Potential for Using *Fusarium* to Control *Fusarium* Disease in Forest Nurseries

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Abstract: The taxon *Fusarium oxysporum* contains a complex of fungi that are very important pathogens of many plant species worldwide, including seedlings grown in forest nurseries. All members of this complex appear very similar morphologically, and can often be differentiated only on the basis of genetic analyses. Strains of *F. oxysporum* may be pathogenic or nonpathogenic and both types often occupy the same environments and readily infect plant roots. Because of their similar requirements, nonpathogenic strains of *F. oxysporum* have been exploited as biological controls of pathogenic *Fusarium* strains on several types of crops. Although nonpathogenic strains infect plants, they do not induce disease symptoms. All previous nonpathogenic *F. oxysporum* strains have been obtained from, and used for, particular agricultural systems. We have obtained several isolates that are nonpathogenic on conifer seedlings and are genetically distinct from highly virulent isolates. Three of these are currently being tested on container Douglas-fir seedlings within a greenhouse to evaluate their efficacy for controlling root disease caused by virulent *F. oxysporum* isolates.

Keywords: nursery diseases, biological control, *Fusarium*, microorganism interactions

Background

Fusarium oxysporum Schlechtend:Fr. has been associated with important diseases in forest nurseries for decades. This fungal species causes several different types of diseases, including pre- and post-emergence damping-off, cotyledon blight of young germinants, stem and root decay of young seedlings, and root disease of older seedlings (Bloomberg 1971; James 1986, 1987; James and others 1991). These diseases are often very difficult to control (Bloomberg 1971, 1976; James and others 1991; James 2004) and some losses can usually be expected during each seedling crop. Most forest and conservation nursery crop species are susceptible to, and often damaged by, *F. oxysporum*. Damage has especially been severe on pine species, including ponderosa (*Pinus ponderosa*), lodgepole (*P. contorta*), and western white (*P. monticola*), as well as Douglas-fir (*Pseudotsuga menziesii*), and true firs (*Abies* spp.) (James 1986; James and others 1991). However, most other conifer, hardwood, and brush species can also be impacted by these fungi.

In bareroot nurseries, *F. oxysporum*-induced diseases have traditionally been controlled by pre-plant soil fumigation with non-selective biocides (James 1989; James and others 1990b; James and others 1996). Chemicals used as fumigants include methyl bromide, chloropicrin, metam-sodium, and dazomet (James 1989). These have usually effectively decreased soil populations of *F. oxysporum* and several other pathogens (James and others 1990b; James and others 1996). However, soil fumigation has not always resulted in adequate disease control (James and Beall 1999). One major problem is that fumigants can potentially kill all soil organisms, including fungi, bacteria, Actinomycetes, nematodes, and insects (James 1989). Fumigants do not preferentially kill pathogenic fungal strains, and can also greatly reduce or eliminate beneficial microorganisms, including bacteria and nonpathogenic fungi. When most microorganisms are killed by chemical soil fumigants, the resulting soil becomes a vacuum that can readily be colonized by the first introduced organisms (James 1989). Introduced organisms can greatly increase their populations due to lack of competition from other organisms. For example, if pathogenic isolates of *F. oxysporum* are introduced into fumigated soil on infested seeds, contaminated equipment, or blowing soil, subsequent disease severity may be much higher than if the soil had never been fumigated (James 1989). Chemical and running water

seed treatments prior to sowing can significantly reduce pathogen inoculum (James 1985b, 1986, 1987; James and others 1991). It is nearly impossible, however, to exclude all pathogens following fumigation (James 1989). Nursery growers hope that beneficial, nonpathogenic microorganisms initially colonize fumigated soil at high levels, and effectively exclude establishment by pathogens.

Recent increases in container production of forest seedlings have occurred throughout western North America. Unfortunately, *Fusarium oxysporum* may also be associated with diseases of container seedlings. In particular, damping-off due to infested seeds can be severe (James 1986, 1987). In general, peat-based growing media are usually not contaminated with potential *Fusarium* pathogens (James 1985a). Some pathogen inoculum can be introduced into new container seedling crops on re-used plastic or Styrofoam™ containers (James and others 1988). Fortunately, most growers have instituted effective container sterilization procedures, especially immersion in hot water (James and Woolen 1989; Dumroese and others 2002), which eliminates most potential pathogen inoculum. Although pathogen inoculum can reside and remain viable within greenhouses, proper sanitation procedures, such as treating surfaces within growing areas with sterilants (bleach), can greatly reduce potential for pathogen introduction into new seedling crops (James and others 1990a). In addition to *F. oxysporum*, another *Fusarium* species (*F. proliferatum* (Matsushima) Nirenberg) has been shown to be an important pathogen on container seedlings (James and others 1997). It is especially associated with root diseases that tend to occur near the end of the growth cycle.

The Nagging Problem

Fusarium oxysporum is actually a taxon encompassing several different *Fusarium* species that are characterized by specific, consistent morphological characteristics (Gordon and Martyn 1997; Kistler 1997; Baayen and others 2000; Skovgaard and others 2001). For example, some members of this species complex are aggressive pathogens that cause either vascular wilts or root and stem decay (Gordon and Martyn 1997; Fravel and others 2003). Other isolates, however, are strictly saprophytic and do not elicit disease symptoms on infected hosts (James and others 1991; Gordon and Okamoto 1992; Gordon and Martyn 1997). Saprophytic isolates can often be isolated from both healthy and diseased plants (James and others 1991; Gordon and Martyn 1997). Although they look identical in culture, pathogenic and nonpathogenic isolates may have different genetic characteristics (Gordon and Okamoto 1992; Kistler 1997; Stewart and others 2004, 2005). Pathogenic isolates have traditionally been identified by their ability to elicit plant disease symptoms. However, such tests are expensive, time-consuming, and require several weeks or months for completion (James 1996).

Fungi within the *F. oxysporum* species complex produce three kinds of spores that can be delimited microscopically: multi-celled macroconidia, smaller (usually unicellular) microconidia, and resting spores called chlamydospores (Nelson and others 1983; James and others 1991). Another important taxonomic characteristic of this complex is the production of microconidia within groups called false heads at the end of short, unbranched monophialides (Nelson and others 1983). Some isolates produce varying shades of blue-purple pigments in culture, particularly on a full nutrient medium such as potato dextrose agar. Particular isolates will also produce blue sclerotia, especially in older cultures (James and others 1991).

A serious problem in dealing with these fungi is that different isolates having similar morphology can exhibit wide ecological variability (Gordon and Martyn 1997). When we isolate *F. oxysporum* from plants or soil in forest nurseries, we cannot differentiate pathogenic from nonpathogenic strains based on isolate morphology. Therefore, we cannot predict disease impacts because we do not know what portion of the *F. oxysporum* population are aggressive pathogens.

Solving the Problem

Fortunately, recent work has indicated that pathogenic and nonpathogenic strains from forest nurseries can often be separated on the basis of genetic characteristics (Stewart and others 2004, 2005, 2006). Nonpathogenic strains may have metabolic differences from pathogenic strains, such as reduced production of plant-susceptible toxins (Amraoui and others 2005). Some pathogenic strains within the *F. oxysporum* complex have been reclassified as a new species called *F. commune* sp. nov. (Skovgaard and others 2003). We hope that molecular probes may soon be developed that can be used to identify pathogenic isolates within host plants, particularly before disease symptoms become evident (Kelly and others 1988), and within nursery soils (Stewart and others 2004).

Fusarium oxysporum as a Biological Control Agent in Other Agricultural Settings

Because pathogenic and nonpathogenic strains of *F. oxysporum* exhibit wide genetic diversity (Correll and others 1986; Appel and Gordon 1994; Kistler 1997; Vakalounakis and Fragkiadakis 1999; Edel and others 2001; Lori and others 2004), some strains of this fungus can be used as biological control agents, either directly on unwanted pest plant species (Hebbar 1996) or indirectly to control disease-causing pathogens on important crops (Alabouvette and others 1993; Fravel and others 2003). For example, certain strains effectively control broomrapes (Thomas and others 1998; Amsellem and others 2001) and witchweeds (Ciotola and others 2000), which can be important pests on certain agricultural crops. Other strains effectively control undesirable narcotic plants such as coca, opium poppy, and hemp (Connick and others 1998). Strains utilized as mycoherbicides are quite host-specific and will only target the undesirable plants (Hebbar 1996; Connick and others 1998).

Because of the wide diversity within *F. oxysporum*, scientists began testing the potential of nonpathogenic strains to control plant diseases caused by *Fusarium*. This process of biological control was called "cross protection" because nonpathogenic strains protected plants from pathogenic strains (Hillocks 1986; Louter and Edgington 1990; Huertas-Gonzalez and others 1999). Nonpathogenic *F. oxysporum*

strains with potential as biological control agents have often been isolated from disease-suppressive soils (Alabouvette and others 1984; Tamietti and others 1993; Larkin and others 1996), that is, soils within which specific diseases do not occur even though pathogens may be present. Some microorganisms within suppressive soils may limit development of pathogens by their effects either directly on the pathogens or indirectly on host plants (Liu and others 1995; Larkin and Fravel 1999).

One of the most studied nonpathogenic strains of *F. oxysporum* (designated Fo47) was isolated several years ago from disease-suppressive soil in France (Alabouvette and others 1993; Larkin and Fravel 1999; Benhamou and Garand 2001; Cotxarrera and others 2002). This strain has been effective against root diseases, especially those caused by pathogenic strains of *F. oxysporum* (Fuchs and others 1997, 1998; Duijff and others 1998; Duijff and others 1999; Fravel and others 2003). This strain has effectively controlled diseases on a variety of crops, including tomatoes (Fuchs and others 1997, 1998; Olivain and Alabouvette 1997; Larkin and Fravel 1998; Steinberg and others 1999a, b; Bolwerk and others 2005), peas (Benhamou and Garand 2001), asparagus (Blok and others 1997), carnations (Postma and Rattink 1992), and *Eucalyptus* seedlings (Salerno and others 2000). However, in a small laboratory test (James 2002), strain Fo47 was ineffective in controlling *Fusarium* damping-off of young Douglas-fir germinants. Strain Fo47 is currently marketed in France as "Fusaclean" (Natural Plant Production, Nogueres, France) (Benhamou and Garand 2001).

Another highly-effective nonpathogenic strain (designated CS-20) has effectively controlled *Fusarium* root diseases on tomatoes, muskmelon, basil, and watermelon plants (Larkin and Fravel 1999; Fravel and Larkin 2002; Fravel and others 2005). Strains Fo47 and CS-20 have not yet been registered for use in the United States. However, another strain (251/2) is currently undergoing registration as a biological control agent on specific agricultural crops (Guillino and others 1995). Several additional strains of nonpathogenic *F. oxysporum* that exhibit potential as biocontrol agents include CS-1, Fo-B2, 70T01, MT 0062, and Fop2 for tomatoes (Yamaguchi and others 1992; Tamietti and others 1993; Larkin and Fravel 1999; Shishido and others 2005), Fo90105 for chickpeas (Hervas and others 1997), and Fo47b10 for carnations (Lemanceau and others 1993). These strains have shown biocontrol efficacy in specific experiments on particular crops, but are currently unavailable for commercial use.

Several different mechanisms have been identified by which nonpathogenic *F. oxysporum* strains can elicit biological control. Probably the most common is the induction of host plant resistance (Damicone and Manning 1982; Alabouvette and others 1993; Hervas and others 1995), primarily through two processes: induced systemic resistance (ISR) (Fuchs and others 1997; Duijff and others 1999; Larkin and Fravel 1999; Freeman and others 2002) and systemic acquired resistance (SAR) (Kubota and Abiko 2001; He and others 2002). Each of these processes result in induction of different chemicals within host plants to prohibit either infection or development of pathogenic fungi. For example, ISR induces production of pathogenesis-related proteins such as chitinases and ß-1-3 glucanases (Benhamou and Garand 2001), whereas SAR causes formation of other proteins such as peroxidase and phenylalanine ammonia-lyase (He and others 2002). These chemicals are distributed systemically within host plants, causing resistance to root, foliage, and stem pathogens (Benhamou and Garand 2001; He and others 2002). Induction of resistance usually requires plants to be initially exposed to the biocontrol agent before pathogens are present (Alabouvette and others 1993; Fuchs and others 1999) and often at much higher inoculum levels than pathogens (Bolwerk and others 2005). In some cases, resistance is induced by nonpathogenic endophytic organisms residing within host plants (Nejad and Johnson 2000). Systemic resistance occurs quickly, but may not remain for long time periods (Hervas and others 1998).

Another major mechanism of biological control by nonpathogenic *F. oxysporum* strains is competition with pathogens (Mandeel and Baker 1991). Competition usually involves nutrients required by both pathogens and nonpathogens and infection sites (niches) that may be common for both groups of organisms (Schneider 1984; Alabouvette and others 1993; Guillino and others 1995; Fuchs and others 1999; Cotxarrea and others 2002; Freeman and others 2002; Fravel and others 2003; Bolwerk and others 2005). If most available nutrients are initially utilized by nonpathogens, they become limiting to pathogens and subsequently reduce pathogen development (Steinberg and others 1999a; Fravel and others 2003). Likewise, if most infection sites are occupied by nonpathogens, pathogens cannot successfully infect hosts (Alabouvette and others 1993; Fravel and others 2003). This is especially true in plant roots (Fravel and others 2003; Bolwerk and others 2005).

Other related biocontrol mechanisms have also been identified. For example, nonpathogens can act as plant growth-promoting organisms, inducing plants to overcome effects of pathogens by rapid growth and development (Liu and others 1995; Koike and others 2001). Some nonpathogens can reduce pathogen metabolic activity (Duijff and others 1999) and inhibit pathogen chlamydospores germination (Fravel and others 2003) within soil.

Studies have thus far failed to identify antibiosis (production of antibiotic chemicals) (Fravel and others 2003; Bolwerk and others 2005) and mycoparasitism (one fungus parasitizing another fungus) (Bolwerk and others 2005) as mechanisms of biological control exhibited by nonpathogenic strains of *F. oxysporum*. Such mechanisms have been reported, however, for other biological control agents that may be effective against pathogenic strains of *Fusarium*, including several bacteria (Lemanceu and Alabouvette 1991; Hervas and others 1997; Hervas and others 1998; Larkin and Fravel 1998; Duijff and others 1999; Bapat and Shah 2000; Bora and others 2004) and fungi (Hock and Fuller 1977; Hervas and others 1998; Larkin and Fravel 1998; De Cal and others 2000; Cotxarrera and others 2002; Harveson and others 2002). Also, at least one strain of *F. oxysporum* has been identified as a mycoparasite against other fungi (Vajna 1985); another strain effectively infects and kills soilborne nematodes (Mennan and others 2005). In many cases, several different modes of action have been identified in plant disease biocontrol systems (James and others 1993).

Several organisms other than nonpathogenic *F. oxysporum* have been tested for biocontrol efficacy of *Fusarium* diseases in forest nurseries (Dumroese and others 1996, 1998; Mousseaux and others 1998; James 2000). Unfortunately, not all

of these agents have performed satisfactorily in controlled greenhouse tests. One reason may be that most commercial biocontrol agents have been developed for use on specific agricultural crops and may not be effective on forest nursery seedlings. Thus, efficacy on a wide range of very different plants may be limited because biocontrol strains are not readily adapted to particular plants or cropping systems (Fravel and others 2003). This same limitation may apply to some nonpathogenic strains of *F. oxysporum*, particularly those initially isolated from agricultural soil or the rhizosphere of annual crop plants.

Nonpathogenic *F. oxysporum* strains might exert more specific biocontrol against pathogenic *Fusarium* because they occupy the same niches, compete for the same nutrients, and use the same root infection sites. Therefore, if the plant growing environment is initially occupied by nonpathogenic strains, pathogenic strains will have difficulty becoming established or causing plant infection (Alabouvette and others 1993; Fuchs and others 1999). Unfortunately, some studies have indicated that much higher populations of nonpathogenic strains must be present to effectively restrict pathogens (Alabouvette and others 1993; Larkin and Fravel 1999; Fravel and others 2003; Bolwerk and others 2005). One way to enhance efficacy of nonpathogenic strains would be to combine them with specific fungicides which they can tolerate but pathogens cannot (Guillino and others 1995; Reid and others 2002; Fravel and others 2005). Another way is to mix nonpathogenic strains of *F. oxysporum* with bacteria to enhance biocontrol efficacy (Park and others 1988; Olivain and others 2004).

Testing Nonpathogenic *F. oxysporum* as Biological Control in Forest Nurseries

Based on the potential of nonpathogenic strains of *F. oxysporum* to control plant diseases and their relative safety in crop production (Guillino and others 1995), we have initiated a study to evaluate the potential of three selected strains to control root disease of container Douglas-fir seedlings. Our test is also designed to evaluate effects of another potential biological control agent (*Bacillus subtilis* GB03) on disease.

We selected three strains of *F. oxysporum* that were nonpathogenic in previous tests (James and others 2000). These isolates were also genetically differentiated from pathogenic isolates on the basis of AFLPs (Stewart and others 2004, 2005, 2006). One isolate (Q-12) was initially obtained from the roots of a diseased western white pine seedling, another (Q-76) from the roots of a healthy appearing western white pine seedling, and the last isolate (Q-103) from the roots of a diseased Douglas-fir seedling. These isolates will be incorporated singly or in combination into peat-based growing media. Douglas-fir seeds will be germinated on, and seedlings grown within, inoculated media. After 10 weeks, selected seedlings will be transplanted into media amended with an inoculum of a highly-virulent isolate from the *F. oxysporum* complex, which was previously identified in controlled pathogenicity tests (James and others 2000) and is recognized by some as *F. commune* (Stewart and others 2006). Efficacy of the three tested nonpathogenic *F. oxysporum* strains and *Bacillus subtilis* GB03 to control root disease caused by highly virulent isolates from the *F. oxysporum* complex will be evaluated. If one or more of the nonpathogenic *F. oxysporum* strains exhibit biocontrol potential, further tests will be conducted on other *Fusarium* pathogens, particularly *F. proliferatum*, a common aggressive pathogen of container seedlings (James and others 1997).

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