Responses to Selection for Resistance to *Sclerotinia trifoliorum* in Alfalfa by Stem Inoculations

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ABSTRACT

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Plants of alfalfa cultivar Delta were screened for resistance to *Sclerotinia trifoliorum* by inoculating tips of intact or excised stems and measuring the extent of necrosis that developed basipetally after 2 wk. Four of 494 plants that exhibited low levels of necrosis in repeated tests were cloned and polycrossed. Seed of these selections were harvested by parental source, and the four half-sib families were evaluated for resistance to *S. trifoliorum* by both stem and whole-plant inoculations. Three of four families were significantly ($P = 0.05$) more resistant than the parental cultivar when tested with stem inoculations. The same three families also were significantly ($P = 0.05$) more resistant than the parental cultivar when tested with whole-plant inoculations. The fourth half-sib family gave intermediate responses with both inoculation methods. Plants of the three most resistant families were combined and transplanted at two field sites, along with the parental cultivar, into naturally infested soils during two winter seasons. In each of four experiments, significantly ($P = 0.05$) less natural disease developed in plants of the progeny than in the parental cultivar. These results demonstrate that resistance to *S. trifoliorum* in alfalfa, as identified by the stem inoculation technique, is heritable and is also expressed as whole-plant resistance to controlled inoculations and to natural infection in the field.

Sclerotinia crown and stem rot (SCSR), caused by *S. trifoliorum* Eriks., is one of the most important constraints to production of fall-seeded alfalfa (*Medicago sativa* L.) and other forage legumes in the southeastern United States. Disease develops from late fall to early spring and is favored by frequent rainfall, prolonged high humdity, and mild temperatures during winter months. Entire stands of alfalfa may be destroyed by SCSR or rendered uneconomical for hay production under prolonged optimal conditions for disease (13,18,21).

Alfalfa cultivars vary in susceptibility to SCSR (12,13,17,21), but no cultivar or germ plasm with effective resistance or tolerance to this disease has been developed. Certain fungicides may give effective control of SCSR in the field (14,19), but these are not registered for use on alfalfa in the United States. Spring planting is an effective control practice because mature plants of alfalfa are relatively resistant to *S. trifoliorum* (2,6,13). However, fall planting of forage crops is a preferred practice in the southeastern United States, and it enables earlier harvests the following year in the absence of severe disease.

The lack of progress in development of alfalfa cultivars with resistance to SCSR may be attributed partly to the lack of an effective procedure to screen for resistance (21). Several artificial inoculation methods have been used to compare the susceptibility of alfalfa cultivars to *S. trifoliorum* or to screen for resistance in alfalfa and other forage legumes (5,7,11,12,17,21). However, most of these methods have not been critically evaluated for effectiveness as screening procedures by testing progenies from selections. Most methods also do not facilitate clear identification of the most resistant plants at low frequencies in a population (5,11,12) or repeated measurements of individual plant responses to verify their resistance or susceptibility (7,11,12,17,21).

In 1991, Pratt and Rowe (10) described a new technique for inoculating individual stems of alfalfa with *S. trifoliorum* or *S. sclerotiorum* (Lib.) de Bary. Similar techniques had been previously described for *S. sclerotiorum* on bean, soybean, and *Brassica* spp. (1,4,15,16), but not for *Sclerotinia* spp. on forage legumes. Inoculum was applied near apices of growing stems, and the extent of basipetal necrosis was measured after 2 wk. Significant differences in the length of necrosis induced in stems of 19 randomly selected alfalfa clones were observed with both pathogens.

Results of the previous study (10) suggested that the stem inoculation technique might be used to screen alfalfa for resistance to *S. trifoliorum*. However, neither the heritability of stem responses nor their relationship to whole-plant resistance was determined. Therefore, the objectives of this study were to select alfalfa plants for phenotypic resistance to *S. trifoliorum* by the stem inoculation technique and to evaluate progeny of selections for resistance to stem inoculations, whole-plant inoculations, and natural infection in the field.

MATERIALS AND METHODS

Primary screening by stem inoculations. Seed were germinated for 2 days on 2% water agar and planted individually in cone-shaped plastic containers (12 cm high, 45 cm^3 capacity; Fir Cell, Stuewe & Sons, Inc., Corvallis, OR) containing sand. Compatible *Rhizobium* inoculum was applied to radicles at planting or dusted over seeds and watered in after planting. Plants were grown in the greenhouse and fertilized weekly with Jensen's nutrient solution minus N (20) for 9–12 wk prior to stem inoculations. Inoculations were performed only on actively growing stems with a minimum height of 23 cm and prior to inception of flowering or dormancy. Growth rates of stems differed among plants, so primary screening involved a series of inoculation experiments with varying numbers of plants in each.

Stems were inoculated by the method of Pratt and Rowe (10). Pieces of absorbent cotton (0.04–0.05 g) were rolled into loose balls by hand, sterilized by autoclaving, saturated in sterile 20% (v/v) V8 juice, and placed on margins of growing colonies of *S. trifoliorum* for 3 days on cornmeal agar or for 1 day on 20% V8 juice agar. Each piece of cotton then was removed, spread into a flat pad (approximately 2.0 × 2.25 cm) with forceps, and applied to the inside surface of a piece of masking tape (2.7 × 3.8 cm). Three or four plastic cones with plants were placed in a clay pot (10.5 cm diameter) and held erect by adding sand to the pot. Apices of stems were held together and sealed within the infested cotton and tape. Each pot was set in a plastic cup (8 cm high, 560 cm^3 capacity), and the cup was set in a transparent plastic bag (42 cm high, 4.3 L capacity) containing approximately 180 ml of water. The bag was drawn up over the stems and sealed to create a saturated atmosphere. Plants were incubated for 4 days in a growth chamber at 15–18 C

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with fluorescent growth lights (145–290 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity) on a 12-hr photoperiod. Bags then were removed and plants were incubated in the growth chamber for 10 additional days. The length of basipetal necrosis caused by *S. trifoliorum* was measured from the bottom edge of the tape to the margin of healthy tissue on each stem.

Secondary and tertiary screening by stem inoculations. Potentially resistant plants retained after primary screening were transplanted to clay pots (16 cm diameter) with a greenhouse potting mixture (1:1, sand:peat + vermiculite + limestone; Pro-mix A, Premier Brands, Inc., Stamford, CT) and fertilized weekly with macronutrients (5-11-26 or 20-20-20, N-P-K) and biweekly with micronutrients and trace elements (Peter's S.T.E.M., W. R. Grace & Co., Fogelsville, PA). Foliage was clipped to promote growth of multiple stems. Secondary and tertiary screenings were performed with excised stems that were inoculated individually with smaller pieces of cotton (approximately 0.01 g) and tape (2.5 × 3.2 cm). Stems excised 23–25 cm below apical meristems were placed in 500-cm^3 flasks (two to 10 stems per flask) containing 400 ml of water and inoculated individually with infested cotton and tape. Ten stems were inoculated per plant in each experiment. Plastic bags were inverted over stems and sealed around flasks to create a saturated atmosphere. Flasks were incubated in the growth chamber at 15–18 C, bags were removed after 4 days, and the length of necrosis on each stem was measured after 10 more days as in primary screening. Secondary screening was performed in a series of experiments, as in primary screening, because of the different growth rates of stems. Tertiary screening was performed in a single final experiment.

Progeny evaluations by stem inoculations. Plants for progeny testing were planted directly into plastic cones with the greenhouse potting mixture, grown in the greenhouse, and fertilized as described previously. Stems were excised, incubated in flasks with water, and inoculated and evaluated as in secondary and tertiary screening experiments. Experiments were performed in a growth room at 17–20 C with fluorescent growth lights (80 $\mu E \cdot m^{-2} \cdot s^{-2}$ intensity) on a 12-hr photoperiod. Each flask contained one stem of each entry (one stem per plant) and was considered a complete block in statistical analysis. Fifteen flasks were included in each of two experiments. Results of experiments were combined in statistical analysis after homogeneity of variance was determined by chi-square tests.

All inoculation experiments for primary, secondary, and tertiary screening and for progeny evaluations were performed with a single isolate of *S. trifoliorum* (10) that was maintained and cultured as described for clover isolates (9). Stems with uninfested cotton were not included in experiments, because earlier studies showed that necrosis did not develop in stems following application of sterile cotton and V8 juice or infested cotton that had been autoclaved.

Progeny evaluations by whole-plant inoculations. Whole-plant inoculations were performed with inoculum produced from wheat and oat grain (7) infested with the same isolate used for stem inoculations. Inoculum was infested, dried, comminuted, sieved, and recombined as previously described (9). Plants grown in plastic cones in the greenhouse for 3 wk were selected for uniform size. Eight cones with plants were placed in a clay pot and held upright with sand. Each pot was placed in a plastic cup and the cup was placed in a plastic bag. Water was added to the bag external to the cup to prevent saturation of roots. One replicate pot of each treatment was randomized on a cart to provide a complete block. Foliage in all pots was sprayed evenly with a fine mist of sticker (Pel-Gel Nutrient Adhesive, Liphatech, Inc., Milwaukee, WI) (3.33 ml per pot) (9), and inoculum (1.88 g per pot) was dusted evenly over foliage of all plants. Bags were pulled up over foliage and sealed to provide a saturated atmosphere. Pots of each block were randomized in a single row on a bench in the growth room under the conditions previously described. Bags were unsealed, folded down, and resealed at 4-day intervals. Numbers of surviving plants in each pot were counted 24 days after inoculation. Twelve blocks were included in each of two experiments. Results of experiments were combined in statistical analysis after homogeneity of variance was determined in chi-square tests. Pots with uninfested grain were not included in experiments because no symptom development or mortality occurred when uninfested grain or autoclaved inoculum was applied to plants.

Progeny evaluations in naturally infested soils in the field. Field experiments were conducted in naturally infested soils on the Leveck Animal Research Center at Mississippi State University. Two experiments were established at different sites in early October during each of the 1991–1992 and 1992–1993 growing seasons. In each experiment, severity of naturally occurring disease caused by *S. trifoliorum* was compared in plots of alfalfa cultivar Delta and a population (STR) that consisted of a mixture of equal numbers of plants of the three most resistant half-sib families as determined by stem and whole-plant inoculations (Tables 1 and 2). Plots were arranged in a randomized complete block design with eight replications in each experiment. Each plot was established by transplanting 36 plants, grown 4–5 wk in plastic cones in the greenhouse, into a 6 × 6 arrangement with 12-cm intervals between plants. All plots were separated by alleys 60 cm wide seeded to annual ryegrass. In early to mid-April, each plant was scored for disease severity by a four-point system based on numbers of stems killed: 1 = healthy plant (no stems killed), 2 = <50% of stems killed to crowns, 3 = 50–99% of stems killed, and 4 = all stems killed (plant dead). The score for each replicate plot was the mean for the 36 plants. Death of stems due to infection by *S. trifoliorum* was confirmed by presence of sclerotia on dead stem tissue near crowns.

Table 1. Lengths of necrotic lesions induced in excised stems of alfalfa cultivar Delta and four half-sib families following inoculation with *Sclerotinia trifoliorum*[y]

Alfalfa population	Mean length of necrotic lesion[z] (cm)
Delta	4.9 a
Family No. 1	4.0 ab
Family No. 2	3.5 b
Family No. 3	3.4 b
Family No. 4	3.4 b

[y] For each of two experiments, 15 actively growing stems from different plants of each treatment were excised and incubated in flasks with water in a randomized complete block design with one replicate stem per treatment in each flask (block). Stems were inoculated by sealing pieces of cotton infested with *S. trifoliorum* around apices with masking tape.

[z] Mean length of necrosis induced by *S. trifoliorum* basipetally from inoculation point 14 days after inoculation. Means are from combined experiments, and those not followed by the same letter differ significantly at $P = 0.05$ according to Duncan's new multiple range test.

Table 2. Survival of plants of alfalfa cultivar Delta and four half-sib families following whole-plant inoculation with *Sclerotinia trifoliorum*[y]

Alfalfa population	Mean plant survival[z] (no.)
Delta	2.5 a
Family No. 1	3.7 ab
Family No. 2	4.3 bc
Family No. 3	5.3 cd
Family No. 4	5.7 d

[y] For each of two experiments, 12 replicate pots per treatment with eight plants per pot were inoculated with dried-grain inoculum of *S. trifoliorum* at 3 wk of age in a randomized complete block design. The five pots in each block were inoculated simultaneously by dusting inoculum over foliage.

[z] Mean number of plants per pot surviving inoculation with *S. trifoliorum* at 24 days after inoculation. Means are from combined experiments, and those not followed by the same letter differ significantly at $P = 0.05$ according to Duncan's new multiple range test.

RESULTS

Selection and polycrossing of resistant phenotypes. During primary, secondary, and tertiary screening, plants were not discarded or selected for potential resistance on the basis of any defined standards of phenotypic resistance or susceptibility. Rather, plants with the lowest levels of necrosis in single or multiple inoculated stems, in comparison to other plants in each experiment or at each level of screening, were retained for further testing or crossing.

Initially, 494 plants of cultivar Delta were evaluated during primary screening, with seven to 105 plants in each of nine experiments. Fifty-four plants that did not develop symptoms of infection in stem tissue beneath or within the tape enclosing inoculum were considered potential escapes and discarded because highly resistant or immune responses to *S. trifoliorum* appeared unlikely in an unselected alfalfa population. Twenty-seven of 440 symptomatic plants, with relatively little necrosis (0.5–1.5 cm below the tape), were retained and transplanted to pots. Secondary screening was performed in six experiments, with two to nine plants in each, depending on when sufficient numbers of stems reached the appropriate length for inoculation. Ten plants were retained and reevaluated in a single tertiary screening experiment. Four plants with the lowest mean lengths of necrosis were retained as resistant parental phenotypes and cloned by rooting stem cuttings.

Equal numbers of pots of the four cloned plants were grown to flowering and randomized in a cage with honey bees (*Apis mellifera* L.) for polycrossing. Seed was harvested by parental source to give four half-sib families of polycross seed.

Evaluation of progeny by stem inoculations. Responses of the four half-sib families and Delta to stem inoculations were compared in two experiments. Plants of two half-sib families had significantly ($P = 0.05$) less necrosis than Delta in one individual experiment, whereas treatments did not differ significantly ($P = 0.05$) in the second. In analysis of combined experiments (Table 1), plants of half-sib families Nos. 2, 3, and 4 had significantly less necrosis than Delta, and treatment × experiment interaction was not significant. Plants of family No. 1 had less necrosis than Delta in both experiments, but differences were not significant in either individual or combined experiments.

Evaluation of progeny by whole-plant inoculations. Responses of the four half-sib families and Delta to whole-plant inoculations with dried-grain inoculum were compared in two experiments. Half-sib families Nos. 2, 3, and 4 had significantly greater ($P = 0.05$) survival of plants in both experiments (Table 2). Family No. 1 had significantly greater survival in one experiment, but differences in the second experiment and in combined experiments were not significant. In analysis of combined experiments, treatment × experiment interaction was not significant.

Evaluation of progeny in the field. Symptoms of infection by *S. trifoliorum* appeared in nearly all plants of both experiments during both years by early spring, and 91–99% of plants had one or more stems killed at crown level. Mean disease scores for Delta and the STR population and frequency distributions of plants in the four scoring classes are given for each experiment in Figure 1. Mean scores of the STR population were significantly lower than those of Delta at $P = 0.05$ in three experiments and at $P = 0.01$ in one experiment. In all experiments, more STR plants received scores of 1 and 2 (most resistant) and more Delta plants received scores of 3 and 4 (most susceptible).

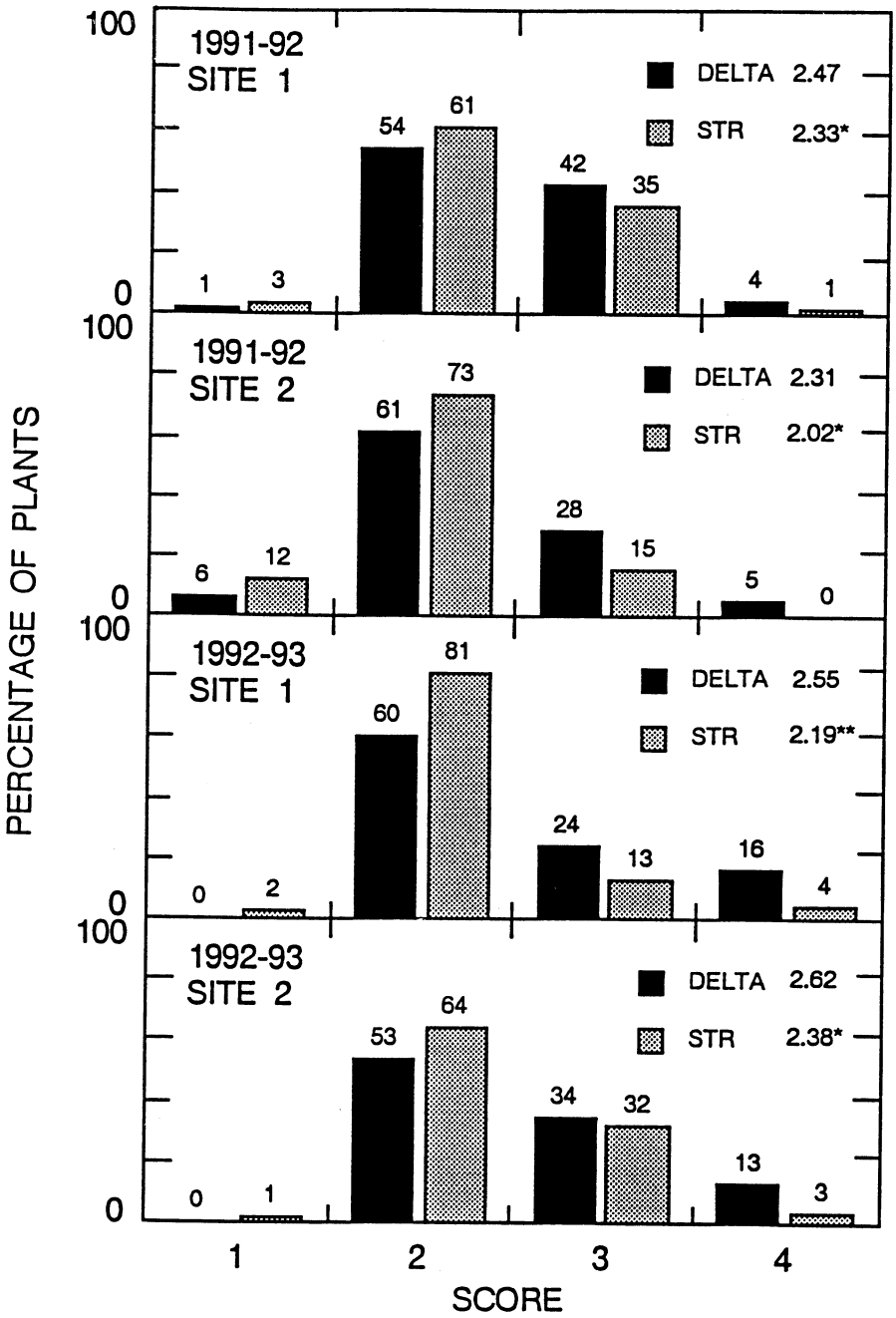

Fig. 1. Mean scores and frequency distributions of scores for severity of disease caused by *Sclerotinia trifoliorum* in alfalfa cv. Delta and a resistant population (STR) in two field experiments (site 1 and site 2) in naturally infested soils during each of two seasons (1991–1992 and 1992–1993). Score: 1 = healthy plant, 2 = <50% of stems killed to crowns, 3 = 50–99% of stems killed, 4 = all stems killed (plant dead). Means (upper right) and frequency distributions in each experiment are based on 36 plants in each of eight replicate plots. Mean score of STR significantly less than that of Delta at * = $P = 0.05$, ** = $P = 0.01$.

DISCUSSION

Polycross progeny from three of four alfalfa plants, selected for resistance to *S. trifoliorum* by the stem inoculation technique, exhibited significantly greater resistance than the parental cultivar in combined experiments with stem inoculations and in repeated individual experiments with whole-plant inoculations. Composited progeny of these three plants also manifested resistance, in comparison to the parental cultivar, to natural infection in the field in four experiments conducted during two growing seasons. These results indicate that phenotypically resistant responses to *S. trifoliorum* in alfalfa stems are heritable in at least some plants. Furthermore, resistant responses in stems are associated with resistance to whole-plant inoculations under controlled conditions and to natural infection and disease development in the field.

Progeny of the fourth selected plant (family No. 1) were significantly more resistant than the parent cultivar in one whole-plant inoculation experiment, and values for disease severity in all stem and whole-plant inoculation experiments were numerically less than those for the parental cultivar (Tables 1 and 2). These results indicate that plants of family No. 1 also exhibited some resistance, but at a lower level than in the other three families, and that this resistance could not be demonstrated consistently by the experimental designs used.

An important finding of this study is that progeny of plants selected for resistance by the stem inoculation technique manifested increased resistance, in comparison to the parental cultivar, in four field experiments conducted during two growing seasons (Fig. 1). Although numerous artificial inoculation techniques for screening for resistance to *S. trifoliorum* in alfalfa have been described (5,10–12,17,21), this appears to represent the first instance in which progeny of plants selected for resistance by an artificial inoculation technique manifested increased resistance to natural infection in the field. This fact is especially important, because selections in the laboratory were based on plant responses to a single isolate of *S. trifoliorum*, whereas resistance in the field was expressed against the total pathogen genotype present at each test location. Differences in virulence of isolates and host specialization in pathogenicity are known in *S. trifoliorum* (3,8,9), and such variability in the pathogen might potentially cause discrepancies in host responses to single vs. multiple pathogen genotypes. Results of this study, however, demonstrate that screening for resistance to a single isolate of *S. trifoliorum* can significantly improve the performance of a population in the field where multiple pathogen genotypes are presumed to be present. The improved performances of progeny are considered to reflect genetic resistance rather than tolerance because they are based on levels of disease severity rather than on indirect parameters such as forage yield.

In evaluations of progeny by the stem inoculation technique, only two families differed significantly from the parental cultivar in one individual experiment, and none differed significantly in the second, on account of high variability within treatments. However, when analysis of variance was performed with combined experiments, three families differed significantly from Delta (Table 1). These results are consistent with those of a previous study: No significant differences in stem responses of 19 alfalfa genotypes to *S. trifoliorum* or *S. sclerotiorum* were noted in individual experiments with 12 stems per entry, but numerous differences to both pathogens were demonstrated by analysis of combined experiments (10). Variability in stem responses may be caused partly by differences in stem morphology. Within all families and the parental cultivar, plants differed in the rate of growth and succulence of stems, and necrosis caused by *S. trifoliorum* tended to be greatest in the most succulent stems. Irrespective of the causes, however, the variability in alfalfa stem responses to *S. trifoliorum* is similar to that observed with *S. sclerotiorum* on soybean. Boland and Hall (1) encountered high variability in stem responses of soybean cultivars, and analysis of combined experiments was required to demonstrate significant differences. Chun et al (4) also described a high level of unexplained variability in soybean stems that caused a low reproducibility of results between experiments.

The stem inoculation technique may require more time and effort than would be practical for screening large numbers of plants for resistance in an applied breeding program. Nevertheless, an important positive feature of the technique is that it enables repeated measurements of responses of individual plants to *S. trifoliorum*. Most previously described techniques appear to allow only a single determination of the response of each plant (7,11,12,17,21). Therefore, the stem inoculation technique may be most suitable for evaluating responses of smaller numbers of plants in genetic studies or for verifying the resistance of plants selected from large populations by other methods. Resistant populations developed by the stem inoculation technique, such as the three families described here, also may be used as resistant controls to facilitate development of new methods for identifying host resistance to *S. trifoliorum* in alfalfa and other forage legumes.

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