PATHOGENICITY OF FUSARIUM PROLIFERATUM IN CONTAINER-GROWN DOUGLAS-FIR SEEDLINGS[1]

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ABSTRACT

Fusarium proliferatum (teleomorph: *Gibberella fujikuroi*) is one of the most commonly-isolated fungi from roots of both diseased and healthy container-grown conifer seedlings within nurseries in the Pacific Northwest of the United States. This fungus colonizes root cortical tissues and is particularly common on seedlings several months old. From evaluations at container nurseries, we found that most isolates of *F. proliferatum* were very similar morphologically. Much less pathogenic diversity on conifer seedlings occurred within this species than was often found with other fusaria, particularly *F. oxysporum*. Most tested isolates were very aggressive on conifer seedlings, causing damping-off and root disease. Genetic diversity among conifer seedling isolates needs evaluation. Disease-associated losses are variable among nurseries and maintaining high seedling vigor is important for limiting damage.

INTRODUCTION

Recently, production of container-grown conifer seedlings for reforestation has greatly increased in the Pacific Northwest of the United States. Conifer seedlings can be produced quickly (6-8 months) and usually perform when outplanted as well as bareroot seedlings which may take 2-3 years to produce. Unfortunately, conditions ideal for growing container seedlings are also ideal for infection and damage by several disease-causing fungi (James 1984b), particularly foliage pathogens like *Botrytis cinerea* Pers. ex Fr. (James 1984a) and root diseases caused by *Fusarium* spp. (James et al. 1987).

Several years ago we began investigating root diseases of container-grown seedlings because losses were often extensive and growers needed tools for reducing these losses. Initial efforts involved isolating and identifying root-colonizing fungi associated with diseased seedlings at various stages throughout the greenhouse growth cycle. Several different types of fungi were isolated, but the most common were in the anamorphic genus *Fusarium* (James et al. 1987). Although several different *Fusarium* spp. were encountered at different nurseries (James et al. 1989a), one species was usually represented at high numbers in all sampled nurseries. This species produced common *Fusarium*-like macroconidia in sporodochia, chains of microconida borne either in mono- or polyphialides, lacked typical chlamydospores and often produced various shades of violet pigment in pure culture. Superficially, cultures of this species resembled *F. oxysporum* Schlecht., a species commonly encountered causing root disease of conifer seedlings, especially bareroot stock (James et al. 1989a, 1991). Based on descriptions of Nelson et al. (1983), we classified this fungus in the section *Liseola* and designated it in the anamorphic taxon *F. proliferatum* (Matsushima) Nirenberg.

Over several years, at different container nurseries, we collected many isolates of *F. proliferatum* that appeared quite similar morphologically when grown in pure culture on potato dextrose agar (PDA). The only morphological variability we encountered was level of violet pigmentation and extent of polyphialide production. This lack of variability contrasts with other fusaria we have commonly isolated, particularly those classified as *F. oxysporum* (James et al. 1989a), which varies widefly in extent of aerial mycelium, pigmentation, sporodochial production, and microconidial morphology.

We found that *F. proliferatum* was more commonly isolated later in the crop cycle when seedlings were several months old, rather than on very young seedlings (James et al. 1995). We also found that this species was infrequently isolated from conifer seed (James et al. 1995). Apparently, the species was

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introduced at high levels to seedling crops in some other way.

Since most nurseries use containers for several seedling crops, we began to look at reused containers as a potential source of root disease inoculum. Most nurseries superficially washed containers with high-pressure water (sometimes steam) to remove residual organic debris (growing media, seedling roots) remaining after seedling extraction. This type of "cleaning" was unsatisfactory because relatively high levels of fungal inoculum remained, particularly *F. proliferatum*, on the inner walls of both styroblock and plastic cell containers (James et al. 1988). Although chlamydospore production is lacking in *F. proliferatum* (Nelson et al. 1983), the fungus is capable of surviving within containers for several months, probably colonizing organic debris remaining after "cleaning" (James et al. 1995). Immersing containers in hot water renders most potential disease inoculum, including *F. proliferatum*, nonviable (James et al. 1988; James and Woollen 1989).

Despite treating containers with hot water, we still encountered *F. proliferatum* on roots of container seedlings by the end of the growth cycle, even those without typical disease symptoms (James et al. 1995). *F. proliferatum* may be disseminated throughout greenhouses on bodies of adult fungus gnats (James 1994), but we suspect that passive dissemination of this fungus is most common. In particular, the fungus produces long chains of microconidia that dislodge upon drying to be carried by air currents. It is also possible that some inoculum may be brought into greenhouses from outside sources. Because *F. proliferatum* commonly infects apparently healthy seedlings, we hypothesized that some isolates might be more pathogenic than others, similar to the situation that occurs with *F. oxysporum* (Gordon and Okamoto 1992; James et al. 1989b). The remainder of this paper describes our efforts to test our hypothesis and discusses our findings in the context of what we know about *F. proliferatum* epidemiology.

MATERIALS AND METHODS

One laboratory and three greenhouse experiments were conducted to evaluate pathogenicity of *F. proliferatum*. In all experiments, Douglas-fir (*Pseudotsuga menziesii* var. *glauca* [Beissn.] Franco) seeds (seedlot FN, University of Idaho, Moscow, Idaho) were used. All seeds were soaked in a two-part bleach and three-part water solution for 10 min (Wenny and Dumroese 1987), rinsed 48 h in running tap water, and stratified 21 days at 2-3°C.

Inoculum for the experiments was prepared using the techniques of Miles and Wilcoxon (1984). Basically, this techniques uses perlite, an inert, inorganic, siliceous rock of volanic origin, commonly used in potting mixtures, as the matrix for fungal growth. To provide nutrients for fungal growth, cornmeal (150g) was moistened with warm 1% potato dextrose agar (PDA)(300ml) and added to perlite (75g). Perlite/cornmeal/PDA mixtures were inoculated with 10ml spores suspensions of selected *Fusarium* isolates (prepared by adding sterile, distilled water to 14-day-old cultures) and incubated in the dark at about 24°C for at least 21 days, after which the fungus had thoroughly colonized the perlite/cornmeal substrate. After incubation, inoculum was dried in open petri plates within a cabinet. After 5-7 days, inoculum was dry and uncontaminated because the food base was completely colonized by the inoculated fungus. Inoculum was stored in plastic vials under refrigeration until needed, remaining viable for at least 2 years.

For all greenhouse experiments, Douglas-fir seedlings were grown in a 1:1 peat:vermiculite growing medium (W. R. Grace & Co., Portland, Oregon), commonly used to grow conifer seedlings, using a standard growing regime (Wenny and Dumroese 1992).

Laboratory experiment:

Twenty-four isolates of *F. proliferatum* obtained from roots of diseased or non-diseased container-grown seedlings were tested for pathogenicity using a previously described lab technique (James 1996). Basically, glass vials (23ml) were filled to about two-thirds capacity with a dried coconut:vermiculite medium (Grace/Sierra Horticultural Productes, Milpitas, CA) and autoclaved 60 min at 121°C. For each isolate, inoculum was ground to a fine powder with mortar and pestle and 0.05g was added to each of 24 vials of autoclaved medium, resulting in an aproximately 1:50 (w/w) mixture of inoculum to medium. Inoculum was distributed throughout the medium by shaking. For each pathogenicity test sequence, one set of 24 control vials was made using non-inoculated perlite instead of inoculum.

Douglas-fir seeds, after stratification, were placed on moistened sterile filter paper (Whatman No. 3, Whatman International Ltd., Maidstone, England) within sterile petrie dishes and incubated under 12 hr. diurnal fluorescent light at about 24°C. Germinants, selected when their primary root was at least 3mm long, were carefully placed with their primary root pointing downward, one per vial. Four ml sterile

water were added to each vial just before germinant placement. We incubated vials with germinants under cool, fluorescent, diurnal light (12 h photoperiod) at 22-24°C. Germinants within inoculation vials were examined daily for disease symptoms (damping-off and hypocotyl rot). Two ml sterile water were added to germinants surviving 7 days. Tests concluded at 14 days, and all surviving germinants were removed and examined for disease symptoms. Reisolations were made from all inoculated germinants on a selective agar medium (Komada 1975) to determine if infection by inoculated isolates occurred.

A numerical rating system was developed to compare levels of *Fusarium* virulence (James 1996). The rating system awarded points based on duration of germinant survival within inoculated vials, occurrence and type of disease, reisolation of inoculated fungal isolate, and primary root growth within the vial. Germinants received one point for each day of survival from day 3-14 (12 points maximum), another 1, 2 or 4 points depending on type of disease apparent (maximum points if non-diseased), an additional 1, 2 or 4 points based on fungal reisolation (maximum points if no organism reisolated), and 3 points more if the root grew to the bottom of the vial. The range of possible points was 3-23, with higher point values reflecting less aggression by the tested isolate against the germinant. To convert points to a score in which higher numbers represent greater virulence, we devised a reciprocal rating. Ratings ranged from 0 to 100; 0 indicating germinants were uninfected within 14 days and 100 indicating all germinants killed within 3 days by the inoculated isolate.

Greenhouse experiment:

We conducted three greenhouse pathogenicity experiments on seedlings of various ages: germinants, 2 months old, and 5 months old. Inoculum was prepared for two isolates each of *F. proliferatum* and *F. oxysporum*. The first *F. proliferatum* isolate (8629G) was obtained from non-diseased Douglas-fir seed (seedlot FN), while the second isolate (8656M) was from roots of a non-diseased Douglas-fir seedling (seedlot FN). Both *F. oxysporum* isolates were obtained from roots of diseased Douglas-fir seedlings growing at the USDA Forest Service nursery, Coeur d'Alene, Idaho (isolate 8649S from seedlot 6070 and isolate 8675B from seedlot 2741). Results of some of these tests using other *F. oxysporum*, *F. acuminatum* Ell. & Ev., *F. avenaceum* (Fr.) Sacc., and *F. sambucinum* Fuckel isolates were previously reported (James et al. 1989b).

Germinant experiment:

Metal trays (38cm x 23cm x 5cm) were filled with peat:vermiculite, autoclaved 60 minutes at 121°C, and allowed to cool. Inoculum was mixed thoroughly into the autoclaved medium at a 1:25 or 1:50 (w/w) basis. Controls consisted of autoclaved growing medium amended with uninoculated perlite at the same two treatment concentrations. The inoculum-growing medium mixture was moistened with sterile distilled water until the mixture was at field capacity.

Seeds were treated as described above, but after the bleach treatment and before the 48 hr. running water rinse, seeds were also soaked 3 minutes in a 70% ethanol solution. After stratification, 200 seeds were sown into each tray and covered with 5mm layer of silica grit. Trays were then placed inside the greenhouse.

Germination and seedling emergence were monitored in each tray; post-emergence damping-off was determined by examining trays for seedlings with stem lesions. Damped-off seedlings were carefully removed and used to isolate inoculated fungal isolates to confirm pathogenicity. All isolations were made on a selective medium for *Fusarium* spp. (Komada 1975).

Germination was considered complete 30 days after sowing and ten non-diseased seedlings, if that many were still available, were then collected from each tray for root isolations. Also, ten ungerminated seed were carefully removed from each tray for determination of infection by inoculated fungal isolates. Isolations were made onto the selective medium and emerging fungi were compared with inoculated isolates to confirm root and seed infection.

Two-month-old seedling experiment:

Seedlings were grown in 66ml Spencer-LaMeire Root Trainers for 7 weeks and then carefully removed from their containers. Roots were gently and thorougly washed to removed adhering particles of growing medium and repotted in Ray Leach pine cells (66ml) with a mixture of peat:vermiculite amended with inoculum at a concentration of 1:50 (w/w). Twenty-five seedlings were inoculated per isolate; controls consisted of repotting seedlings in an uninoculated perlite-growing medium mixture. As seedlings died, we removed them for root isolations to determine if infection was by inoculated isolates. All remaining seedlings were rated for se-

verity 84 days after inoculating using a five-point system describing extent of foliar chlorosis and necrosis (James et al. 1989b). The higher the rating, the more severe the disease symptoms. After rating, isolations were made from all seedlings' roots to determine infection by inoculated isolates.

Five-month-old seedling experiment:

Seedlings were grown in Ray Leach pine cells for 5 months and then carefully removed from their containers. Roots were gently and thoroughly washed to remove adhering particles of growing medium and repotted into Ray Leach super cells (144ml) with a mixture of peat:vermiculite amended with inoculum at a concentration of 1:50 (w/w). Ten seedlings were inoculated per test isolate; controls consisted of repotting seedlings in an uninoculated perlite-growing medium mixture. As seedling died, we removed them for root isolations to determine if infection was by inoculated isolates. Seedlings were rated using the five-point disease severity system, and roots isolated as described above 68 days after inoculation.

RESULTS AND DISCUSSION

Laboratory experiment:

All tested *F. proliferatum* isolates had virulence ratings of high or moderate on young Douglas-fir germinants (Table 1). For some isolates, three different tests were conducted and, virulence was usually quite similar in all tests. No apparent effects of host source or disease condition of host was noticed on relative virulence of these isolates. In general, most *F. proliferatum* isolates evaluated were consistently high in virulence with little variation among all the tested isolates. This contrasts similar tests of soilborne and conifer seedling root tissue isolates of *F. oxysporum*, which consistently show a very wide range of virulence from non-pathogenic to highly virulent (James, unpublished).

Greenhouse experiments:

Comparisons of selected *F. proliferatum* and *F. oxysporum* isolates indicated that the former species was more pathogenic than the latter in tests evaluating Douglas-fir seed germination and post-emergence damping-off (Table 2), and disease severity on young (Table 3) and older (Table 4) seedlings. *F. proliferatum* test isolates resulted in reduced germination and higher post-emergence damping-off than typical *F. oxysporum* isolates (Table 2). Likewise, the same *F. proliferatum* isolates resulted in greater mortality of young Douglas-fir seedlings and overall higher disease ratings (Table 3), as well as higher disease ratings of 5-month-old seedlings (Table 4). Although only a few isolates were evaluated in these latter tests, the data suggests that *F. proliferatum* may be more virulent in controlled pathogenicity tests than typical *F. oxysporum* isolates.

From these experiments, we conclude that anamorphic isolates of *F. proliferatum* are important, potentially highly virulent pathogens of container-grown conifer seedlings. Douglas-fir is especially susceptible to damage, although many other conifer species are also affected. Reducing damage caused by *F. proliferatum* may be difficult because by the time disease symptoms occur on seedlings, most root systems are thoroughly colonized with the pathogen. Fungicide applications when disease symptoms become noticeable are usually ineffective (Dumroese et al. 1990), probably because of the extent of root colonization and the fact that much of the active ingredient is incapable of reaching root systems in enough concentration to eliminate the pathogen. Likewise, biological control agents have thus far proved ineffective (Dumroese et al. 1996). We recommend prevention as the most reliable means of reducing damage. Prevention requires an aggressive program of sanitation, including sterilizing containers which may be reused for additional seedling crops (James et al. 1988), cleaning greenhouse benches, floors and walls with a surface sterilization agent such as bleach, and monitoring disease carefully, including removal of diseased seedlings to reduce potential secondary pathogen spread (James et al. 1991). Such techniques have worked well in some nurseries to reduce disease severity.

More work is needed in understanding the epidemiology of *F. proliferatum*- associated diseases. We need to know more about inoculum sources, factors affecting seedling root infection, and potential ameliorating effects of other microorganisms that might induce antagonism. As chemical pesticide use continues to decrease because of environmental and worker safety concerns, growers will have to rely on cultural disease controls as well as implementing effective biological controls as part of integrated pest management systems to reduce impacts of root diseases caused by *F. proliferatum*.

Table 1. Virulence of selected *Fusarium proliferatum* isolates on young Douglas-fir germinants in the laboratory.

	Source[1]			Percent			
Isolate	Host	Conditon	Test No.	Percent Diseased	Avg. [2] Survival	Avg. Score	Virulence[3] Rating
9036B	DF	Diseased	1 2 3	100 100 100	5.4 4.8 3.1	87 90 99	High High High
9061C	DF	Healthy	1 2	100 100	6.6 3.0	81 98	High High
9104C	DF	Healthy	1 2	100 100	3.5 3.0	96 98	High High
9108B	WL	Diseased	1 2	93 79	9.8 8.3	63 70	Mod. Mod.
9112F	BS	Diseased	1	100	5.8	84	High
9125D	WL	Diseased	1 2	100 100	3.3 3.9	98 94	High High
9131A	LP	Diseased	1 2 3	93 92 100	9.5 8.2 5.2	65 69 85	Mod. Mod. High
9131B	LP	Diseased	1 2 3	80 63 87	9.2 10.5 7.9	61 52 72	Mod. Low Mod.
9139H	WB	Diseased	1	100	4.4	92	High
9201E	GN	-	1 2 3	100 100 100	5.6 3.3 3.4	86 98 98	High High High
9202C	CT	-	1 2	100 92	3.8 5.6	94 85	High High
9202D	CT	-	1 2	100 100	3.8 3.7	95 96	High High
9202F	CT	-	1 2 3	100 100 100	3.9 3.1 3.1	95 99 99	High High High
9202H	CT	-	1 2	100 100	4.5 3.0	93 99	High High
9202I	CT	-	1	100	4.2	93	High
9202J	CT	-	1 2	100 92	5.6 5.2	85 87	High High
9202-O	CT	-	1	100	4.0	94	High
9202T	CT	-	1 2 3	100 100 100	4.0 3.2 3.3	93 99 99	High High High

Table 1 continued

	Source[1]			Percent			
Isolate	Host	Conditon	Test No.	Percent Diseased	Avg. [2] Survival	Avg. Score	Virulence[3] Rating
9223F	AS	Diseased	1	100	3.0	100	High
9224A	DF	Diseased	1	100	3.1	98	High
9225P	WB	Diseased	1	100	4.0	92	High
9249P	DF	Healthy	1	100	3.8	96	High
9306C	WP	Diseased	1	100	3.2	99	High

[1] All hosts were container-grown seedlings except GN = fungus gnats trapped in greenhouses and CT = styroblock containers used to grow seedlings. DF = Douglas-fir. WL = western larch (*Larix occidentalis* Nutt.), BS = Colorado blue spruce (*Picea yungens* Englem.). LP = lodgepole pine (*Pinus contorta* Dougl.), WB = whitebark pine *Pinus albicaulis* Englem.), AS = aspen (*Populus tremuloides* Michx.). WP = western white pine (*Pinus monticola* Dougl.).

[2] Average number of days germinants survived; all tests ran for 14 days.

[3] Virulence scores ranged from 0 to 100; the higher the number, the more virulent the isolate. See James 1996 for complete descriptions of the numerical point system and derivation of virulence scores.

Table 2. Effects of selected *Fusarium proliferatum* and *F. oxysporum* isolates on germination of Douglas-fir seed and post-emergence damping-off of young seedlings in the greenhouse.

Species	Isolate	Inoculum Concentration	Percent Germination	Percent Damping-off
F. proliferatum	8629G	1:25 1:50	15 15	97 97
	8656M	1:25 1:50	6 17	100 94
F. oxysporum	8649S	1:25 1:50	25 43	33 32
	8675B	1:25 1:50	29 24	35 46
Controls		1:25 1:50	72 69	1 1

Table 3. Effects of selected *Fusarium proliferatum* and *F. oxysporum* isolates on disease severity in young, inoculated Douglas-fir seedlings in the greenhouse.

Species	Percent Isolate	Mortality	Average Disease Rating*	Percent Root Colonization**
F. proliferatum	8629G 8656M	92 96	4.7 4.9	100 100
F. oxysporum	8649S 8675B	24 32	2.8 3.0	100 100
Controls		0	1.6	0

* Rating system from 1-5; higher numbers mean more severe disease symptoms.
** Seedlings not killed by either *Fusarium* spp. within 84 days of inoculation. Root colonization is the percent of sampled root system colonized by inoculated isolates.

Table 4. Effects of selected *Fusarium proliferatum* and *F. oxysporum* isolates on disease severity in older inoculated Douglas-fir seedlings in the greenhouse.

Species	Isolate	Average Disease Rating*	Percent Root Colonization**
F. proliferatum	8629G 8656M	3.0 3.5	85 99
F. oxysporum	8649S 8675B	2.0 1.6	77 87
Controls		2.0	25

* Rating system from 1-5; higher numbers mean more severe disease symptoms.
** Seedlings not killed by either *Fusarium proliferatum* or *F. oxysporum* within 68 days of inoculation. Root colonization is the percent of sampled root system colonized by inoculated isolates (for controls: percent of sampled root system colonized by any *Fusarium* spp.).

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