Virulence, Legume Host Specificity, and Genetic Relatedness of Isolates of *Fusarium oxysporum* from Red Clover

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ABSTRACT

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Fusarium oxysporum is the most prevalent fungal pathogen recovered from symptomatic red clover (*Trifolium pratense*) plants in Wisconsin and contributes to stand loss and reduced productivity. Three field isolates and 44 single-conidium isolates of *F. oxysporum* were recovered from red clover plants with vascular wilt symptoms and assessed for virulence, host specificity, and source of variation in host reaction. In addition, genetic relatedness of selected isolates was determined by vegetative compatibility groups (VCG). Twenty-one populations of red clover, two populations of alfalfa (*Medicago sativa*), and one population each of alsike clover (*Trifolium hybridum*), ladino clover (*Trifolium repens*), and birdsfoot trefoil (*Lotus corniculatus*) were tested for their reaction to isolates of *F. oxysporum*. Host populations differed significantly in their reaction to specific field isolates or single-conidium isolates. Isolates differed significantly in their ability to elicit reactions both among and within host species. VCGs were not useful in predicting host reaction because isolates from distinct groupings elicited similar host reactions. The reaction of red clover to *F. oxysporum* is attributable to genetic diversity in isolate virulence as well as to variation within the host population for resistance.

Red clover (*Trifolium pratense* L.) is an important perennial forage crop in North America, with 4–5 million ha producing an annual yield of 4.0 t/ha (20). Botanically considered a perennial, red clover seldom persists beyond 2 or 3 yr, and in the humid southeastern United States, it is considered a biennial or annual. Stand deterioration is a chronic problem, and increasing the persistence of red clover is a major breeding objective (19). The impact of diseases on red clover stand life and productivity has been estimated at 10 (12) to 50% (6) of the crop value. Fungal pathogens, particularly systemic root and vascular diseases caused by species of *Fusarium*, are major components of the disease complex that impacts red clover persistence (5,12,13). *Fusarium oxysporum* Schlectend.:Fr. is the most common, economically important, and studied species (1,14) and is the most prevalent isolate recovered from diseased red clover plants in Wisconsin, especially during the first year of growth (11,22). Red clover cultivars differ in their reactions to this pathogen, and resistance is related to cultivar persistence (23).

Development of a red clover breeding program to enhance resistance to *F. oxysporum* requires a basic understanding of the source of variation in host reaction. If the primary variation in reaction to the pathogen is due to genetic variation in host population resistance, then maximum resistance in the final population could be achieved by screening numerous host populations with one or a few highly virulent isolates. Conversely, if the primary variation in the reaction of the host is due to variation in the pathogen, selection for resistance to one or a few isolates would not result in resistance to other isolates. Developing red clover with resistance to *F. oxysporum* in this latter scenario would necessitate the screening of a red clover population with many genetically divergent isolates of the pathogen.

The objectives of this study were to determine the virulence, host specificity, and genetic relatedness of heterogeneous and single-conidium isolates of *F. oxysporum* pathogenic to red clover and to identify the primary source of variation in the reaction of red clover to *F. oxysporum*.

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MATERIALS AND METHODS

Fungal isolates. Three field isolates of *F. oxysporum* causing vascular wilt symptoms were recovered from diseased red clover plants at the Ashland Agricultural Research Station, Ashland, WI, in 1989. Symptomatic root or stem tissue was cut to 1 cm lengths, washed in distilled water, and soaked in a solution of 60% sterile water, 8% ethanol, and 32% household bleach (0.525% NaOCl) for 3 min. A 0.5-cm subsection was cut from the center of each sterilized tissue section and placed in 100- × 15-mm plastic petri plates containing potato-dextrose agar (PDA; Difco Laboratories, Detroit). Plates were placed under fluorescent light (12-h photoperiod; 60μE m^{-2} s^{-1}) at 21 C for 7 days. Field isolates from these plates were transferred to fresh PDA for an additional week prior to identification.

The three field isolates are designated F1, F2, and F3 (the composite of all three isolates is F0). Fifteen single-conidium subcultures were derived from F1 (SC1–15), and three single-conidium subcultures were derived from F2 (SC16–18). Three single-conidium isolates each were recovered from alfalfa (*Medicago sativa* L.) and alsike clover (*T. hybridum* L.) plants inoculated with F0. These isolates are identified as SC19–21 and SC22–24, respectively. Races 1 and 2 of *F. oxysporum* Schlectend.:Fr. f. sp. *pisi* (J.L. Hall) W.C. Snyder & H.N. Hans. and an array of single-conidium isolates of *F. oxysporum* identified and collected from Arlington, Ashland, and Marshfield, WI (18), also were used. Representative isolates were sent to the Fusarium Research Center, The Pennsylvania State University, University Park, for cataloging and storage.

Isolates were stored on silica gel crystals (3,16) at 5 C until needed. Crystals with viable conidia were placed on PDA at 21 C for 7 days under fluorescent lights (12-h photoperiod; 60μE m^{-2} s^{-1}). Mycelial plugs (4 mm in diameter) were taken from the colony margin and placed in potato-dextrose broth (Difco) in half-filled baffle-sided Erlenmeyer flasks (two plugs per 75 ml). Flasks were placed on a rotary shaker (150 rpm) at 21–23 C for 7 days under cool-white fluorescent light (12-h photoperiod; 60μE m^{-2} s^{-1}). Conidia were strained through two layers of cheesecloth, quantified with a hemacytometer, and final

concentrations were adjusted with sterile 0.1% water agar.

Inoculation procedure. Isolates of *Fusarium* associated with alfalfa and red clover penetrate roots directly (2,21), but wounding of the roots significantly increases colonization of the host (9,24). Therefore, the procedure used in this study was to cut seedling roots prior to inoculation, similar to the standard Fusarium wilt test used to characterize alfalfa cultivars (15).

Red clover seeds were planted in 12- × 12- × 8-cm plastic trays containing sterile planting medium (1:1:2, v/v/v, sand:silt loam soil:vermiculite). Seedlings were maintained in the greenhouse with 16 h of daylight (190–1,000 μE m^{-2} s^{-1}), 20/15 C day/night temperatures, and fertilized biweekly (all purpose soluble plant food, 20-20-20 N-P-K, Peters Professional Plant Food, W.R. Grace & Co., Fogelsville, PA).

Six-week-old seedlings were uprooted and washed free of excess planting medium. The roots were clipped to a length of approximately 4 cm, and the stems were clipped to a height of approximately 3 cm. Roots were completely immersed in a conidia suspension (>1 × 10^6 microconidia per milliliter) of respective *F. oxysporum* isolates for 20 min. The volume of conidia suspension was adjusted to maintain a ratio of 1 ml of suspension per inoculated seedling. Control plants were immersed in an equivalent amount of sterile water. After inoculation, seedlings were immediately transplanted into 35- × 50- × 10-cm flats containing sterile planting medium. Each flat contained 240 plants arranged in 12 30-cm-long rows of 20 plants each.

Eight weeks after inoculation, plants were uprooted and washed. Roots were cut longitudinally, and root vascular symptoms were rated visually on a disease severity index (DSI) scale of 1–5 as follows: 1 = no visible evidence of vascular discoloration; 2 = visible evidence of vascular discoloration (<33% of root diameter discolored); 3 = definite vascular streaking evident (33–66% of root diameter discolored); 4 = severe vascular discoloration accompanied by necrosis of the root and crown (>66% of root diameter discolored); 5 = dead plant. DSI scores were subjected to an analysis of variance or general linear model, and least significant differences were calculated (SAS Institute, Cary, NC).

Virulence of isolates. Three tests were undertaken to confirm and evaluate isolate virulence on red clover populations. In the initial test, a composite of three field isolates of *F. oxysporum* was tested against 21 populations of red clover. The design was a randomized complete block with 20 plants in each of five replications for each population.

In the second and third tests, the reactions of red clover cultivars Arlington and Chesapeake were measured. The second test consisted of 24 single-conidium isolates (SC1–24), three field isolates (F1–3), and a composite of the field isolates (F0). A randomized complete block was used in a factorial arrangement of treatments (cultivar and isolates) with two replications of 10 plants per treatment.

The third test was conducted with 17 single-conidium isolates of *F. oxysporum* collected and identified from Arlington, Ashland, and Marshfield, WI (18), two isolates each of races 1 and 2 of *F. o. pisi*; and isolate SC16. Isolates were randomized within three replications of 15 plants, and inoculum concentration was 4 × 10^7 microconidia per milliliter of 0.1% water agar.

Host specificity of isolates. Five red clover cultivars (Lakeland, Arlington, Marathon, Chesapeake, and Wisconsin Common), one experimental population (C11), and one cultivar each of alfalfa (Blazer), birdsfoot trefoil (*Lotus corniculatus* L. 'Norcen'), alsike clover (Common), and ladino clover (*T. repens* L. 'Common') were inoculated in tests four and five to determine their reaction to isolate F0. A randomized complete block was used in each test. The fourth test consisted of four replications of 10 plants for each legume, and the fifth test consisted of three replications of 20 plants for each legume. Arlington was not included in the fourth test. Inoculum concentrations were 3 × 10^6 and 2.7 × 10^7 microconidia per milliliter of 0.1% water agar respectively. Isolates of *F. oxysporum* were recovered from diseased

Table 1. Mean disease severity index (DSI) and reaction distribution of 21 red clover populations to a composite of three field isolates of *Fusarium oxysporum* recovered from symptomatic red clover plants at Ashland, WI

Population	Percent plants in DSI[z]					Mean
	1	2	3	4	5	
C11	43	24	13	13	7	2.17
Marathon	37	20	19	11	13	2.42
C191	24	34	14	17	11	2.57
C815	21	32	17	22	8	2.64
C814	32	15	19	18	16	2.70
C145	23	21	13	25	17	2.92
Arlington	18	24	18	23	15	2.93
Penn Pers 2	15	27	15	20	22	3.07
Bombi (2×)	12	21	20	31	16	3.18
St 970 (4×)	14	19	21	20	25	3.23
Fus Early	15	17	16	27	23	3.27
Lakeland	12	22	16	21	27	3.30
Fus Late	18	7	15	37	21	3.37
C192	19	9	14	28	30	3.41
Kenstar	4	15	22	42	15	3.50
Penn Pers 1	10	18	19	16	37	3.52
WT 2×4	7	16	16	21	39	3.70
Pennscott	6	16	15	26	37	3.72
C816	8	10	13	34	34	3.77
Chesapeake	3	9	21	23	49	3.96
Hedda (4×)	3	6	11	19	59	4.28
LSD (0.05)						0.51

[z] Disease severity index: 1 = no symptoms; 5 = dead plant.

Table 2. Reaction of red clover cultivars Arlington (Arl.) and Chesapeake (Ches.) to three field isolates of *Fusarium oxysporum* recovered from symptomatic red clover plants at Ashland, WI, and 24 single-conidium isolates derived from the isolates

Isolate	DSI[y]		Mean
	Arl.	Ches.	
F2	3.05	4.30	3.68
SC08	3.35	3.90	3.63
SC17	3.55	3.55	3.55
SC16	3.35	3.50	3.43
SC13	3.05	3.75	3.40
SC15	3.45	3.30	3.38
SC20	2.95	3.80	3.38
SC06	2.60	4.10	3.35
SC23	2.80	3.75	3.28
F3	2.80	3.65	3.23
F1	2.90	3.55	3.23
F0	3.00	3.30	3.15
SC01	2.85	3.35	3.10
SC12	2.85	3.30	3.08
SC09	2.50	3.55	3.03
SC05	2.90	3.10	3.00
SC19	2.80	3.15	2.98
SC24	2.65	3.25	2.95
SC02	2.80	3.10	2.95
SC18	2.40	3.40	2.90
SC07	2.55	3.25	2.90
SC21	2.40	3.35	2.88
SC10	2.55	3.15	2.85
SC22	2.55	3.05	2.80
SC04	2.15	3.40	2.78
SC03	2.30	2.35	2.33
SC11	2.50	2.85	2.33
SC14	2.30	2.30	2.30
Noninoculated control	1.60	1.98	1.74
LSD (0.05)			0.62
Mean[z]	2.69	3.27	2.98

[y] Disease severity index: 1 = no symptoms; 5 = dead plant.

[z] The mean LSD ($P = 0.05$) for cultivars is 0.16.

tissue of alfalfa (isolate SC20) and alsike clover plants (isolate SC23) that scored a DSI ≥ 3. These isolates were used in a sixth test.

In test six, red clover cultivar Chesapeake and experimental population C11, alsike clover, the alfalfa germ plasm MNGN-1 (15; selected for susceptibility to *F. oxysporum* Schlectend.:Fr. f. sp. *medicaginis* (J.L. Weimer) W.C. Snyder & H.N. Hans.), and the alfalfa cultivar Agate (a resistant control) were evaluated for their reactions to the single-conidium isolates SC16 (a subculture of F2), SC20 (recovered from alfalfa plants inoculated with F0), and SC23 (recovered from alsike clover plants inoculated with F0).

Treatments consisted of each isolate alone and in all combinations plus a noninoculated control. Inoculum concentration was adjusted to 2.3×10^7 microconidia per milliliter of 0.1% water agar, and subsamples of respective isolates were combined for multiple isolate treatments. A randomized complete block was used with a split-plot arrangement of treatments in three replications of 10 plants per treatment combination. Eight whole plots were seven *Fusarium* isolate treatments and one noninoculated control. Subplots were the five legume populations.

Genetic relatedness of isolates. Vegetative compatibility was used as a measure of genetic relatedness using the methods developed by Pulhalla (17). The procedure consisted of generating nitrate-nonutilizing mutant phenotypes by plating isolates of *F. oxysporum* onto 1.5% $KClO_3$ PDA. Isolates SC16, SC20, SC23, *F. o. pisi* race 2, and six isolates collected from Arlington, Ashland, and Marshfield, WI, were characterized for vegetative compatibility group (VCG).

RESULTS AND DISCUSSION

Virulence of isolates. Significantly different reactions to the composite of the three field isolates of *F. oxysporum* were observed among the 21 red clover populations (Table 1) with mean DSI values ranging from 2.17 to 4.28. The experimental germ plasm C11 and cultivar Marathon were significantly lower in reaction than all but the experimental populations C191, C815, C814, and C145. The most susceptible populations in the study were the Swedish tetraploid cultivar Hedda and the cultivar Chesapeake.

Significant differences in virulence were observed among single-conidium isolates when evaluated on cultivars Arlington and Chesapeake (Table 2). The range for DSI among all isolates was 2.15–4.30, with Chesapeake expressing significantly less resistance than Arlington. There was no interaction between isolates and cultivars. It is interesting that the range in this test was almost identical to the range of the previous test (Table 1) in which the reactions of 21 red clover populations to a mixture of three field isolates from one location were measured. From the results of the first test, it could be inferred that the variation in reaction was due to the divergent host populations tested, but differences in reaction to single-conidium isolates in the second test indicated that much of the variation in reaction may have been due to the heterogeneous nature of the field isolates used.

In the third test, significant differences were observed among single-conidium isolates recovered from tissue and soil collected at several Wisconsin locations (data not shown). There was no significant isolate-cultivar interaction, and the range of mean DSI values, from 2.69 to 3.40, was not as great as in the previous two tests. However, all isolates elicited a reaction from red clover. The reduced range of virulence may be due to the fact that these isolates were previously screened for virulence on red clover (18), and only the more virulent ones were selected for this study. Of particular interest were the *F. o. pisi* isolates of races 1 and 2, which were effective in eliciting a host reaction.

Host specificity of isolates. The reaction of red clover and four other legume forages to inoculation with F0 is summarized in Table 3. Ladino clover and Norcen birdsfoot trefoil showed little or no reaction in either test. When inoculated with the lower inoculum concentration, alfalfa, alsike clover, ladino clover, and trefoil showed little reaction and were all significantly less affected than the red clovers. However, at the higher inoculum concentration, the reactions of alfalfa, alsike clover, and ladino clover increased and were not significantly different from Marathon red clover. Birdsfoot trefoil continued to show little or no response to any of the isolates.

Significant isolate, legume, and isolate-legume interactions were observed when alsike clover, alfalfa, and red clover were subjected to the *F. oxysporum* isolates SC16, SC20, and SC23. All of these isolates were virulent on red clover (Table 2). The susceptible red clover cultivar Chesapeake and the susceptible alfalfa germ plasm MNGN-1 had consistently higher mean DSI values (Table 4). Alsike clover expressed little reaction to any of the isolates or isolate combinations, and its mean DSI was significantly lower in reaction than the other legumes tested. The more resistant red clover germ plasm C11 had significantly lower DSI (2.40) than the resistant alfalfa cultivar Agate (2.84) when averaged across isolates. However, differences between C11 and Agate were only significant for isolate SC20 and the combination of SC16 and SC20.

Table 3. Reaction of red clover, alfalfa, alsike clover, ladino clover, and birdsfoot trefoil to a composite of three field isolates of *Fusarium oxysporum* recovered from symptomatic red clover plants at Ashland, WI

	DSI[x]	
Forage legume	**Test 4[y]**	**Test 5**
Red clover		
Chesapeake	2.99	3.25
WI Common	3.17	2.60
Lakeland	3.13	2.55
Arlington	...[z]	2.38
C11	2.17	2.08
Marathon	2.19	1.95
Other legumes		
Blazer alfalfa	1.41	2.02
Alsike clover	1.23	1.92
Ladino clover	1.00	1.53
Birdsfoot trefoil		
Norcen	1.00	1.07
LSD (0.05)	0.47	0.50

[x] Disease severity index: 1 = no symptoms; 5 = dead plant.
[y] Inoculated at two concentrations: 3×10^6 (test 4) and 2.7×10^7 (test 5).
[z] Not included.

Table 4. Summary of reaction of red clover, alfalfa, and alsike clover to three closely related single-conidium isolates of *Fusarium oxysporum* alone and in combination

	DSI[x,y]					
	Alfalfa		Alsike clover	Red clover		
Isolate[w]	**MNGN-1**	**Agate**	**Common**	**C11**	**Chesapeake**	**Mean**
SC16	3.23 a	2.87 cb	1.00 d	2.40 c	3.67 a	2.63 ab
SC20	3.30 a	3.00 a	1.20 c	2.03 b	3.43 a	2.60 ab
SC23	2.73 b	2.50 b	1.20 c	2.10 b	3.70 a	2.45 b
SC16 + 20	3.50 ab	2.90 b	1.33 c	2.03 c	3.97 a	2.75 ab
SC16 + 23	3.83 a	2.47 b	1.23 c	2.50 b	3.73	2.76 a
SC20 + 23	3.03 ab	2.77 b	2.10 b	2.17 b	3.87 a	2.79 a
SC16+ 20 + 23	3.00 b	2.97 b	1.23 c	2.93 b	4.07 a	2.84 a
Noninoculated control	2.37 a	2.23 a	1.13 b	1.77 b	2.09 b	1.92 c
Mean[z]	3.13	2.84	1.30	2.40	3.57	

[w] Isolate SC16 derived from red clover, SC20 from alfalfa, and SC23 from alsike clover.
[x] Disease severity index: 1 = no symptoms; 5 = dead plant.
[y] Means within a column followed by the same letter are not significantly different ($P = 0.05$) using LSD.
[z] LSD (0.05) = 0.24 for legume means.

Although there were no significant differences in virulence for individual isolate treatments averaged over all germ plasm, these single-conidium isolates did differ in their ability to elicit a reaction among and within host species. Since these isolates were closely related and had only one cycle of host selection, the variation in reaction is intriguing. Isolate SC20, recovered from alfalfa, and isolate SC23, recovered from alsike clover, failed to elicit a differential reaction in the two alfalfa populations even though these alfalfa populations are used as susceptible and resistant checks in standard testing of alfalfa for *F. oxysporum* resistance (15). However, isolate SC16, recovered from red clover, did differentiate between susceptible and resistant populations of alfalfa and red clover.

Wide variation in reaction to a specific isolate of *F. oxysporum* exists within and between host species. These isolates are closely related; therefore, much of the variation in reaction may be due to different mechanisms of resistance among the legume species as well as variation in kind and degree of resistance within a species. The genetic mechanism of resistance employed by a host may quickly select for races of *F. oxysporum* that circumvent that particular resistance mechanism, as is illustrated by the virulence of isolate SC20 on both populations of alfalfa. It is conceivable that Agate and MNGN-1, chosen for their differing reaction to *F. o. medicaginis*, possess similar susceptibilities that were quickly exploited by a different race of the pathogen. Selection for resistance to a specific *F. oxysporum* isolate in a specific host population would not result in consistent performance when the host is subjected to heterogeneous populations of *F. oxysporum*.

The naturally occurring pathogen variation is such that pathogen mutation would not be a necessary corollary for breakdown of resistance in a host if the host population were not developed with the incorporation of several mechanisms of defense. Conversely, selection within a genetically narrow host population using many isolates might not identify all the potential mechanisms of resistance that exist within the host species.

Genetic relatedness of isolates. Isolates SC16, SC20, and SC23 were in the same VCG and were compatible with an isolate recovered from soil in which red clover had been grown near Ashland, WI (SC70; Table 5). Two isolates from Marshfield, WI, SC81 from soil in which red clover had been grown and SC71 from a field in which alfalfa had been grown, were compatible. The Ashland red clover soil isolate (SC70) was compatible with a red clover tissue isolate from Marshfield (SC97). However, SC97 was not compatible with SC16, SC20, and SC23, which were derived from Ashland red clover tissue isolates.

Since *F. oxysporum* has no known sexual cycle, parasexual recombination via hyphal anastomosis is the proposed mechanism for generating genetic diversity within the species (1). Such recombination depends on the ability of strains to form heterokaryons with one another, i.e., they must be vegetatively compatible. When Pulhalla (17) first introduced his technique to determine compatibility, he also proposed an evolutionary model for the origin of formae speciales and races of *F. oxysporum*.

It was suggested that *F. oxysporum* once had a normal sexual stage with meiotic recombination that was subsequently lost, and the loci determining vegetative compatibility and virulence became fixed, resulting in isolated asexual inbreeding groups (VCGs). Although VCGs are useful in determining genetic relatedness of isolates, Pulhalla's model has not yet been verified. Our results indicate that the number of genes controlling compatibility is higher than the number of virulence genes, because several distinct VCGs, including two races of *F. o. pisi*, all demonstrated similar symptoms on red clover. In addition, isolates from separate geographic areas were compatible, which is not expected if these populations were geographically isolated for any length of time. Numerous studies, undertaken with other host crops, demonstrate similar findings (4,7,8,10).

Given the heterogeneous state of naturally occurring populations of *F. oxysporum* and the wide host range of the pathogen, several virulent single-conidium isolates should be used for screening red clover populations for resistance. Isolates should be pretested for virulence on a range of red clover populations, and the specific red clover population(s) targeted for improvement should be tested against each individual isolate. If the target population does not have individuals resistant to each isolate, the breeder should consider additional red clover populations for expanding host resistance. Plants selected for resistance to each isolate should be recombined prior to rescreening. Our results indicate that screening with a combination of isolates did not result in significantly increased reactions and, in fact, may result in a reduced range of resistance if isolate competition results in preferential invasion of the host by a given isolate. The dynamics of isolate competition within a heterogeneous population of *F. oxysporum* deserves more research.

Table 5. Vegetative compatibility groups of selected isolates of *Fusarium oxysporum* pathogenic[z] to red clover, alsike clover, alfalfa, and pea

Isolate	SC16	SC20	SC23	SC70	F82	SC81	SC91	SC97	SC50	SC71
SC16	+	+	+	+	–	–	–	–	–	–
SC20		+	+	+	–	–	–	–	–	–
SC23			+	+	–	–	–	–	–	–
SC70				+	–	–	–	+	–	–
F82					+	–	–	–	–	–
SC81						+	–	–	–	+
SC91							+	–	–	–
SC97								+	–	–
SC50									+	–
SC71										+

[z] Tested for virulence in prior studies.

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