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Identity, pathogenicity, and comparative virulence of *Fusarium* spp. related to stand declines of leafy spurge (*Euphorbia esula*) in the Northern Plains[1]

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Abstract:

Strains of *Fusarium* spp. causing root disease and crown rot on leafy spurge in natural epidemics and in association with stand declines were identified as *F. oxysporum*, *F. solani*, and *F. proliferatum*. These species predominated among *Fusarium* spp. isolated from stunted and diseased feeder roots and crowns. There were significant differences in virulence for strains among and within each species. Two strains of *F. oxysporum* belonged to the same vegetative compatibility group. Ten of 11 strains of *Fusarium* originated from noncropped or wild areas. These results indicate that *Fusarium* spp. capable of causing disease on leafy spurge vary as to species, virulence, and compatibility group and are found in a number of sites where this troublesome perennial weed occurs.

Additional keywords:

Rangeland, weeds.

The noxious rangeland weed, leafy spurge (*Euphorbia esula* L.), is pervasive in the Northern Plains of the U.S. and prairie provinces of Canada, infesting more than 1.4 million ha in the U.S. (2). Leafy spurge spreads rapidly through aggressive root proliferation, is toxic to cattle, and competes with native vegetation (13), thus threatening the natural beauty of wild areas and reducing the grazing potential of rangelands. Agricultural and

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other economic losses due to proliferation of this weed are estimated to be in the hundreds of millions of dollars (2). Biological control of leafy spurge with plant pathogens is being investigated as a means of control in situations and areas where application of herbicides is inadvisable, such as riparian zones in wildlife refuges. Soilborne pathogens that have been investigated as candidates for control of leafy spurge are *Rhizoctonia solani* (3, 6), and *Agrobacterium tumefaciens* (4). *Rhizoctonia solani* in association with *Fusarium* spp. is connected with stand declines of leafy spurge. Such stand declines are exceedingly rare based on observations made in extensive surveys made by the authors and cooperating local personnel and thus are notable as sources of potential biological control strains. There is a lack of existing data on the identification, pathogenicity, comparative virulence, and vegetative compatibility group of *Fusarium* spp. associated with natural stand declines of leafy spurge. The objective of this study was to investigate these aspects, and relate the results to the presence of other plant pathogens and insect biological control agents that may affect the pace and extent of stand decline or disease severity at specific sites.

Materials and methods

Collection and processing of disease samples

Surveys were undertaken in 1991 to 1994 in cooperation with local weed control personnel to identify diseased leafy spurge, either from stands that were declining in density, or in patches or clusters of diseased plants within otherwise apparently healthy populations of the weed. Locations where collections were made, collection dates, and the nature of the disease on leafy spurge are given in Table 1. Samples collected were plants exhibiting chlorosis or stunting that occurred within or at the periphery of disease patches. Isolation of *Fusarium* spp. from samples was often done in conjunction with isolation procedures for other soilborne pathogens, with methods and media described previously (6). Samples were either transported under refrigeration, sent immediately by overnight mail by field personnel, or processed at a local facility as described below. Samples were stored at 5º C and were usually processed within 48 to 72 hours of collection. Roots and crowns were examined for symptoms of disease, and thoroughly washed under running tap water to remove all soil. Samples were then soaked for 10 to 15 minutes in a solution of 0.5% sodium hypochlorite, or wiped vigorously with pieces of cotton soaked with the hypochlorite solution prior to rinsing for 1 minute with running tap water, and were blotted dry with sterile paper towels.

Diseased tissue was dissected with a sterile scalpel and pieces of root, root bud (adventitious shoots), and crown tissue at the margins of necrotic tissue were plated on acidified potato dextrose agar (APDA) and Nash and Snyder (10) media. These cultures were incubated at 20 to 25º C in the dark for 10 to 21 days.

Table 1. **Origin of strains of *Fusarium* spp., and symptoms and observations of disease on leafy spurge in the field.**

Strain	Location	Sample collection date	Symptoms and notes
Lyman Creek 4A	Bozeman, MT	6-1-91	Isolated from roots damaged by larvae of *Aphthona flava* along with *Rhizoctonia solani* AG-4 (3)
Sidney 3Y	Sidney, MT	5-16-91	Stand decline; isolated from crowns also infected with *R. solani* AG-4 (3)
Fairy Soil 3V	White Sulphur Springs, MT	6-11-91	Stand decline; isolated from crowns of diseased leafy spurge at periphery of fairy rings associated with decline (6)
Fairy Soil 3B	White Sulphur Springs, MT	6-26-91	Stand decline; isolated from crowns of diseased leafy spurge at periphery of fairy rings associated with decline
McLain 2	Stillwater County, MT	8-15-92	Stand decline, root and crown rot, *R. solani* also isolated
ND94-5	Theo. Roosevelt Natl. Park, ND, South Unit	5/25/94	Wilting and dying plants with crown rot
ND94-6a	Theo. Roosevelt Natl. Park, ND, South Unit	5/25/94	Root and crown rot of dead and dying plants at periphery of patch with dead leafy spurge
ND94-8	Theo. Roosevelt Natl. Park, ND, South Unit	5/25/94	From root of stunted, chlorotic and wilted plants with damage caused by larvae of *Aphthona nigriscutis*
ND94-10	Theo. Roosevelt Natl. Park, ND, South Unit	5/25/94	Spots with dead plants: distorted foliar growth, necrotic root buds; *R. solani* also isolated
WY94-11-4	Devils Tower Natl. Monument, WY	6/14/94	Crown also infected with *R. solani*
MT94-20	Two Dot, MT	6/17/94	Crowns also infected with binucleate *Rhizoctonia* spp.

Culture and storage of strains

Single-spore strains were prepared by standard methods (11, 14), either from cultures isolated from plants collected in the field or from greenhouse pathogenicity tests, and used to prepare inoculum. Briefly, macroconidial suspensions were diluted, spread on agar amended with 50 to 100 μg per ml each of streptomycin and chloramphenicol (WA + S/C) (1), and incubated at 20 to 28° C. Single spores were selected from plates of WA + S/C, transferred to petri dishes containing carnation leaf agar (CLA) (11) for the production of characteristic macroconidia and other structures necessary for identification, and incubated in an incubator set at day/night temperatures of 25 and 20° C, with a 12-hour photoperiod under six 20-W full-spectrum fluorescent tubes; cultures were also incubated at lab temperatures of 20 to 28° C under three 40-W full-spectrum fluorescent tubes and one 40-W blacklight tube with a 14-hour photoperiod. Representative strains that exhibited a colony type that predominated among the several single-spore strains

from each sample were used in pathogenicity tests. All strains were stored at -80° C in nutrient media containing 15% glycerol, and at -20° C on sterile carnation leaves and sterile toothpicks partially colonized by the strains.

Pathogenicity tests and comparative virulence of strains

Inocula for pathogenicity tests were grown in a liquid medium containing 2% (wt/vol) Dietfiber (Lauhoff Grain Co., Danville, IL). Inoculum was thoroughly mixed in a pasteurized greenhouse potting medium (1:1:1, vol/vol, of Bozeman silt loam, peat, and sand, PH 6.6) to achieve approximately 150 CFU per g of air-dried soil mix. Populations of *Fusarium* spp. strains were determined by plating fourfold dilutions of soil on Nash and Snyder medium and processing the data to obtain the most probable number of CFU (7).

Nine-week-old rooted stem cuttings of leafy spurge propagated from plants collected at a single location in northeast Montana were used in all pathogenicity tests. The stem cuttings were planted in infested soil, three per pot, five pots per strain (which constituted a treatment), and grown in the greenhouse at 20 to 28° C. Treatments were arranged in a randomized complete block design. Controls were leafy spurge planted in noninfested soil. Plants were harvested after 10 weeks and assessed for overall root development, root lesions, and crown rot. Disease was assessed by applying the following 0 to 6 rating scale: 0 = no disease; 1 = evident stunting and root discoloration; 2 = as 1, with root lesions and/or root necrosis; 3 = root lesions, necrosis and stunting, moderate overall stunting, and evident chlorosis; 4 = root lesions, root necrosis, overall severe stunting with chlorosis, and crown rot; 5 = death of plant after 8 weeks; and 6 = death of plant within 8 weeks. The test was repeated twice. Data were analyzed by Waller and Duncan's exact Bayesian *k*-ratio least significant difference rule ($P = 0.05$), with data pooled from all trials. Discolored root and crown tissue was plated on Nash and Snyder medium, transferred to APDA or PDA prepared with fresh potatoes (NPDA) (11,14), identified as described below, and compared with cultures used to prepare inoculum.

Identification of *Fusarium* strains from leafy spurge

Cultures growing on CLA for 7 to 14 days were examined microscopically for macroconidial shape, conidiophore morphology, and the occurrence and morphology of chlamydospores. Mycelial plugs were transferred from CLA cultures to plates of NPDA for assessment of such cultural characteristics as colony color, the color of the underside of the culture, the formation of sporodochia, and the presence of sclerotia.

Vegetative compatibility group testing of *Fusarium oxysporum* strains

Several *Fusarium*-defined nitrogen source (FDNS) media were prepared by amending a minimal medium (MM) containing 3% sucrose and 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco, Detroit, MI) (12; and V. Miller, personal communication) with one of several different nitrogen sources (8). A medium containing

2% sucrose, 0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.1% yeast extract, 2% sodium nitrate, and 5% chlorate (YC) was used to generate nitrate nonutilizing (*nit*) mutants (12). FDNS media were used to phenotypically characterize *nit* mutants and the nitrate-FDNS was used to recognize *nit* mutants, and for complementation (hetero-karyon) tests. *Nit* mutants were generated by placing small mycelial plugs of *Fusarium* strains on YC medium and observing for fast-growing sectors over 4 to 5 days. The *nit* mutants were then grown on FDNS media to phenotypically characterize the mutants. Three different *nit* mutants, *Nit*l, *Nit*3, and *Nit*M, were obtained from each strain of *F. oxysporum* and *F. proliferatum*. No *nit* mutants were obtained from strains of *F. solani* in the present study. All *nit* mutants from each strain of *F. oxysporum* and *F. proliferatum* were paired in all possible combinations on nitrate-FDNS media, and pairings scored as vegetatively compatible when wild-type mycelial growth occurred within 2 weeks at colony interfaces between mutant strains. All complementation tests were made at least twice.

Results

Pathogenicity, comparative virulence and identification of *Fusarium* strains

Eight of 11 strains in the present study were collected from sites not previously cropped. Two strains (Sidney and ND9420) were collected from sites that bordered cropped areas but had not received cultivation. One strain, McLain 2, was collected at a site that had been cropped to small grains until the 1950s.

Strains were identified according to Nelson *et al*. (11). Eight of 11 strains were identified as *F. oxysporum* (Table 2) on the basis of the presence of abundant macroconidia that were delicate with a blunt apical cell, abundant single-celled kidney-shaped or oval microconidia, and abundant chlamydospores formed singly or in pairs. Cultural characteristics were fast growth on PDA and white to pink mycelia with faint purplish pigment apparent when cultures were viewed from the underside, abundant tan or orange sporodochia, and the frequent occurrence of blue sclerotia in culture. Two strains were identified as *F. solani* on the basis of large, generally cylindrical macroconidia exhibiting thick walls that were parallel for most of the length of the spore, the presence of chlamydospores and microconidia, and cream-colored sporodochia. Furthermore, macroconidia of *F. solani* had blunt or pointed apical cells, wedge-shaped foot cells, and three to five septa. Oval to kidney-shaped microconidia were home on elongated monophialides. Colonies were fast growing and mycelium was a bluish cream color on NPDA. One strain was identified as *F. proliferatum* on the basis of abundant club-shaped microconidia borne in short chains, polyphialides, and the absence of chlamydospores. Macroconidia were abundant, nearly straight or slightly curved with thin walls, with a basal foot cell, and typically had three to five septa.

Table 2. Identification and comparative virulence of *Fusarium* spp. on leafy spurge.

Strain	Species[y]	Mean disease rating[z]
ND94-8	*Fusarium oxysporum*	5.4 a
Lyman Creek 4A	*F. oxysporum*	4.7 ab
Fairy Soil 3B	*F. oxysporum*	4.6 abc
McLain 2	*F. oxysporum*	4.2 bcd
Sidney 3Y	*F. proliferatum*	3.7 cde
Fairy Soil 3V	*F. solani*	3.4 def
ND94-10	*F. oxysporum*	3.0 efg
MT94-20	*F. oxysporum*	2.6 fgh
ND94-5	*F. oxysporum*	2.2 ghi
ND94-6a	*F. solani*	2.0 hi
WY94-11-4	*F. oxysporum*	1.4 i
Control		0.2 j

[y] Strains were identified with the key of Nelson *et al*. (11).

[z]A scale of 0 to 6: 0 = no disease; 1 = evident stunting and root discoloration; 2 = as 1, with root lesions and/or root necrosis; 3 = root lesions, necrosis and stunting, moderate overall stunting, and evident chlorosis; 4 = root lesions, root necrosis, overall severe stunting with chlorosis, crown rot; 5 = death of plant after 8 weeks; and 6 = death of plant within 8 weeks. Means with the same letter are not significantly different according to Waller and Duncan's exact Bayesian *k*-ratio least significant difference rule ($P = 0.05$), with data pooled from two trials, each with five replicates per treatment.

Leafy spurge plants harvested 10 weeks after planting in infested soil exhibited root stunting (Fig. 1) with numerous dead feeder roots. Internal discoloration was often evident in crown tissue of plants grown in infested soil. All strains caused some degree of root stunting and discoloration. The strains designated ND94-8, Lyman Creek 4A, Fairy Soil 3B, and McLain 2 were the most virulent (Table 2). Lyman Creek 4A and Fairy Soil 3B caused severe crown rot of leafy spurge. In descending order of virulence were the remaining strains, designated Fairy Soil 3V, Sidney 3Y, ND94-10, MT94-20, ND94-5, ND94-6a, and WY94-11-4, respectively. The two most virulent strains, ND94-8 and Lyman Crk 4A, were associated with the presence of the insect biological control agents *Aphthona nigriscutis* and *A. flava*, respectively, and were isolated from roots or crowns damaged by the feeding of the larvae of these species. The following four strains ranking lower in virulence were isolated from plants found at the periphery of stand declines that have been in progress over several years, and the remaining strains were isolated from plants within or at the periphery of small (0.5 to 2 m in diameter) patches of diseased plants of leafy spurge. Strains of *R. solani* were isolated along with *Fusarium* spp. from plants from five of the sites where the six most virulent *Fusarium* spp. strains were isolated. *R. solani* was isolated from diseased plants at only two of the sites from which the remaining five strains of *Fusarium* spp. were isolated.

Two *F. oxysporum* strains, McLain 2 and WY94-11-4, were vegetatively compatible. No other strains belonged to the same vegetative compatibility group. These two strains were significantly different in comparative virulence (Table 2).

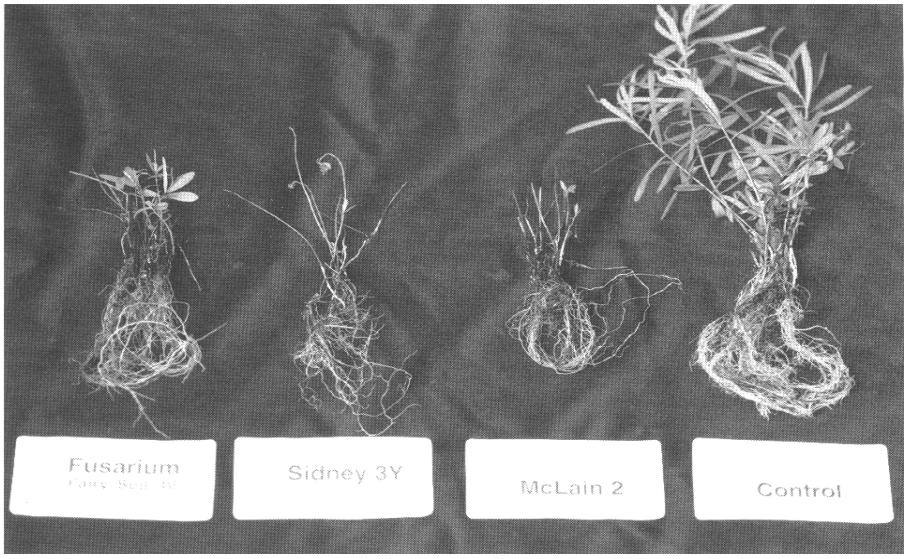

Fig. 1. Reduction in root density caused by destruction of feeder roots by (from left to right) strains of *Fusarium solani*, *F. proliferatum*, and *F. oxysporum,* respectively.

Discussion

The present study is the first to our knowledge to document the pathogenicity, comparative virulence, identity, and vegetative compatibility of *Fusarium* spp. associated with rare domestic occurrences of diseases and stand declines of leafy spurge.

Nine of 10 sites where active stand declines or epidemics of root disease of leafy spurge occurred were short-grass rangeland and had not previously been cropped. *F. oxysporum* and *F. solani* are *Fusarium* spp. that predominated isolations from soils and roots of grasses and forbs in Minnesota prairies (15). *F. proliferatum* (previously synonymous with *F. moniliforme*) also was isolated from leafy spurge. This species has been shown, similarly to the above two species, to have a high ability to colonize host residues (9), resulting in relatively high inoculum potentials. These three species may occur as a collection of either broad-host range pathogenic strains combined with nonpathogenic strains, or pathogenically-specialized strains, or both. Other research indicates that specialized strains increase over time (10, 15) with continued presence of the host. It is critical for weed biological control to determine which scenario applies to the present strains. It is notable that two members of an identical VCG differed widely in virulence. This may indicate the overall potential of strains pathogenic to leafy spurge for variation in virulence and other traits. Future research will assess the host ranges of the *Fusarium* spp. pathogenic to leafy spurge to determine if there is sufficient variability in host range and virulence, as with *Rhizoctonia solani* pathogenic to leafy spurge (3), to suggest the potential for selecting narrow-host-range, highly virulent strains for biological control.

The association of *F. oxysporum* and other *Fusarium* spp. with *Rhizoctonia* (3, 6) and *Pythium* spp. (A. J. Caesar, unpublished) in stand declines occurs both domestically and in Eurasian stands of *Euphorbia* spp., and there is also a consistent relationship of insect root damage with the impact of these soilbome pathogens (A. J. Caesar, unpublished).

The two strains (ND94-8 and Lyman Crk 4A) found to be most virulent were isolated from sites with established and intense activity of larvae of the flea beetle *Aphthona*, released as a biological control agent. It is possible that insect-caused damage to roots at these two sites has created conditions selective for strains capable of aggressively colonizing such root tissue. There are, however, numerous domestic sites where root-attacking insect biological control agents have established on leafy spurge with little apparent effect on plant density. Thus, studies of virulence of the predominant strains at such low-impact sites are needed in comparison with strains from other sites where greater reductions in leafy spurge are seen. Studies are also needed on inoculum density/disease incidence relationships, and comparisons of inoculum densities in rare domestic disease sites and in Eurasian stands of *Euphorbia esula/virgata*, respectively, where diseased plants are found with great frequency. The strain ranking third in virulence is associated with a stand decline that is related to the occurrence and expansion of fairy rings (5), where several other soilbome pathogens have been isolated from declining spurge plants including *Rhizoctonia* (6) and *Pythium* spp. (A. J. Caesar, unpublished). The strains ranking fourth and fifth in virulence are both associated with stand declines, the effects of which are attributable to *R. solani* (6) in addition to *Fusarium.* Thus, a practical control strategy for leafy spurge might best be served by establishing a complex of pathogens and insects similar to those that exert the strong natural control of *Euphorbia* in Eurasia, where the species occurs only in sparse stands, and which seem to be approximated at only a few domestic sites.

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