Field Reaction of Artificially Inoculated Alfalfa Populations to the Fusarium and Bacterial Wilt Pathogens Alone and in Combination

F. I. Frosheiser and D. K. Barnes

Research Plant Pathologist and Research Plant Geneticist, Agricultural Research Service, U.S. Department of Agriculture, Department of Plant Pathology and Department of Agronomy and Plant Genetics, respectively, University of Minnesota, St. Paul, MN 55108.

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ABSTRACT

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Plants of 12 alfalfa cultivars and experimental lines were inoculated with *Fusarium oxysporum* f. sp. *medicaginis* and *Corynebacterium insidiosum* alone and in combination and then transplanted into the field. Differences in reaction to *F. oxysporum* and *C. insidiosum* were observed among the entries. The internal root symptoms of Fusarium and bacterial wilts could be differentiated by the color and outline of the discolored areas in cross section. Fusarium wilt severity was about equal in plants inoculated with *F. oxysporum* alone and with the mixed inoculum. Bacterial wilt was less severe when inoculated with the mixed inoculum than with *C. insidiosum* alone. Resistance in a cultivar to bacterial wilt, Phytophthora root rot (caused by *Phytophthora megasperma*), or anthracnose (caused by *Colletotrichum trifolii*) had no apparent effect on the development of Fusarium wilt. The procedure described for inoculation and culture of the alfalfa plants appeared satisfactory for evaluating their reaction to *F. oxysporum* and for screening alfalfa populations for resistance to the pathogen.

Additional key words: *Medicago sativa*.

Weimer (6) first described Fusarium wilt of alfalfa (*Medicago sativa*) and identified the causal organism as *Fusarium oxysporum* var. *medicaginis*. According to Snyder and Hanson (5) the pathogen should be designated as *F. oxysporum* Schl. f. sp. *medicaginis* (Weimer) Snyd. & Hans.

The external symptoms of Fusarium wilt are rapid wilting of some or all the stems which often is accompanied by yellowing or reddening of the foliage. The plants eventually die, sometimes suddenly. Top symptoms can be confused with those caused by root-rotting fungi. The woody cylinder of the root has one or more dark- or reddish-brown streaks, which may enlarge or merge until the entire cylinder is discolored and the plant dies (Fig. 1).

The disease usually progresses slowly in natural alfalfa stands and only scattered plants show symptoms at any one time. However, considerable losses in stand may occur over a period of several years. The disease occurs in most areas of the United States, but is most severe in southern areas (4).

The only known report of breeding for resistance to Fusarium wilt of alfalfa was by Wilson and Melton (7). Presently no standard evaluation techniques are available for evaluating Fusarium wilt resistance in alfalfa.

In glasshouse investigations we found that alfalfa plants could be infected with the pathogen by immersing the roots in an aqueous spore suspension before transplanting them. This procedure is similar to the method usually used in field tests to inoculate alfalfa with *Corynebacterium insidiosum* (McCull.) H. L. Jens, the bacterial wilt pathogen (2, 3). We then initiated a field study with the objectives of determining: (i) whether the methods used in the glasshouse were satisfactory for large-scale field investigations, (ii) whether plants can be infected by inoculation with a mixture of the Fusarium and bacterial wilt pathogens, (iii) whether symptoms of Fusarium wilt and bacterial wilt can be readily distinguished in the same roots, (iv) whether alfalfa cultivars differ in their reactions to the fungus, and (v) whether resistance to other alfalfa root and crown pathogens affect the reaction of a plant to *F. oxysporum*.

MATERIALS AND METHODS

Seven alfalfa cultivars and five experimental lines, each having high levels of resistance to one or more of the following: *C. insidiosum*, *Phytophthora megasperma*, and *Colletotrichum trifolii*, were chosen for the study (Table 1). Cultivars Agate, Ramsey, and Vernal and the MnPL experimental lines are winterhardy alfalfas developed within and for the Minnesota-Wisconsin area. Cultivars Team, Arc, WL318, and the experimental lines Team WR-3 and 69-T10 are moderately winterhardy and were developed predominantly in the Maryland area. Salton is a nonwinterhardy alfalfa which was developed in the desert valleys of southern California.

The study was organized as a split-plot design. Main plots were four inoculation treatments (*F. oxysporum*

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alone, *C. insidiosum* alone, a mixture of both pathogens, and a noninoculated control). Subplots were the 12 cultivars and experimental lines. The study was replicated three times with 60 to 80 plants per treatment per replication.

The Fusarium inoculum was prepared from three *F. oxysporum* f. sp. *medicaginis* isolates obtained in Minnesota from alfalfa roots. The cultures had been maintained in an autoclaved, sand-loam mixture in test tubes. The isolates were increased individually by adding a few infested soil particles to sterile nutrient broth (2.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_20$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g yeast extract, and 15 g sucrose in 1.0 liter of distilled water). The broth was incubated on a shaker for 4 days in the laboratory. Abundant microconidia and minimal mycelial fragments were produced. The three isolates were combined and the resulting inoculum was diluted to about 1.5×10^6 conidia/ml as estimated from hemocytometer counts.

The inoculum of *C. insidiosum* was prepared by soaking infected alfalfa root tissue, 50 g/liter, in tap water for about 30 min with frequent agitation. The infected roots had been selected the previous September, washed, ground, and stored in plastic bags at −18 C. After the soak period the inoculum was strained through cheese cloth to remove plant debris.

The mixed inoculum was prepared by adding the concentrated conidial suspension of *F. oxysporum* f. sp. *Medicaginis* to the bacterial suspension, both prepared as described above. The final concentration of each pathogen in the mixture was about equal to that in the individual inocula.

Alfalfa seedlings were grown in benches filled with sand in the glasshouse. The seedlings were lifted in mid-June, when they were about 10-wk old; their roots were washed in tap water, and the plants for each plot were tied in a bundle. The roots were kept in tap water until plants for all plots in a replicate were prepared. Then the roots were immersed in the desired inoculum for 20 to 30 min. After inoculation the tops were clipped to within 4 cm of the crown and the roots were clipped to about 12 cm from the crown. Plants in plots that received the same inoculation treatment were grouped together, wrapped in paper towels with wet vermiculite, and refrigerated until transplanted the next day.

TABLE 1. Reaction of alfalfa entries to *Corynebacterium insidiosum*, *Phytophthora megasperma*, and *Colletotrichum trifolii*

Entry	Disease reaction[a]		
	Bacterial[b] wilt	Phytophthora[b] root rot	Anthracnose[c]
Agate	R	R	MR
Arc	MR	S	R
MnPL-1	R	S	S
MnPL-2	R	S	S
MnPL-4	R	MR	S
Ramsey	R	MR	MS
Salton	S	MR	S
Team	S	S	MR
Team WR-3	R	S	MR
Vernal	R	S	MS
WL 318	R	R	R
69-T10	MR	S	MS

[a]Abbreviations: R = resistant, MR = moderately resistant, MS = moderately susceptible, and S = susceptible.
[b]Unpublished data of D. K. Barnes and F. I. Frosheiser, ARS, USDA, University of Minnesota, St. Paul, MN 55108.
[c]Unpublished data, J. H. Elgin and T. E. Devine, ARS, USDA, Beltsville, MD 20705.

TABLE 2. Average severity index and percentage of dead plants in alfalfa entries 3 mo after inoculation with either *Fusarium oxysporum* alone, *Corynebacterium insidiosum* alone, or the two pathogens combined

Entry	ASI[a]/treatment				Dead plants per treatment[b]		
	F. oxysporum alone	*C. insidiosum* alone	Combined inoculum	Noninoculated control	*F. oxysporum* alone (%)	*C. insidiosum* alone (%)	Combined inoculum (%)
Agate	2.15	1.36	2.65	0.48	31	1	40
Arc	2.91	2.24	3.16	0.29	44	1	40
MnPL-1	3.74	1.42	3.85	0.44	63	0	62
MnPL-2	3.69	1.02	4.00	0.54	64	1	70
MnPL-4	4.05	1.27	3.86	0.44	76	1	70
Ramsey	2.82	1.68	3.18	0.25	48	4	52
Salton	2.01	3.29	2.86	0.60	27	3	19
Team	3.09	2.77	3.54	0.35	40	1	43
Team WR-3	3.28	1.56	3.34	0.43	46	1	51
Vernal	3.58	1.83	3.48	0.64	62	1	54
WL 318	2.67	1.78	2.50	0.34	33	1	31
69-T10	3.44	2.19	3.90	0.47	56	0	63
Avg.	3.12	1.87	3.36	0.44	49	1	50
LSD P=0.05	0.54	0.33	0.64	NS	13	NS	14
P=0.01	0.73	0.45	0.88		17		19

[a]Average severity index based on a 0-5 scale. 0 = no internal root discoloration, 5 = stele entirely discolored or plant dead.
[b]No noninoculated plants died.

A modified tobacco transplanter was used to transplant the plants about 20 cm apart in rows spaced 1.0 m apart. To avoid contamination as much as possible, all plots of the noninoculated control were planted first, then all plots inoculated with *C. insidiosum* alone, then all plots inoculated with *F. oxysporum* alone, and finally the plots inoculated with the mixed inoculum. The field was irrigated when necessary.

The numbers of established plants were recorded 2 wk after transplanting and counts of live plants were recorded periodically during the season. The surviving plants were lifted in mid-September and each tap root was sectioned and rated for both Fusarium wilt and bacterial wilt severity according to the amount of internal discoloration. The ratings were based on the following scale: 0 = no discoloration, 1 = small dark strands in the stele, 2 = small discolored areas on the cut surface, 3 = partial ring of discolored tissue under the cortex, 4 = complete ring of discolored tissue under the cortex, and 5 = stele completely discolored or plant dead. Both diseases were rated on the same scale. The occurrence of symptoms of both diseases in the same root was noted. The percentages of plants in each disease-severity class and average disease-severity indexes (ASI) were calculated. The ASI values were subjected to the analysis of variance, and least significant differences were calculated.

RESULTS

Infection and disease development were satisfactory in the treatments with *F. oxysporum* alone, and also with *C. insidiosum* alone. The differences among entries in severity of bacterial wilt (Table 2) were similar to those observed in previous evaluations (Table 1). The differences observed in severity of Fusarium wilt were similar to results previously obtained in the glasshouse. Therefore, we concluded that the present field methods were satisfactory for evaluating resistance to Fusarium wilt.

Fusarium oxysporum killed plants more quickly than did *C. insidiosum*. Very few plants were dead from *C. insidiosum* 3 mo after transplanting, but 90% of the plants killed by *F. oxysporum* died within 5 wk after transplanting.

The symptoms of Fusarium wilt could be distinguished readily from those of bacterial wilt in taproot sections by the difference in color and outline of the discolored areas. Fusarium wilt was characterized by a dark- or reddish-brown discoloration and bacterial wilt was characterized

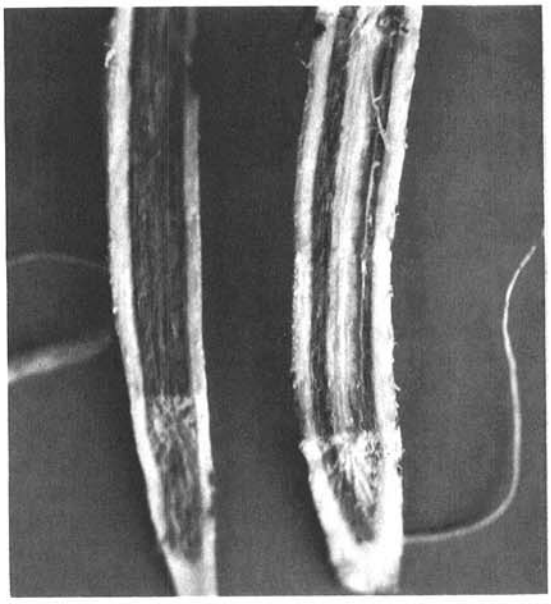

Fig. 1. Alfalfa tap roots with advanced stages of Fusarium wilt caused by *Fusarium oxysporum* f. sp. *medicaginis*. (Left) Stele entirely discolored. (Right) Outer ring of stele discolored.

TABLE 3. Percentage of plants scored 0 to 1 (resistant) and a percentage of surviving plants with root symptoms of either pathogen, in alfalfa entries inoculated with *Fusarium oxysporum* alone, *Corynebacterium insidiosum* alone, and the two pathogens combined

Entry	Resistant plants per treatment			Surviving plants with root symptoms (scored 2-5)/treatment			
				Fusarium wilt symptoms		Bacterial wilt symptoms	
	F. oxysporum alone (%)	*C. insidiosum* alone (%)	Combined inoculum (%)	*F. oxysporum* alone (%)	Combined inoculum (%)	*C. insidiosum* alone (%)	Combined inoculum (%)
Agate	54	58	42	15	13	42	8
Arc	33	25	23	23	25	74	22
MnPL-1	19	57	13	18	19	43	11
MnPL-2	21	75	15	15	12	25	6
MnPL-4	18	63	19	6	10	37	2
Ramsey	41	52	30	11	15	44	8
Salton	58	7	22	15	12	90	51
Team	24	13	9	36	31	87	29
Team WR-3	23	54	21	33	20	46	8
Vernal	22	44	21	16	15	55	14
WL 318	34	42	39	32	17	57	17
69-T10	25	31	11	18	19	69	15
Avg.	31	43	22	20	17	56	16

by a yellowish-brown discoloration in the stele. The discolored areas were more sharply defined in Fusarium wilt than in bacterial wilt.

In advanced stages of Fusarium wilt the entire outer ring of the stele or the entire stele was discolored (Fig. 1). This degree of disease development was seldom seen at the final reading because by then the plants usually had died. Live *Fusarium*-infected plants often had small, partial, or entire discolored rings within the stele in cross section, indicating that the discoloration was limited to vascular bundles or groups of bundles. Discoloration due to bacterial wilt was more diffuse and not particularly limited to certain vascular tissue in the stele. In advanced stages of bacterial wilt the entire stele was discolored. The root cortex usually was not discolored by either disease. A small percentage of the roots had symptoms of both Fusarium wilt and bacterial wilt.

The average ASI for all entries inoculated with the mixed inoculum was slightly higher than the ASI for all entries inoculated with *F. oxysporum* alone and much higher for all entries inoculated with *C. insidiosum* alone (Table 2). The average percentage of plants that died from the mixed inoculum was similar to that from *F. oxysporum* alone. The percentage of plants that died from *C. insidiosum* alone was negligible.

The percentage of live plants with symptoms of Fusarium wilt was nearly equal for entries treated with *F. oxysporum* alone and entries treated with the mixed inoculum. However, the percentage of live plants with symptoms of bacterial wilt was much lower in treatments given the mixed inoculum than in treatments given the bacterial inoculum alone (Table 3). *Fusarium oxysporum* apparently suppressed either the infection by *C. insidiosum*, the development of bacterial wilt symptoms, or both.

The MnPL-4 alfalfa population, which is highly resistant to bacterial wilt, was the most susceptible to Fusarium wilt; and Salton, which is highly susceptible to bacterial wilt, was the most resistant to Fusarium wilt (Table 2 and 3). The increased resistance to anthracnose of Arc over its parent, Team (Table 1), did not enhance its resistance to Fusarium wilt. It appeared that PRR resistance might be associated with resistance to Fusarium wilt because both Agate and WL318 had resistance to PRR and had resistance to Fusarium wilt. However, in a more recent study (Frosheiser and Barnes, *unpublished*) MnGN-1, an experimental line with more resistance to PRR than any other alfalfa line available, was the most susceptible to Fusarium wilt of 81 entries that were tested.

DISCUSSION

The results of this study demonstrates the practicality of evaluating alfalfa populations for reaction to *F. oxysporum* f. sp. *medicaginis* in the field. The procedure also appears preactical for use in a large-scale breeding program for the development of resistant cultivars. The large differences in disease severity among entries suggests that resistance to Fusarium wilt is heritable and that resistant genes are common in alfalfa. It should be possible to increase the level of resistance to Fusarium wilt with a recurrent selection breeding program.

Symptoms of Fusarium wilt were distinct from those of bacterial wilt in roots of plants infected with both pathogens. Since inoculation procedures are similar for both, it would seem advantageous to mix the bacterial and Fusarium inocula and screen simultaneoulsy for both pathogens. However, we do not recommend this because of evidence that *F. oxysporum* f. sp. *medicaginis* may inhibit *C. insidiosum* or have some type of competitive advantage. Further studies are needed to understand the interaction between the two pathogens.

Based on the reactions of the different alfalfa entries it was apparent that the level of resistance to Fusarium wilt was not associated with the level of resistance to anthracnose, Phytophthora root rot, or bacterial wilt. We believe that adding resistance to Fusarium wilt in alfalfa cultivars with resistance to other root and crown pathogens will help increase stand productivity and longevity in many alfalfa-growing areas.

Our *Fusarium* sp. isolates were obtained from infected alfalfa plants growing in Minnesota. Variability in pathogenicity of the fungus has not been thoroughly investigated, and it is possible that isolates from other areas could differ. Armstrong and Armstrong (1) observed that *F. oxysporum* f. sp. *vasinfectum* races 1 and 2 from cotton (*Gossypium* spp.) and *F. oxysporum* f. sp. *cassia* from *Cassia tora* were equal to *F. oxysporum* f. sp. *medicaginis* in pathogenicity to alfalfa and caused identical symptoms. We suggest that, until more is known about the wilt-causing strains of *F. oxysporum*, alfalfa cultivars being developed for specific areas should be screened against isolates from those areas.

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