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Fusarium oxysporum Protects Douglas-fir (*Pseudotsuga menziesii*) Seedlings from Root Disease Caused by *Fusarium commune*

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Fusarium root disease can be a serious problem in forest and conservation nurseries in the western United States. *Fusarium* inoculum is commonly found in most container and bareroot nurseries on healthy and diseased seedlings, in nursery soils, and on conifer seeds. *Fusarium* spp. within the *F. oxysporum* species complex have been recognized as pathogens for more than a century, but attempts to distinguish virulence by correlating morphological characteristics with results of pathogenicity tests were unsuccessful. Recent molecular characterization and pathogenicity tests, however, revealed that selected isolates of *F. oxysporum* are benign on Douglas-fir (*Pseudotsuga menziesii*) seedlings. Other morphologically indistinguishable isolates, which can be virulent, were identified as *F. commune*, a recently described species. In a replicated greenhouse study, inoculating Douglas-fir seedlings with one isolate of *F. oxysporum* prevented expression of disease caused by a virulent isolate of *F. commune*. Moreover, seedling survival and growth was unaffected by the presence of the *F. oxysporum* isolate, and this isolate yielded better biological control than a commercial formulation of *Bacillus subtilis*. These results demonstrate that an isolate of non-pathogenic *F. oxysporum* can effectively reduce Fusarium root disease of Douglas-fir caused by *F. commune* under nursery settings, and this biological control approach has potential for further development.

Keywords **:** biological control, *Fusarium commune*, *Fusarium oxysporum*, nursery management, root disease, *Pseudotsuga menziesii*

The association of *Fusarium* spp., particularly those in the *F. oxysporum* Schlechtend.:Fr. taxon, with damping-off and root disease in conifer reforestation nurseries of the western United States has been recognized for nearly a century (Spaulding, 1914). These fungi are important pathogens in bareroot and container nurseries (Bloomberg, 1971; Dumroese and James, 2005), affecting a variety of plant species including conifers (Kim et al., 2012). Foliar chlorosis, necrosis, and wilting can be above-ground symptoms of this root disease (Dumroese and James, 2005). Although the epidemiology has been discerned (James et al., 1987) and integrated pest management techniques recommended (Dumroese et al., 2002), *Fusarium* spp. continue to cause damage to seedling crops, especially Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco) and western white pine (*Pinus monticola* Dougl. ex D. Don). Chemical pesticides are generally in effective, especially late in the crop production cycle. Known potential biological control agents are also often ineffective, likely because they were developed for use in agronomic, rather than forestry systems (Fravel et al., 2003).

Efforts to improve disease control have been hampered because of an inability to correlate *F. oxysporum* populations with subsequent disease potential (Stewart et al., 2012). Morphologically similar isolates of *F. oxysporum* can exhibit wide ecological variability (Gordon and Martyn, 1997). For example, isolates classified as *F. oxysporum* based on morphology were found to have a wide range of virulence on Douglas-fir (Stewart et al., 2006). Recent molecular studies using amplified fragment length polymorphism (AFLP) and DNA sequencing confirmed that selected non-virulent *Fusarium* were *F. oxysporum*; whereas, virulent isolates were identified as *F. commune*, a recently described species (Skovgaard et al., 2003; Stewart et al., 2006).

Fungi classified as *F. commune* and *F. oxysporum* are morphologically indistinguishable, but genetically very distinct (Stewart et al., 2006), showing disparate levels of virulence on Douglas-fir seedlings (Stewart et al., 2012). Both fungi are apparently well-adapted to forest nursery environments. Previous studies using crops other than Douglas-fir in nurseries have shown that non-pathogenic *F. oxysporum* can prevent disease by pathogenic *F. oxysporum* (Fuchs et al., 1999; Fravel and Larkin, 2002; Huertas-Gonzalez et al., 1999; Larkin and Fravel, 2002; Shishido et

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al., 2005). We therefore hypothesized that some non-pathogenic isolates of *F. oxysporum* may have the potential to protect conifer seedlings from virulent isolates of *F. commune*. Our study objectives were to determine if 1) non-pathogenic *F. oxysporum* could infect Douglas-fir seedlings without causing adverse effects, such as reducing growth; and 2) non-pathogenic *F. oxysporum* can potentially protect Douglas-fir seedlings against a highly virulent isolate of *F. commune*. We believe this paper is the first to describe biological control of *F. commune* with *F. oxysporum*, and the first to employ non-pathogenic isolates from forest and conservation nurseries in the western United States against a common pathogen in those same systems.

Based on previous (Stewart et al., 2006) and subsequent (unpublished) AFLP and DNA sequencing results, we selected three *F. oxysporum* isolates (Fo-Q12, Fo-Q76, and Fo-N16; hereafter Q12, Q76, and N16) from diverse phylogenetic clades and a *F. commune* isolate (Fc-H14; hereafter H14). *Fusarium oxysporum* isolates Q12, Q76, and N16 were initially obtained from the roots of a diseased western white pine seedling, an asymptomatic western white pine seedling, and a diseased Douglas-fir seedling, respectively. The highly virulent isolate (H14) was obtained from a diseased Douglas-fir seedling and was originally identified as *F. oxysporum* based on morphology as described by Nelson et al. (1983). Although H14 was previously used in pathogenicity tests, it was only recently identified as *F. commune* based on molecular genetic analyses (Stewart et al., 2006). Following isolation, all isolates were maintained on carnation leaves in sterile water (Fisher et al., 1982). We evaluated pathogenicity of all isolates using traditional Koch's postulates with Douglas-fir seedlings as hosts. These tests revealed that the 3 *F. oxysporum* isolates were non-pathogenic and the *F. commune* isolate was highly virulent (data not included).

Inoculum for pathogenicity testing was prepared as described by Miles and Wilcoxson (1984) because similar inoculum was effective in previous pathogenicity tests on conifer seedlings (James et al., 1989). Perlite, an inert, inorganic, siliceous rock of volcanic origin commonly used in soilless planting media in conifer container nurseries, was the matrix for fungal growth. Inoculum consisted of 150 g of yellow cornmeal moistened with 300 ml warm 1% potato dextrose agar (PDA), mixed with 75 g of perlite. This mixture was autoclaved for 60 min at 121 °C (1.3 kg cm^{-2}). After cooling, the perlite-cornmeal mixture was inoculated with mycelium from 14-day-old cultures of each *Fusarium* isolate grown on PDA. Inoculum was incubated in the dark at about 24 °C for 20 to 24 days. Following incubation, the inoculum was air dried within a cabinet for at least 3 days and kept refrigerated in plastic bags until needed. Similar inoculum was previously stored this way for several months without loss of viability (James et al., 1989).

Our experiment employed a 6 inoculation treatments (described below) × 2 levels of *F. commune* isolate H14 (present or absent) × 3 replications × 10 seedlings factorial design. The 6 inoculation treatments were as follows. Treatment 1 was the control: non-inoculated Pro-Mix® HP [ca 70% (v:v) *Sphagnum* peat moss and ca 30% (v:v) horticulture coarse-grade perlite; Premier Horticulture, Quakertown, Pennsylvania, USA]. Treatment 2 was Pro-Mix® BX, the same medium as Pro-Mix® HP but commercially inoculated with the proprietary biofungicide Subtilex®, a strain of *Bacillus subtilis* (GB03). Treatments 3 through 5 each included inoculum of one *F. oxysporum* isolate (Q12, Q76, or N16, respectively) mixed (1:50, w:w) with the same Pro-Mix® HP used in Treatment 1. Both Pro-Mix® media had a moisture content of 42% when inoculum was added. Treatment 6 was a combination of all three *F. oxysporum* isolates (Q12, Q76, and N16); inoculum for each isolate was added to Pro-Mix® HP at a rate of 1:16 (w:w). For each inoculation treatment × *F. commune* level × replication combination we filled 10 individual containers (Ray Leach Cone-tainers™, Stuewe & Sons, Inc., Tangent, OR, USA; 2.5-cm diameter, 16-cm length, 66-ml volume, 1076 containers m^{-2}) often used to grow conifer seedlings (360 containers total). A subsample of each potting medium was collected for assay of potentially pathogenic fungi. Once filled in mid April, containers were sown with 3 seeds each of interior Douglas-fir (*P. menziesii* var. *glauca* Beissn.) from the Flathead National Forest in western Montana USA (Hungry Horse Ranger District; 1675 m elevation) that had been stratified for 28 days. Seeds were covered with about 1 cm of fine rock mulch (1.2- to 4.8-mm particles; Target® Forestry Nursery Grit, Target Products Ltd., Burnaby, British Columbia, Canada) and placed on metal tables inside a fully-controlled greenhouse. Target greenhouse temperatures and photoperiod followed the recommendations of Wenny and Dumroese (1992). Most seeds germinated between 7 and 14 days after sowing; emerged seedlings were thinned to a single seedling per container. Beginning 3 weeks after sowing and continuing throughout the experiment, each inoculation treatment × *F. commune* level × replication combination was fertigated when container weights were 85% of field capacity (Landis et al., 1989). The fertigation was performed with a soluble fertilizer containing the following (mg/L): 150 N (100 as NO_3^- and 50 as NH_4^+), 50 P, 170 K, 45 Ca, 20 Mg, 62 S, 20 Fe, and 15 S.T.E.M.® (micronutrients; The Scotts Co., Marysville, Ohio). Containers were systematically rearranged at regular intervals to minimize microclimate variation.

Three months after sowing (mid July), we carefully

removed seedlings from their containers and gently shook the media from roots. All seedlings in the "absent" *F. commune* H14 treatments were transplanted into non-inoculated Pro-Mix® HP (180 total), whereas seedlings in the "present" *F. commune* H14 treatments (180 total) were transplanted into Pro-Mix® HP inoculated (1:50, w:w) with the highly virulent *F. commune* H14 as described above. A subsample of the Pro-Mix®–inoculum mixture was collected to assay for viable *F. commune*.

Four weeks after transplanting (mid August), seedlings in each inoculation treatment × *F. commune* level × replication were rated for their severity of foliar symptoms using a 1 to 5 system as described in James et al. (1989). In this experiment, however, seedlings fell into only three of the classifications. Seedlings with a rating of 1 were asymptomatic, lacking foliar chlorosis or necrosis; seedlings with a rating of 3 had evidence of necrosis on < 50% of their foliage. Dead seedlings received a rating of 5. All seedlings were carefully removed from their containers and their roots were gently washed to remove adhering potting medium. Samples of the potting media from control seedlings (no *F. oxysporum* or *Bacillus*) later exposed to *F. commune* were retained for assay of the pathogen. Healthy seedlings were measured for height (root collar to tip of terminal bud) and root collar diameter. For all seedlings (diseased and healthy), 10 lateral roots were randomly selected and 2- to 3-mm-long tips were aseptically served and incubated on Komada's medium, a selective medium for *Fusarium* spp., especially *F. oxysporum* (Komada, 1975). Remaining roots and shoots from healthy appearing seedlings were dried at 60 °C until constant weight to determine biomass. The peat-based growing medium was assayed using a standard soil-dilution technique (James et al., 1996). After a 7- to 10-day incubation, fungal isolates from roots and media were observed; the most common colonies were selected from each treatment combination for DNA extraction and sequencing as described in Stewart et al. (2006) to confirm that the isolates were the same that were inoculated onto seedlings.

Our study design tested six inoculation treatments (control; *F. oxysporum* Q12, Q76, and N16; a combination of all 3 isolates; and *Bacillus subtillus* GB03) with either the presence or absence of the highly virulent isolate (*F. commune* H14). After confirming homogeneity of variance, we used separate ANOVAs (Statistical Analysis System Institute, Gary, NC, USA) to compare height, root collar diameter, and biomass of apparently healthy seedlings among potential biological control treatments with and without the presence of the highly virulent isolate. When the *F*-tests were significant at $P = 0.05$, we used least squared means (lsmeans) to separate means. For survival of seedlings after exposure to the highly virulent isolate, we analyzed data with a Chi-square test and calculated the *P* value using the Monte Carlo estimation (Agresti et al., 1979).

Seedlings inoculated with only *F. oxysporum* (Q12, Q76, N16, or the combination) were found to have roots colonized with *F. oxysporum*, whereas seedlings inoculated with only H14 were found to be colonized by *F. commune* based on DNA sequences of the elongation factor 1-α and mitochondrial small subunit rDNA (data not shown). Similarly, after inoculation with both *F. oxysporum* and *F. commune*, subsequent asymptomatic seedlings yielded both species.

We detected no significant treatment differences in height, root-collar diameter, or biomass of asymptomatic seedlings when inoculated with the potential biological controls, even when subsequently exposed to *F. commune* (Table 1). In the absence of *F. commune*, no seedlings died in the control or had necrotic foliage when exposed to any of the *F. oxysporum* isolates or *Bacillus subtilis* GB03. Seedling survival was significantly different, however, after control and bio-control-inoculated seedlings were exposed to *F. commune* ($X = 24.3770$; $P = 0.0050$). Seedlings initially inoculated

Table 1. Means (± S.E.) of morphological characteristics for Douglas-fir seedlings inoculated with potential biological controls and subsequently challenged with *Fusarium commune*

Treatments					Challenged with *Fusarium commune* Fc-H14[a]			
	n	Height (cm)	Root-collar diameter (mm)	Biomass (g)	n	Height (cm)	Root-collar diameter (mm)	Biomass (g)
Control	30	9.0 (0.2)	1.39 (0.03)	0.29 (0.01)	17	9.3 (0.3)	1.52 (0.04)	0.30 (0.02)
Bacillus subtillus GB03	30	8.7 (0.2)	1.41 (0.03)	0.28 (0.01)	16	8.0 (0.3)	1.38 (0.04)	0.24 (0.02)
Fusarium oxysporum Fo-Q12	30	8.5 (0.2)	1.45 (0.03)	0.28 (0.01)	30	8.6 (0.2)	1.51 (0.03)	0.29 (0.01)
F. oxysporum Fo-Q76	30	8.0 (0.2)	1.42 (0.03)	0.27 (0.01)	20	8.7 (0.3)	1.44 (0.04)	0.28 (0.01)
F. oxysporum Fo-N16	30	8.2 (0.2)	1.38 (0.03)	0.27 (0.01)	18	8.2 (0.3)	1.43 (0.04)	0.26 (0.02)
F. oxysporum combination (Fo-Q12 + Fo-Q76 + Fo-N16)	30	8.6 (0.2)	1.43 (0.03)	0.28 (0.01)	25	8.2 (0.3)	1.44 (0.03)	0.27 (0.01)
P value		0.0602	0.0530	0.7889		0.0899	0.0737	0.0804

[a]Only asymptomatic seedlings evaluated.

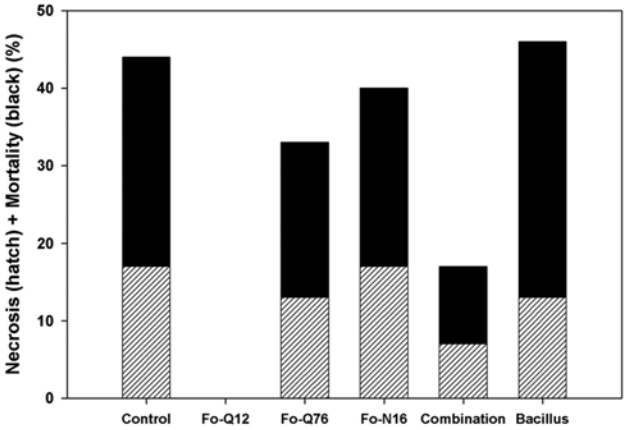

Fig. 1. Seedlings were grown 3 months in the presence of *Fusarium oxysporum* isolates Fo-Q12, Fo-Q76, and Fo-N16; a combination of all 3 isolates; *Bacillus subtillus* GB03; or a non-inoculated control. The hatched portion of the bars shows the percentage of seedlings that developed necrotic needles (but < 50% of foliage was affected; classification 3 as described in James et al. (1989)) after being transplanted into medium containing *Fusarium commune* Fc-H14; whereas the black portion indicates the percentage that died. *Fusarium oxysporum* Fo-Q12 had significantly less necrosis (zero) and mortality (zero) than the other treatments, which were similar in response.

with *F. oxysporum* Q12 and subsequently exposed to *F. commune* exhibited no necrosis or mortality (Fig. 1). No significant differences were observed, however, among the other treatments exposed to *F. commune* with respect to incidence of necrotic foliage or mortality.

In this study, *Bacillus subtilis* GB03 was ineffective in protecting Douglas-fir from a pathogenic isolate of *F. commune*. We have previously found that a commercially available isolate of the biocontrol fungus *Gliocladium virens*, marketed to protect seedlings against Fusarium root disease, was also ineffective (Dumroese et al., 1996). Our experience is that application of potential biological control fungi developed from agronomic environments for specific crops show little (Dumroese et al., 1998) to modest (Mousseaux et al., 1998) benefit with conifer seedlings. A isolate of *F. oxysporum* (Fo47) has been an effective biological control against pathogenic *F. oxysporum* on many species (*e.g.*, Blok et al., 1997; Fuchs et al., 1997) including some tree seedlings (Salerno et al., 2000). In limited laboratory tests, however, this isolate, which is closely related to our *F. oxysporum* isolates, did not protect young Douglas-fir germinants from pathogenic *Fusarium* spp. (James, 2002).

The non-pathogenic *F. oxysporum* Q12 provided excellent protection of Douglas-fir seedlings against the virulent *F. commune* H14 without adversely affecting seedling growth. The biocontol efficacy of *F. oxysporum* Q12 is perhaps attributable to its collection from a specialized nursery environment. Cropping system adaptation associated with increased biocontrol efficacy has been previously described for *Fusarium* (Fravel et al., 2003). Our ability to routinely isolate both *F. oxysporum* and *F. commune* from Douglas-fir roots indicates that these species likely occupy similar rhizosphere niches and may compete for available nutrients and/or the same root infection sites. If so, prior inoculation of Douglas-fir seedlings with certain non-pathogenic *F. oxysporum* strains may have restricted *F. commune* access to roots (*e.g.*, Alabouvette et al., 1993; Fuchs et al., 1999). Induced systematic resistance has been reported in *Fusarium* (Fuchs et al., 1997; Larkin and Fravel, 1999; Mandeel and Baker, 1991). Perhaps, *F. oxysporum* Q12 is able to induce systematic resistance more effectively than either *F. oxysporum* Q76 or N16. Moreover, this may be why *F. oxysporum* Q12 was an effective biocontrol agent when applied to seedlings before exposure to *F. commune* at the same concentration (*i.e.*, 1:1). Other studies indicated that much higher concentrations of the biocontrol agent (*e.g.*, 10:1 to 100:1 for Fo47) must be present to effectively restrict disease when plants are exposed to the pathogen and biocontrol agent simultaneously (Alabouvette et al., 1993; Bolwerk et al., 2005; Larkin and Fravel, 1999). Biocontrol activities can be attributed to diverse factors, such as antibiosis, competition, induced host resistance, interactions with other microbes, or other mechanisms (Fravel et al., 2003; Hadelsman and Stabb, 1996). Further studies are needed to determine the mode of action for the biocontrol activity of *F. oxysporum* Q12 against highly virulent *F. commune* H14.

Although we successfully controlled root disease caused by *F. commune* by prior inoculation with a non-pathogenic isolate of *F. oxysporum*, only one virulent isolate was tested and only one non-pathogenic isolate offered control. Clearly, additional work is needed to determine the scope of efficacy, particularly with additional conifer species. Testing additional non-pathogenic *F. oxysporum* isolates, which are genetically similar to Q12, may reveal other isolates with biocontrol potential. Additionally, the efficacy of *F. oxysporum* Q12 against diverse *F. commune* isolates also warrants testing.

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