.58	5.94 cd
Mean	4.13	3.70	3.93	7.09	6.92	7.01
F value for genotype differences[z]	1.72 NS	1.60 NS	2.54**	1.56 NS	1.61 NS	2.19**

[x] Stems of rooted cuttings of genotypes were inoculated at tips with an isolate of each *Sclerotinia* sp., and length of basipetal necrosis induced in each stem was measured 2 wk later. Data within experiments are means of four replicate pots, three stems per pot. Data in combined experiments are means of eight pots for each *Sclerotinia* sp. and were compared by analysis of variance with a randomized block design, with four replicates per block. Means in a column not followed by the same letter differ significantly at $P = 0.05$ by Duncan's multiple range test.

[y] Mo = selection from cultivar Moapa '69, Na = selection from Narragansett, I1313 = from PI 143369.

[z] NS = not significant at $P = 0.05$, ** = significant at $P = 0.01$.

ticular, the secretion of oxalic acid and pectinolytic enzymes in host tissue have been considered as potential mechanisms for both species (11,13,18,19). However, the overall lack of similarity in responses of alfalfa genotypes to the two pathogens in this study, as evidenced by a low correlation of results, suggests that oxalic acid or enzyme production alone, or any other single mechanism of pathogenesis, is not likely to totally account for the parasitism of both species.

The symptomatology and stages of disease development caused by *S. trifoliorum* on alfalfa in the field have not been described as precisely as for clovers (19). However, Gilbert and Bennett (8), in their original report of the disease on alfalfa, noted that the earliest manifestations of mycelial development are within the stems and that infection progresses down stems to crowns before plants are killed. These observations suggest that the stem-inoculation technique may simulate natural disease development more closely than whole-plant (6,13,23) or crown (22) inoculations. Selection for slow progression of infection by *S. trifoliorum* down stems might slow the spread of disease within and between plants in the field and thereby increase chances for survival of plants until warm conditions in spring terminate disease development.

The stem-inoculation technique is advantageous for selection purposes because inoculated stems may be severed below points of infection so that plants may be retained for additional evaluations or crosses. Further studies are needed to evaluate the heritability of alfalfa stem responses to *S. sclerotiorum* and *S. trifoliorum*, the consistency of responses to different isolates, and relationships of stem responses to physiological states of the host and to the severity of natural disease in the field.

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