Wound-Related Modifications of Penetration, Development, and Root Rot by *Fusarium roseum* in Forage Legumes

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ABSTRACT

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The role of wounding in root rot of forage legumes was examined by using two closely related fungi that differ in virulence and three forage legumes that differ in susceptibility. Plants of alfalfa, red clover, and crown vetch were grown either in conventional or in gnotobiotic slant-board nutrient solution culture. Unwounded or scalpel-wounded roots were inoculated at restricted sites with isolates of *Fusarium roseum* 'Acuminatum' and *F. roseum* 'Avenaceum.' Wounding increased the incidence and severity of root rot caused by both fungi and accelerated penetration and colonization of the roots in the three species that were examined. All fungi penetrated epidermal cells in close proximity to the wound; only rarely were hyphae observed entering the wound directly. In addition, wounding altered the type of growth of *F. roseum* 'Acuminatum' in the root cortex by enhancing the formation of distributive hyphae and eliminating the formation of chlamydospores which occurred in unwounded roots. The primary role of wounding was not to provide a breach of the root surface, but to alter the host-pathogen interaction to favor fungal development in the root.

Additional key words: *Coronilla varia*, *Medicago sativa*, *Trifolium pratense*.

Wounding of red clover and alfalfa crown and root tissue has been shown to increase Fusarium root rot. Field incidence of Fusarium root rot increased with wounding of root and crown tissue by insects (3,6,11), winter injury (3,5,11,28), or harvest machinery (7). Inoculations with *Fusarium* plus root-feeding insects (6,12,14) or *Fusarium* plus low temperatures (16) caused higher incidences of root rot than inoculations with *Fusarium* alone. In greenhouse experiments, massive mechanical injury to roots of mature red clover plants during inoculation increased the incidence of Fusarium root rot (6,21,28).

Wounding of plant surfaces is presumed to disrupt a mechanical barrier that obstructs ingress by potential pathogens (3,7,27). Microscopic examination of red clover seedlings and 5-wk-old plants inoculated with pathogenic isolates of *Fusarium roseum* (Lk.) emend. Snyd. and Hans. 'Avenaceum,' however, indicated that these isolates penetrated and colonized plant roots directly (1,19,20). *Fusarium roseum* (Lk.) emend. Snyd. and Hans. 'Acuminatum' also directly penetrated and colonized red clover roots, although it did not always cause symptoms (20). Thus, the role of wounding in this host-pathogen interaction appeared to involve more than merely providing an entry site.

The objective of this research was to examine *F. roseum* 'Acuminatum' and *F. roseum* 'Avenaceum' in wounded and intact roots of several forage legumes to determine how wounding altered the development of root rot and fungal colonization. These fungi were chosen because they differ in virulence and development in red clover (10,20). Crown vetch was included because it is more resistant to Fusarium root rot than red clover and alfalfa (10). The slant-board nutrient solution culture technique (8–10) was used because it permits noninjurious access to roots, controlled placement of inoculum and wounds, and minimal influence of other organisms.

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MATERIALS AND METHODS

All isolates of *F. roseum* 'Acuminatum' and *F. roseum* 'Avenaceum' were originally obtained from diseased forage legume roots and demonstrated to be pathogenic (20). Inoculum was prepared by growing the fungi on autoclaved, white, 100% cotton threads, 1.5 cm long, on the surface of V-8 agar plates (13). After 6 days of incubation in the dark at 18 C, the threads, covered with mycelium and occasional macroconidia were removed and used as inoculum.

Red clover (*Trifolium pratense* L. 'Arlington'), alfalfa (*Medicago sativa* L. 'Saranac AR'), and crown vetch (*Coronilla varia* L.) plants were grown from seeds by using the slant-board culture technique (8). Methods were as previously described (10,20) unless noted. Plants were inoculated at 5 wk after planting for red clover and alfalfa and at 6 wk for crown vetch. Roots from each plant were spread fanlike on individual slant-boards, and nine to 12 roots were inoculated by placing the inoculum thread under an individual root 4 cm proximal to the tip. Roots with water-soaking, cracks, or other signs of injury were not inoculated. Some of the inoculated roots were wounded by carefully nicking the root epidermis on the side opposite the inoculum thread with a sterilized scalpel blade at the point of inoculation. Observations were made 6 days after inoculation, and the length and frequency of necrosis determined.

Effect of wounding. Three sets of inoculations were conducted to study the effect of wounding on induction of cortical rot by the isolates of *Fusarium*. In the first set, separate roots of red clover, alfalfa, and crown vetch were individually inoculated with *F. roseum* 'Acuminatum' isolates 927 or 1055 or *F. roseum* 'Avenaceum' isolate 959. Half of the inoculated roots were wounded at the inoculation site. Five replicates of eight plants per species were examined. In the second set of inoculations, additional isolates of *F. roseum* were compared for ability to cause necrosis in wounded and unwounded red clover roots. Ten isolates were inoculated on each of 48 plants, with one isolate per individual secondary root. In half of the plants, roots were wounded at the inoculation sites. Results of these inoculations were subjected to an analysis of variance of a randomized complete block design, and separate analyses and Duncan's multiple range test ($P = 0.05$) were used to determine differences between plant species, fungal isolates or wounding treatments. In the final set of inoculations, the time interval during which wounding affects root rot development was determined by inoculating individual roots of red clover plants with *F. roseum* 'Acuminatum' isolate 927 or 5207 at 0, 6, 12, 18, 24, 36, and 60 hr after wounding. For each time interval, twelve plants were inoculated. Data were analyzed by using regression analysis.

Microscopy. Excised root tissue was stained by boiling in 0.015% aniline-blue lactophenol for 4 min (25). To study the effect of wounding on fungal colonization of the root cortex, wounded and unwounded roots of alfalfa, crown vetch, and red clover plants (which had been individually inoculated with isolates 927, 959, or 1055) were excised and stained 6 days after inoculation. Roots were observed with a light microscope for cortex colonization and chlamydospore formation. The effect of wounding on the rate of penetration and colonization of red clover roots by isolates 927, 959, and 1055 was examined by harvesting and staining unwounded and wounded inoculated roots 4, 6, 12, 18, 24, 48, 72, 96, and 120 hr after inoculation. Twelve wounded and 12 unwounded roots were observed for each isolate at each time period.

Gnotobiotic inoculations. Because microbial contaminants occur in the slant-board system (10), the effect of wounding on the disease interaction was examined by using pairs of red clover plants grown in gnotobiotic slant-boards (9). When plants were 19 days old, one plant from each slant-board was inoculated, and the second was plated on nutrient agar to test for contamination. Prior to inoculation, roots were moistened with sterile, half-strength Hoagland's solution and separated without apparent injury. Four roots per plant were inoculated at least 2 cm proximal to the root tip with *F. roseum* 'Acuminatum' isolates 927 and 5207 or *F. roseum* 'Avenaceum' isolates 959 and 5215. Roots of half of the plants were wounded by gently pressing the surface of the root with the tip of a sterile dissecting needle at the inoculation site. Plants were observed after 5 days, and the length and frequency of necrosis were determined. Means and standard error of the mean were calculated for each isolate. The root region above the inoculation site was excised and plated on nutrient agar to test for contamination. Inoculated roots were excised, stained with aniline blue and observed at ×400 for cortex colonization. Sixty plants on gnotobiotic slants were inoculated.

RESULTS

Effect of wounding on the frequency and severity of cortical root rot. An analysis of results from inoculations of wounded and unwounded roots of all three legume species revealed that plant species, fungal isolate, wounding treatment, plant × isolate and plant × wounding effects were all significant. Wounding increased both the number of inoculated roots with necrotic lesions and the mean length of necrosis in all three forage legumes regardless of the fungal isolate used for inoculation (Table 1). Nevertheless, the percentage of wounded inoculated crown vetch roots with necrotic lesions (39%) was significantly less than that of wounded inoculated alfalfa roots (99%) and red clover roots (94%).

In a second set of inoculations which compared the effect of root wounding on cortical rot of red clover caused by *F. roseum* 'Acuminatum' versus 'Avenaceum,' the wounding treatment, fungal isolate and wounding × isolate effects were all significant. Wounding significantly increased the percentage of roots with necrotic lesions for all five isolates of *F. roseum* 'Acuminatum' tested and for two of the five isolates of *F. roseum* 'Avenaceum' tested (Table 2). Although isolates of *F. roseum* 'Avenaceum' were capable of causing root rot of unwounded roots, isolates of *F. roseum* 'Acuminatum' caused little or no necrosis of these roots. In wounded roots, there was little difference between isolates of the two groups in the frequency of necrosis.

A nonlinear decrease in the frequency of necrosis of red clover roots inoculated with *F. roseum* 'Acuminatum' isolates 927 and 5207 did occur as the time interval between wounding and inoculation increased (Fig. 1). The effect of wounding was greatest when plants were inoculated within 6 hr of the wounding treatment, but the level of necrosis in roots inoculated at 60 hr after wounding was still greater than the low levels caused by the two fungal isolates in unwounded roots.

Microscopic observations. Red clover and alfalfa roots were observed to have similar patterns of fungal penetration and colonization 6 days after inoculation. All isolates directly penetrated the root epidermal cells of unwounded and wounded roots. Hyphae rarely entered the root through the wound opening. In unwounded roots, *F. roseum* 'Acuminatum' isolates 927 and 1055 formed chlamydospores in the root cortex. In contrast, *F. roseum* 'Avenaceum' isolate 959 extensively colonized the cortex by forming distributive hyphae. In wounded roots, all three isolates formed distributive hyphae in the root cortex. These distributive hyphae grew longitudinally in the intracellular spaces of the root cortex, both acropetally and basipetally from the inoculation site.

The pattern of fungal penetration and colonization of crown vetch roots was less extensive than that of red clover and alfalfa. In unwounded roots, *F. roseum* 'Acuminatum' isolate 927 did not penetrate the root epidermis, and there was limited colonization of the cortex of roots inoculated with *F. roseum* 'Avenaceum' isolate 959 and *F. roseum* 'Acuminatum' isolate 1055. Hyphae of these two

TABLE 1. Frequency and severity of root rot in alfalfa, crown vetch and red clover roots inoculated with isolates of *Fusarium roseum* 'Acuminatum' and 'Avenaceum' with and without wounding[a]

Isolate	Wounding treatment[b]	Alfalfa: Roots with lesions[c] (%)	Alfalfa: Mean length of lesion (mm)	Crown vetch: Roots with lesions (%)	Crown vetch: Mean length of lesion (mm)	Red clover: Roots with lesions (%)	Red clover: Mean length of lesion (mm)
F. roseum 'Acuminatum'							
Isolate 927	Wounded	99*[d]	7.2*	21*	1.5*	98*	4.9*
	Unwounded	23	0.8	0	0.0	18	0.9
Isolate 1055	Wounded	100*	5.2*	62*	4.5*	85*	2.9*
	Unwounded	10	0.3	5	0.3	0	0.0
F. roseum 'Avenaceum'							
Isolate 959	Wounded	100*	12.3*	35*	1.3*	100*	9.6*
	Unwounded	83	6.2	5	0.3	93	7.0

[a] Individual roots were inoculated by placing an inoculum thread of the fungal isolate under the root 4 cm proximal to the tip.
[b] Roots were wounded by carefully nicking the root epidermis on the side opposite the inoculum thread with a sterilized scalpel blade.
[c] Each value is the mean of five replicates with eight plants per replicate.
[d] Asterisks (*) = means for wounded roots differ significantly ($P = 0.05$) from corresponding means for unwounded roots.

isolates directly penetrated the epidermis of unwounded roots and colonized several cortical cells directly below the penetration site, but colonization was limited to these cells. Chlamydospores were occasionally present in the cortex of roots inoculated with isolate 1055.

In wounded crown vetch roots inoculated with isolate 927, half of the roots examined were extensively colonized in the area around the wound; one-quarter had limited colonization with occasional chlamydospores; and one-quarter were not penetrated by fungal hyphae. In wounded roots inoculated with isolate 959, one-third of the roots examined were extensively colonized with distributive hyphae in the cortex, and two-thirds of the roots had limited colonization. Most of the wounded roots that were inoculated with isolate 1055 were extensively colonized in the region around the wound. Distributive hyphae were present in one-third of these wounded roots.

Microscopic observations of red clover roots harvested at time intervals after inoculation indicated that wounding accelerated the rate of penetration of the epidermis and colonization of the cortex by all three isolates tested (Table 3). Penetration occurred within 24 hr after inoculation in the majority of unwounded roots in contrast to 6 hr in wounded roots. In wounded roots, hyphae rarely entered directly into wound opening; instead, epidermal cells, three to five cells away from the wound, were directly penetrated and extensively colonized.

Colonization of the root cortex occurred within 48–72 hr in unwounded roots in contrast to 12 hr in wounded roots (Table 3). Colonization of the cortex was intercellular at first and then intracellular. In wounded roots, all isolates extensively colonized the root cortex at the inoculation site. Within 48 hr of inoculation, all isolates formed distributive hyphae in the cortex of red clover roots that had been wounded at inoculation (Table 3). *F. roseum* 'Avenaceum' isolate 959 formed distributive hyphae in unwounded roots within 96 hr of inoculation. *F. roseum* 'Acuminatum' isolates 927 and 1055 did not form distributive hyphae in unwounded roots during the observation period. Colonization of the root cortex by these two isolates was less extensive in unwounded roots. Chlamydospores occurred in the root cortex of unwounded roots inoculated with isolates 927 and 1055 within 120 hr of inoculation.

Gnotobiotic inoculations. In one-third of the 60 inoculated plants grown on gnotobiotic slant-boards, no bacterial contaminants were found in the sterility checks made prior to inoculations and 6 days after inoculation. The frequency and severity of root rot symptoms of inoculated roots which remained sterile during the entire inoculation process were similar to that of inoculated roots which were contaminated during the procedure (Table 4). In both sterile and contaminated slant-boards, isolates of *F. roseum* 'Acuminatum' caused little or no necrosis in unwounded roots, but they did cause necrotic lesions in wounded roots. Following inoculations with isolates of *F. roseum* 'Avenaceum,' there was little difference between the wounded and unwounded treatments with respect to the percentage of inoculated roots with necrotic lesions and the mean length of the lesions. Microscopic examination of stained inoculated roots showed that isolates of *F. roseum* 'Acuminatum' colonized and formed chlamydospores in the cortex of unwounded roots. These isolates formed distributive hyphae in wounded roots. Isolates of *F. roseum* 'Avenaceum' formed distributive hyphae in both wounded and unwounded roots.

TABLE 2. Comparison of the effect of wounding on the frequency and severity of root rot of red clover caused by *Fusarium roseum* 'Acuminatum' and *F. roseum* 'Avenaceum'

Isolate	Wounding treatment[a]	Roots with necrotic lesions (%)	Mean length of lesion (mm)
F. roseum 'Acuminatum'			
927	Wounded	96*[b]	6.8*
	Unwounded	0	0.0
5207	Wounded	100*	6.8*
	Unwounded	4	0.1
5653	Wounded	92*	2.8*
	Unwounded	4	0.5
5654	Wounded	96*	4.2*
	Unwounded	0	0.0
5655	Wounded	96*	4.8*
	Unwounded	0	0.0
F. roseum 'Avenaceum'			
959	Wounded	100*	9.7*
	Unwounded	67	3.4
5181	Wounded	100*	16.0*
	Unwounded	42	2.5
5215	Wounded	100	19.6*
	Unwounded	96	11.2
5702	Wounded	100	18.0*
	Unwounded	87	5.4
5703	Wounded	100	18.8*
	Unwounded	88	6.0

[a]Individual roots were inoculated 4 cm proximal to the tip by placing an inoculum thread of the fungal isolate under the root. One-half of the roots were then wounded on the side opposite the inoculum thread by carefully nicking the root epidermis with a sterilized scapel blade.

[b]Asterisks(*) = means for wounded roots differ ($P = 0.05$) from corresponding means for unwounded roots.

DISCUSSION

Results of this research showed that wounding of individual roots resulted in localized increases in the incidence and severity of necrosis in all host-pathogen combinations tested. Of the three forage legumes tested, the maximum effect was in red clover and alfalfa. The effect of wounding in crown vetch was limited. Although there was greater fungal development in wounded than unwounded crown vetch roots, wounding did not overcome the basic resistance of this species.

Wounding had a greater overall effect on *F. roseum* 'Acuminatum' than on *F. roseum* 'Avenaceum' by greatly increasing the number of roots with necrotic symptoms.

The main effect of wounding was not to provide an opening through which the fungus could enter. Little entry through the actual wound was observed even though the opportunity was

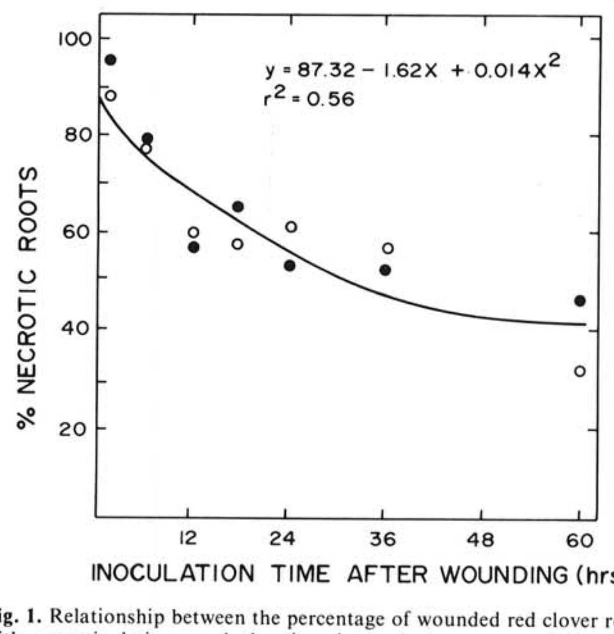

Fig. 1. Relationship between the percentage of wounded red clover roots with necrotic lesions and the time intervals between wounding and inoculation with *F. roseum* 'Acuminatum' isolate 927 (●) or 5207 (O). Each data point is the mean of twelve inoculations observed 6 days after inoculation.

TABLE 3. Time required by isolates of *Fusarium roseum* 'Acuminatum' and 'Avenaceum' to penetrate and colonize wounded and unwounded roots of red clover

	F. roseum 'Acuminatum'				*F. roseum* 'Avenaceum'	
	Isolate 927		Isolate 1055		Isolate 959	
State of host-pathogen interaction	Unwounded (hr)	Wounded (hr)	Unwounded (hr)	Wounded (hr)	Unwounded (hr)	Wounded (hr)
Penetration of epidermis	24[a]	6	24	6	18	6
Colonization of cortex	48	12	72	12	48	12
Distributive hyphae in cortex	Absent	48	Absent	48	96	48
Chlamydospores in cortex	120	Absent	120	Absent	Absent	Absent

[a] Hour of sampling when greater than 50% of inoculated roots were first observed at each stage.

TABLE 4. Frequency and severity of root rot symptoms in red clover plants grown in gnotobiotic slant-boards and inoculated with isolates of *Fusarium roseum* 'Acuminatum' and 'Avenaceum'

		Sterile slant-board[b]		Nonsterile-after inoculation		Nonsterile-prior to inoculation	
Isolate	Wounding treatment[a]	Roots with lesions (%)	Mean length of lesions (mm)	Roots with lesions (%)	Mean length of lesion (mm)	Roots with lesion (%)	Mean length of lesion (mm)
F. roseum 'Acuminatum'							
927	Wounded	100	12.0 ± 3.4[c]	100	13.1 ± 3.0	100	7.2 ± 1.8
	Unwounded	0	0.0	20	2.3 ± 2.0	30	1.7 ± 0.9
5207	Wounded	100	10.0 ± 3.7	100	15.2 ± 4.5	100	9.4 ± 2.9
	Unwounded	10	0.7 ± 0.7	20	0.7 ± 0.5	30	3.0 ± 2.0
F. roseum 'Avenaceum'							
959	Wounded	100	20.6 ± 1.2	100	22.9 ± 1.7	100	17.8 ± 2.1
	Unwounded	100	18.1 ± 6.8	100	19.4 ± 2.1	100	27.1 ± 1.6
5217	Wounded	100	25.8 ± 2.1	100	26.1 ± 1.8	100	20.4 ± 2.0
	Unwounded	100	25.6 ± 2.7	100	23.7 ± 2.4	90	26.0 ± 4.3

[a] Individual roots were inoculated with one fungal isolate 4 cm proximal to the tip. For the wounding treatment, roots of one-half of the plants were gently pressed at the inoculation site with the tip of a sterile dissecting needle.
[b] Results of sterility checks, prior to and after inoculation. For 20 plants, there was no evidence of bacteria prior to and 6 days after inoculation (sterile). For 20 plants, bacteria contaminants were found 6 days after inoculation but not prior to inoculation (nonsterile - after inoculation). For 20 plants, bacteria were found to be present in the slant-board prior to inoculation (nonsterile - prior to inoculation).
[c] Means of ten inoculations measured at 6 days after inoculation plus-minus the standard error of the mean.

present. Instead, wounding, whether by superficial cutting with a scalpel or by pressing young roots with a needle, accelerated fungal penetration and increased development. The extensive cortex colonization of the area surrounding the wound, followed by formation of distributive hyphae in inoculated red clover and alfalfa roots, was similar to the colonization pattern of wounded bean hypocotyls inoculated with *Fusarium solani* f. sp. *phaseoli* (2) and wounded red clover seedlings (1). Earlier penetration and colonization of wounded roots may account for greater lengths of necrosis in wounded roots inoculated with *F. roseum* 'Avenaceum' in comparison to unwounded roots.

In addition, for *F. roseum* 'Acuminatum,' in red clover and alfalfa roots, wounding altered the host-pathogen interaction following penetration by favoring the formation of distributive hyphae rather than chlamydospores. This is the first report of wound-modified fungal development in these host-pathogen combinations and perhaps with any combinations.

Explanations for the effect of wounding must take into account the observed alteration in fungal development. One explanation may be that wounding brings about changes in rhizosphere nutrition which in turn enhances fungal virulence. Concentrations of amino acids and sugars in the rhizosphere of other legume roots (15,18) are known to be highest around seedling roots, at root tips, and at wounds, all areas where *F. roseum* 'Acuminatum' can cause necrosis (10,20). There are also previous experiments which indicate that changes in virulence of isolates of *Fusarium* occur with the alteration of nutrients in the rhizosphere (22,26). Changes in nutrient concentrations in a bathing solution altered the pathogenic development of *F. solani* f. sp. *phaseoli* after penetration (22). Foliar applications of herbicides that increase root exudates increased the incidence of Fusarium root rot of navy bean (26).

Explanations for the formation of chlamydospores by isolates of *F. roseum* 'Acuminatum' in unwounded roots, but not in wounded roots may also aid in understanding the role of wounding. Factors such as nutrient depletion and metabolites of soil microflora, which are known to induce chlamydospore formation from macroconidia in culture (17), could be involved in chlamydospore formation in the cortex of red clover and alfalfa roots. Factors that influence chlamydospore formation by isolates of *F. roseum* 'Acuminatum' may produce different responses in isolates of *F. roseum* 'Avenaceum' which do not form chlamydospores (23).

Although certain soil bacteria induce chlamydospore formation in *Fusarium* (4,24) and increase the incidence of Fusarium root rot in alfalfa (11), the results of experiments done with the gnotobiotic slant-board system indicate that metabolites of the slant-board microflora are not necessary for chlamydospore formation. However, the role of soil microflora in nature cannot be discounted.

Wounding appeared to modify the host-pathogen relationship as evidenced by increases in necrosis and root colonization. In this host-pathogen interaction, wounding provided more than an entry point for the pathogen, but rather promoted earlier penetration and extensive colonization of the host.

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