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Identification, pathogenicity and comparative virulence of *Fusarium* spp. associated with diseased *Euphorbia* spp. in Europe

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Abstract:

Fusarium spp. isolated from diseased *Euphorbia* spp. in Europe were assessed for pathogenicity to North American leafy spurge (*Euphorbia esula/virgata*. Of the nine strains of *Fusarium* spp. isolated either from diseased *E. stepposa* or *E. virgata* in the Caucasus region of Russia and *E. esula/virgata* in southern France, respectively, all were pathogenic to leafy spurge. There were significant differences in virulence among strains. Four strains, including the two most virulent, were identified as *F. oxysporum*. Four of the five other strains were identified as *F. solani* and one was identified as *F. proliferatum*. Three of the four most virulent strains to leafy were isolated from *E. stepposa*. The most virulent strain was associated with root damage caused by insect biological control agents, similar to a previous study on domestic strains of *Fusarium* spp. pathogenic to leafy spurge. Two strains identified as *F. solani* were vegetatively compatible. It was concluded that further screening of a larger set of strains of foreign *Fusarium* spp. under quarantine conditions stateside or in limited overseas facilities is justified and promising.

Introduction

Leafy spurge (*Euphorbia esula* L.) is the most economically damaging weed in the Northern Plains of the United States and Prairie Provinces of Canada (Bangsund). Spurge is difficult to control because of its aggressiveness and the lack of economically feasible chemical measures. Such exotic, invasive weeds constitute a major global change (16). Plant pathogens are under investigation as a primary means of biological control of leafy spurge and other rangeland weeds and with the goal of combining them with insect bio-

logical control or low doses of herbicides. Several soilborne pathogens have been isolated, identified, and assessed for comparative virulence and host range on leafy spurge (3,4,5,6,7). Soilborne fungi constitute the sole means of biological control from which promising agents can be sought domestically as well as from the native range of the target weed. Such candidates are isolated from diseased plants within populations of leafy spurge at sites with epidemics or stand declines (3). Disease outbreaks within leafy spurge stands occur occasionally but are quite rare domestically. In contrast, diseased plants of *Euphorbia esula* or *virgata* are pervasive in Eurasia, the native range of the plant (Caesar unpublished). To investigate the feasibility of a broader study of *Fusarium* spp. as potential biological control agents of leafy spurge, several strains were selected from a large body of strains collected overseas in 1993-95 for identification and assessment for pathogenicity and virulence. Such preliminary studies are necessary to justify any more comprehensive study that would require quite limited overseas greenhouse space or even more limited plant pathogen quarantine space in the U.S.

Collection and processing of disease samples. Locations where collections were made, collection dates, *Euphorbia* host species and the nature of the disease are given in Table 1 (see page 7). Strains were selected for the study on the basis of insect root damage, level of disease observed, and the area of occurrence, which included the Caucasus region of southern Russia. This region is similar to the northern plains in elevation, rainfall and latitude. Plants exhibiting chlorosis and stunting were collected. Isolation of *Fusarium* spp. from samples was often done in conjunction with isolation procedures for other soilborne pathogens, using methods and media described previously (7). Upon collection from the field, samples were transported under refrigeration and processed at a local facility as described below. Samples were stored at 5º C and were usually processed within 48-72 hrs 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 10-15 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 min 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 produced on roots) and crown tissue at the margins of necrotic tissue were plated on acidified potato dextrose agar (APDA) and Nash and Snyder (12) media. These cultures were incubated at 20-25 in the dark for 10-21 days.

Culture and storage of strains. Single spore strains were prepared using standard methods (13,17) both from cultures isolated from plants collected in the field and used to prepare inoculum, and from greenhouse pathogenicity tests. Briefly, macroconidial suspensions were diluted, spread on agar amended with 50-100 μg per ml^{-1} each of streptomycin and chloramphenicol (WA+S/C) (1) and incubated at 20-28º C. Single spores were selected from plates of WA+S/C, transferred to petri dishes containing carnation leaf agar (CLA) (13) 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 20º C, with a 12-hr photoperiod under six 20-Watt full-spectrum fluorescent tubes; cultures were also incubated at lab temperatures of 20º-28º C under three 40-Watt full-spectrum fluorescent tubes and one 40-Watt blacklight tube with a 14-hr photoperiod. Representative strains which exhibited a colony type that predominated among the sev-

eral single-spore strains from each sample were used in pathogenicity tests. All strains were stored at -80º C in nutrient media containing 15% glycerol, and at -20º 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% (w/v) Dietfiber (Lauhoff Grain Co., Danville, IL). Inoculum was thoroughly mixed in a pasteurized potting medium (compost 40%; Sand 10%; Peat moss 20%; Soil 30%) to achieve approximately 150 colony-forming units (cfu) per g of air-dried soil mix. Populations of *Fusarium* spp. strains were determined by plating four-fold dilutions of soil on Nash and Snyder medium and processing the data to obtain the most probable number of cfu (8).

Nine-week old rooted stem cuttings of leafy spurge propagated from plants collected at a single location in NE Montana were used in all pathogenicity tests. The stem cuttings were planted in infested soil, one per pot, five pots per strain, which comprised a treatment, and grown in the greenhouse at 20º-28º C. Treatments were arranged in a randomized complete block design. Controls were leafy spurge planted in noninfested soil. Plants were harvested after 14 weeks, assessed for overall root development, root lesions, and crown rot. Disease was assessed by applying a 0-6 rating scale with 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; 6, death of plant within 8 weeks. The test was repeated once. Data were analyzed using Waller and Duncan's exact Bayesian *k*-ratio LSD rule (P=0.05), using data pooled from both trials. Discolored root and crown tissue was plated on Nash and Snyder medium, transferred to APDA or NPDA and identified as described below and compared with cultures used to prepare inoculum.

Identification of *Fusarium* strains from leafy spurge. Cultures growing on CLA for 7-14 days were examined microscopically for macroconidial shape, conidiophore morphology, and the occurrence and morphology of chlamydospores.

Media used in the identification of various strains of *Fusarium* were potato dextrose agar prepared with fresh potatoes NPDA (11, 14) for the production of characteristic colony colors, sporodochia, and other traits. 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 media (FDNS) were prepared by amending a minimal medium (MM) containing 3% sucrose and 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco) (14, and V. Miller, personal communication) with one of several different nitrogen sources (9). The MM amended with 5% chlorate (YC) was used to generate nitrate nonutilizing (*nit*) mutants (14). FDNS were used to phenotypically characterize *nit* mutants and the nitrate-FDNS was used to recognize *nit* mutants, and for complementation (heterokaryon) tests. *Nit* mutants were generated by placing small mycelial plugs of *Fusarium* strains on YC medium and observing for fast growing sectors over 4-10 days. The *nit* mutants were then grown on FDNS to phenotypically

characterize the mutants. Three different *nit* mutants, *nit*1, *nit*3, and *nit*M were obtained from all four *F. oxysporum* strains and the single strain of *F. proliferatum*. In addition some *nit* mutants were obtained from two of the four strains of *F. solani* in the present study: two *nit* mutants, *nit* 1 and *nit* M, were generated from 94f-15, and a single *nit* M mutant was generated for 94f-24. All *nit* mutants which could be obtained from respective strains of *F. oxysporum* and *F. proliferatum*, and *F. solani* were paired in all possible combinations on nitrate FDNS, and pairings scored as vegetatively compatible when wild-type mycelial growth occurred within two weeks at colony interfaces between mutant strains. All complementation tests were made at least twice.

Results

Pathogenicity, comparative virulence, identification and vegetative compatibility of strains. The strain ranking highest in virulence, 94f-5, was isolated from roots of *Euphorbia stepposa* that had exhibited feeding damage similar to that caused by *Aphthona* spp. (11, Caesar unpublished), although no adults or larvae of any particular insect could be detected. The above strain caused greatly reduced root density in one trial and caused the death of all plants in a second trial. The remaining strains ranked in descending order of virulence were 94f-37, 94f-25, 94f-24, 94f-11, 94f-15, 93f-17, 94f-29, and 94f-30, respectively (Table 2, see page 7). There were significant differences in virulence within this array (P=0.05).

The onset of disease was indicated by chlorosis, progressing to necrosis, of lower leaves. Further progression of disease was indicated by defoliation, stem chlorosis and apical necrosis. Similarly to observations made in the field throughout Eurasia, the number of stems of leafy spurge in greenhouse pathogenicity studies was typically restricted to no more than one or two by all the strains tested. The two most virulent strains in this study, 94f-5 and 94f-37, were identified as *F. oxysporum* 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 ranking lower in virulence, 94f-25 and 94f-24, were identified as *F. solani* on the basis of large, generally cylindrical macroconidia exhibiting thick walls which were parallel for most of the length of the spore, the presence of chlamydospores and microconidia and cream-colored sporodochia. Macroconidia of *F. solani* furthermore had blunt or pointed apical cells, wedge-shaped foot cells and three to five septa. Oval to kidney-shaped microconidia were borne on elongate monophialides. Colonies were fast-growing and mycelium was a bluish cream color on NPDA. Four other strains were identified as either *F. oxysporum* or *F. solani* with the lowest ranking strain identified as *F. proliferatum*. The identity of this single strain was on the basis of abundant club-shaped microconidia borne in short chains, polyphialides, and the absence of chlamydospores. Macroconidia of *F. proliferatum* were abundant, nearly straight or slightly curved with thin walls, a basal foot cell, and typically had three to five septa. Two strains identified as *F. solani*, 94f-15 and 94f-24, isolated from *Euphorbia virgata* and *E. stepposa*, respec-

tively from different areas outside of Spakovskoe sniish, Russia were determined to be vegetatively compatible. No other strains were vegetatively compatible.

Discussion

Although *Euphorbia* spp. in Europe are generally unthrifty as indicated by relatively sparse root density and reduced shoot proliferation, many different levels of disease are observed (Caesar, unpublished). *Fusarium* spp. are isolated from roots and crowns of plants of *Euphorbia* regardless of disease severity. The genus itself is ubiquitous and cosmopolitan, containing many nonpathogenic strains (13). The small body of strains tested in the present study reflects a desire to restrict characterization of strains initially to those obtained under conditions (immediate refrigeration after collection and during transport and rapidity of processing of samples and cultures) that closely approximated those for a previous study on domestic strains of *Fusarium* spp. isolated from diseased leafy spurge (3). The geographic region from which most strains used in the present study were collected, the central and northern Caucasus of Russia, is largely devoid of phenotypes of *Euphorbia* that are similar to North American leafy spurge. However, edaphic conditions in this area are otherwise quite similar to those in major portions of the range of leafy spurge in the Northern Great Plains. The high virulence of strain 94f-5 (isolated from *E. stepposa*) to North American leafy spurge (*Euphorbia esula/virgata*) may at least indicate that the genetic similarity of the original host to the target weed species need not be critical. That three of the four strains ranking highest in virulence to an accession of North American leafy spurge were isolated from *Euphorbia stepposa* would further support this. Whether the ecological origin and therefore adaptation of the strain may generally override the genetics of the host requires further study. Furthermore, whether these data indicate a broad host range of the *Fusarium* strains within or outside taxa that contain the North American *Euphorbia esula/virgata* types is also in need of further study. The finding that the most virulent strain identified in the present study was isolated from insect-damaged roots and crowns is intriguing. The two most virulent strains in a previous study of domestic strains were similarly associated (3). Preliminary results indicate that both foreign and domestic strains are thus far narrow in host range when tested on native grasses and several cultivated species (4). This finding concurs with abundant prior research on *Fusarium* spp. host range. That two strains, isolated in close proximity from *Euphorbia* spp., are of the same vegetative compatibility group is interesting because it indicates the results of the present limited study of strains isolated in Europe and those of a previous study of U.S. strains (3) indicate that *Fusarium* spp. have significant potential as biological control agents of leafy spurge. It was observed in greenhouse tests that, in general, symptom development using foreign strains of *Fusarium* spp. was more rapid in tests overseas than was typically observed with domestic strains (Caesar, unpublished). Studies are needed on comparative virulence of native strains of *Fusarium* spp. pathogenic to leafy spurge versus foreign strains to confirm these observations. The further collection of *Fusarium* spp. strains overseas, especially from plants wounded by insect biological control agents, and their characterization for comparative virulence and host range is justified. Only through such studies can the full potential of *Fusarium* spp. as biological control agents be fully addressed.

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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	Host species	Symptoms and notes
93f-17D	Novomarieskaya, Botanical Reserve, Russian National Forest	6/24/93	*E. stepposa*	Cankers at soil line, chlorosis, vascular discoloration
94f-5	Spakovskoe sniish, Russia	7/3/94	*E. stepposa*	Dead root buds; evidence of insect feeding
94f-11	Agricultural Institute, Spakovskoe sniish, Russia	7/3/94	*E. stepposa*	Dead root buds
94f-15	10 km north of Spakovskoe sniish, Russia	7/4/94	*E. virgata*	Root rot, dead root buds
94-24	9 km east of Spakovskoe sniish, Russia	7/5/94	*E. stepposa*	Dead root buds
94-25	9 km east of Spakovskoe sniish, Russia	7/5/94	*E. virgata*	Cankers at soil line
94-29	Strigament plateau, elevation 831 m, Russia	7/6/94	*E. virgata*	Dead root buds, cankers at soil line
94-30	Tatarka, Russia	7/6/94	*E. virgata*	Dead root buds
94f-37	Cavaillon, France	7/15/94	*E. esula/virgata*	Dead root buds

Table 2. Comparative virulence to North American leafy spurge of *Fusarium* spp. isolated from diseased *Euphorbia* spp. in Europe.

Strain	Mean disease rating[b]	Identification[a]
94f-5	5.5[a]	*Fusarium oxysporum*
94f-37	5.2[a]	*F. oxysporum*
94f-25	5.0[a]	*F. solani*
94f-24	4.4[ab]	*F. solani*
94f-11	3.6[bc]	*F. oxysporum*
94f-15	3.4[bc]	*F. solani*
93f-17D	3.4[bc]	*F. oxysporum*
94f-29	3.2[bc]	*F. solani*
94f-30	2.6[c]	*F. proliferatum*
Control	0.2[d]	—

[a] Strains were identified using the key in Nelson *et al*. (1983).

[b] Disease rating scale of 0-6, means 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 chlorpsis; 4, root lesions, root necrosis, overall severe stunting with chlorosis, crown rot; 5, death of plant after 8 weeks; 6, death of plant within 8 weeks. Means with different letters are significantly different using Waller and Duncan's exact Bayesian k-ratio LSD rule (P=0.05), using data pooled from two trials, each with five replicates per treatment