

**ENDOPHYTIC FUNGAL COMMUNITIES OF *BROMUS TECTORUM*:
MUTUALISMS, COMMUNITY ASSEMBLAGES
AND IMPLICATIONS FOR INVASION**

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

Exotic plant invasions are of serious economic, social and ecological concern worldwide. Although many promising hypotheses have been posited in attempt to explain the mechanism(s) by which plant invaders are successful, there is no single explanation for all invasions and often no single explanation for the success of an individual species. Cheatgrass (*Bromus tectorum*), an annual grass native to Eurasia, is an aggressive invader throughout the United States and Canada. Because it can alter fire regimes, cheatgrass is especially problematic in the sagebrush steppe of western North America. Its pre-adaptation to invaded climates, ability to alter community dynamics and ability to compete as a mycorrhizal or non-mycorrhizal plant may contribute to its success as an invader. However, its success is likely influenced by a variety of other mechanisms including symbiotic associations with endophytic fungi. Cheatgrass populations were sampled across North America and endophytes were isolated from collected plant tissue. Isolation efforts revealed that cheatgrass hosts a high diversity of endophytic fungi. Using one endophyte isolated, *Morchella elata*, we investigated whether the enhanced mutualism hypothesis (EMH) could, in part, explain the invasion success of cheatgrass. Specifically, we determined whether an association with this novel symbiont could enhance cheatgrass fitness. Results from greenhouse and laboratory experiments demonstrated that cheatgrass fecundity, biomass and thermotolerance significantly increased as a result of this symbiosis, providing support in favor of the EMH. From our sampling and isolation efforts, a strong association between a fungivorous nematode, *Paraphelenchus acontoides*, and an endophytic fungus, *Fusarium cf. torulosum* was

discovered. Although plant genotype, environmental conditions and biogeography are typically cited as factors that influence endophytic communities, our discovery prompted an investigation of the role this nematode could have in structuring endophyte communities in cheatgrass. In greenhouse and laboratory experiments, we determined that *P. acantioides* preferred *F. cf. torulosum* to other fungi and that it increased its relative abundance within the endophyte community. No direct effects on plant fitness were observed; however, interactions between these two symbionts did influence community assemblage and, as a result, could indirectly contribute to the success of cheatgrass as an invader.

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Chapter 1: Introduction

1.1. Invasive plants

1.1.1. *Economic and social impacts*

Invasive species are of serious economic and societal concern worldwide (Vitousek *et al.* 1997; Pimentel *et al.* 2005). Within the United States (U.S.), a cost of nearly \$120 billion /year can be attributed to losses, damages and attempts to control invasive plants, animals and microbes; the cost of invasive plants alone is approximately \$35 billion (Pimentel *et al.* 2005). Consequences to the U.S. agricultural industry are extensive. Annual losses are estimated at \$27 billion dollars/year and are attributed to herbicide costs and reduction in crop yields (USBC 2001; Pimentel *et al.* 2005). Exotic invasions in pastures and rangelands is likewise problematic, costing \$1 billion/year in forage losses and \$5 billion/year in control efforts (Babbitt 1998; Pimentel 2005). Similarly, control efforts within natural areas are costly. Exotic plants invade approximately 700,000 ha of wildlands annually within U.S. and the economic costs are immense (Babbitt 1998). For instance, annual losses due to just a single invasive species, European purple loosestrife (*Lythrum salicaria*), were estimated at \$45 million by 1997 (ATTRA 1997).

In addition to the economical impacts of invasives, the societal impacts are equally as immense. Invasive species can threaten human, pet and livestock health and safety. Although introduced pathogens, insects, invertebrates and mammals are often culprits for sickness, disease and death, many introduced plants can also have dire effects. Species such as the Brazilian pepper tree (*Schinus terebinthifolius*) and castor bean (*Ricinus*

communis) can cause anaphylaxis or other allergic reactions (Austin 1978; Challoner & McCarron 1990) whereas consumption castor bean and other invasives such as foxglove (*Digitalis purpurea*), poison hemlock (*Conium maculatum*) and leafy spurge (*Euphorbia esula*) can induce symptoms such as vomiting, convulsions, hallucinations and death (Robbins *et al.* 1941; Kingsbury 1964; Robbins & Johnson 1984; Whitson *et al.* 1991; Scott 1997; Drewitz 2000; Harris 2000). Yellow starthistle (*Centaurea solstitialis*), an invader western North America, produces a chemical toxin that can cause “chewing disease” in horses, which can result in dehydration or starvation (Kingsbury 1964; DiTomaso & Gerlach, Jr. 2000). Other invasives can alter habitat conditions through their growth habit to favor mosquitoes or other vectors for disease (Parsons 1992). Other invaders such as cheatgrass (*Bromus tectorum*) or pampas grass (*Cortaderia* sp.) increase the fire hazard in areas where they have established (Whisenant 1990; DiTomaso 2000a; DiTomaso 2000b). Consequently, areas affected by these infestations have become more increasingly more dangerous; homes and other structures become more at risk and the potential for fire-related injuries and deaths increase.

Aside from the health- and safety-related issues associated with invasives, exotic plant invasions are problematic for a number of other reasons. Because yellow starthistle is typically unpalatable to livestock, ranching has become increasingly difficult and costly in rangelands where it has invaded (Roché & Roché 1988; DiTomaso & Gerlach, Jr. 2000). Exotic plant invasions also can diminish the enjoyment of our surrounding environment in a number of ways. For example, many invasives such as cheatgrass,

yellow starthistle, artichoke thistle (*Cynara cardunculus*) and Canada thistle (*Cirsium arvense*) have sharp awns, thorns or leaves that make hiking and other recreational activities nearly impossible within infested areas. In aquatic systems, recreational activities also can be severely impacted by plant invasions. Plants such as water-hyacinth (*Eichhornia crassipes*) and Florida elodea (*Hydrilla verticillata*) can alter water flow by clogging streams, rivers and irrigation channels (Austin 1978) and boating and fishing can become problematic in lakes where invasives form dense mats (Godfrey 2000).

1.1.2. Ecological impacts

Aside from the extensive economical and societal implications of plant invasions, the ecological consequences are equally as detrimental. Invasive species threaten biodiversity and ecosystem integrity through displacement of natives and interference of ecosystem functions (Heywood 1989). Exotic plants can disrupt native communities via a number of mechanisms including competition for resources (Crooks 2002), alteration of soil environments (Vitousek 1986), hybridization and biotic homogenization (Carroll & Dingle 1996; Cox 1999) and disruption of ecosystem processes (Crooks 2002).

Although competition is a common and natural phenomenon, native plants are forced to further compete for limited resources such as space, water and nutrients with the establishment of exotics. Consequently, exotic plants can disrupt species composition, distribution and abundance within native communities (Schoener 1983). Not only can

density and biomass of native species decrease with the establishment of exotics (Brooks 2000) but also regeneration and community succession can be inhibited or altered (Meekins & McCarthy 1999).

Many exotics can alter the soil environment to a state more desirable for their own and other exotics' establishment and growth; native species are often maladapted to the new conditions (Busch *et al.* 1992; Pickart *et al.* 1998; Bais *et al.* 2003; Klironomos 2002). The ecological consequences can be detrimental; re-establishment of a diverse native assemblage and its associated processes are often challenging when exotics leave an unfavorable legacy within the soil environment (D'Antonio & Meyerson 2002). For many species, nitrogen is a key nutrient and nitrogen-fixing exotics can negatively affect native plant abundance and community dynamics (Pickart *et al.* 1998; Yelenik *et al.* 2004). Other invasives are able to obtain a competitive advantage by altering soil biochemistry and "contaminating" the soil environment, creating conditions unfavorable for natives. These conditions can be achieved through soil salinization (Decker 1961; Vivrette & Miller 1977; Glenn *et al.* 1988; Griffen *et al.* 1989) or through production of allelopathic root exudates (Bais *et al.* 2003; Callaway & Ridenour 2004; Prati & Bossdorf 2004). Some exotics produce allelochemicals that reduce spore germination of arbuscular mycorrhizal fungi (AMF), an important component of the soil microbial community (Roberts & Anderson 2001) whereas other exotics reduce AMF diversity, which is important for plant community biodiversity and productivity (van der Heijden *et al.* 1998; Mummey & Rillig 2006). Consequently, exotic species can disrupt the

association between native flora and their co-evolved microbial communities, negatively impacting growth, productivity and overall abundance of native plants (Callaway *et al.* 2004; Callaway & Ridenour 2004; Wardle *et al.* 2004; Callaway & Hierro 2005). A plant-soil feedback cycle can form, allowing for continued invasions and fostering further disruption (e.g., persistence of exotics, Klironomos 2002; Callaway *et al.* 2004).

Species' introductions to systems with taxonomically similar native species can result in hybridization, which may serve as a significant force toward invasive species' success (Ellstrand & Schierenbeck 2000). If hybridization favors the hybrid, not only is the native confronted with possible extinction but also other taxonomically unrelated species that co-evolved with the native may be at risk of displacement, local extirpation, or other adverse ecological impacts. Through hybridization with native congeners, exotics may be able to accelerate the invasion process through enhanced fitness (e.g., resistance to certain herbivores) that results in an increased ability of hybrids to spread, persist and dominate within their invaded ranges (Vilà & D'Antonio 1998; Weber & D'Antonio 1999; Vilà *et al.* 2000; Blair and Wolfe 2004).

Exotic plant invasions can disrupt natural ecosystem processes in a number of ways including alteration of disturbance regimes, food webs and the physical environment (Whisenant 1990; Crooks 2002; Pimentel 2005). Exotics like cheatgrass or blue gum (*Eucalyptus globulus*) can influence fire patterns, frequency and intensity within invaded areas (D'Antonio & Vitousek 1992; Simberloff & Rejmánek 2011). Natural food webs

can be altered; species like catclaw mimosa (*Mimosa pigra*) and the Brazilian peppertree can create dense monocultures within their invaded ranges, which have a negative impact native plant, avian, amphibian and mammalian abundance and diversity (Braithwaite, Lonsdale & Estbergs 1989; Curnutt 1989). Other invaders such as water hyacinth in Florida provide increased cover for invertebrates and fish and, as a result, reduce hunting success of raptors (O'Hara 1967; Sykes 1987). Other aquatic invasives such as hydrilla and Eurasian water milfoil (*Myriophyllum spicatum*) can alter water movement patterns and reduce light penetration within invaded waters (Schmitz *et al.* 1993). In invaded terrestrial systems, species like tamarisk (*Tamarix* spp.) can impact water table levels and water flows (Vitousek 1986) whereas other species such as Australian pines (*Casuarina equisetifolia*) can increase erosion rates (Schmitz *et al.* 1997).

1.1.3. Invasion hypotheses

To become invasive, a species must be transported to a novel environment (Sakai *et al.* 2001). While invasions are not always mediated by human activity, greater opportunities for introductions have arisen with the increase in worldwide trade and transport (di Castri 1989; Kolar & Lodge 2001; Reichard & White 2001; Sakai *et al.* 2001). Williamson (1996) posited that the potential success of an introduced species is based on a series of low probability introduction and establishment events ("tens rule") and, as a result, less than one percent of introduced species actually become invasive. Most exotics are not suited to novel environments and will not survive to colonize, reproduce and spread (Mack *et al.* 2000, Kolar & Lodge 2001).

However, for exotics that successfully establish, many hypotheses have been developed in attempt to explain their ability to out-compete locally adapted natives (Crawley 1986). Although the probability of a harmful invasion is generally low for any given species, the probability may increase with multiple introductions and their subsequent interactions with other exotics. With potential positive interactions (e.g., mutualisms or beneficial habitat modifications) between exotics in a novel environment, an “invasional meltdown” is possible (Simberloff & Von Holle 1999). Specifically, interactions between exotics may facilitate establishment and persistence of other exotics that, if introduced alone, may or may not have experienced a successful invasion. Because of the potential synergy between exotics, native communities may lose further resistance to future invasions. As a result, invaders often can influence the local environment to favor not only themselves but also a suite of other exotics (Kulmatiski 2006; Parker *et al.* 2006).

It has been posited that potential invaders would likely be related to or have similar traits to already successful invaders in a given region; however, Mack *et al.* (2002) suggest that relatedness is not a reliable indicator. Limited success has been achieved in defining invasive attributes of successful and potential invaders (Kolar & Lodge 2001; Richardson & Rejmánek 2004; McIntyre *et al.* 2005; Cadotte *et al.* 2006). Some focus has also been directed to the importance of “invasibility potential” of ecosystems (Crawley 1987; Sakai *et al.* 2001; Alpert *et al.* 2000; Richardson & Pyšek 2006), which is often associated with native species richness (Burke & Grime 1996; Tilman 1997; Levine 2000; Kennedy *et al.* 2002), latitude and landform (Lonsdale 1999).

The novel weapon hypothesis suggests that some exotic plants can facilitate their own invasion through the exudation of chemicals unfamiliar to naïve native plants (Callaway & Aschehoug 2000; Bais *et al.* 2003; Callaway & Ridenour 2004). Root exudates from some invaders can negatively impact natives that have no defense against the unfamiliar chemicals (Bais *et al.* 2003; Callaway & Ridenour 2004). Furthermore, allelopathic chemicals can reduce growth and competitiveness of natives by triggering changes within the microbial community. Specific associations exist between plants and soil biota (Bever 1994; Klironomos 2002) because the communities have coevolved (Thompson 1999), creating “species-specific rhizosphere biochemistry” (Callaway & Ridenour 2004). Thus, with the invasion of an exotic and its allelopathic chemicals, rhizosphere biochemistry can be altered from its original state, negatively affecting native species.

Another hypothesis, the enemy release hypothesis (ERH), suggests that exotics in novel environments “escape” their natural enemies that serve as biological constraints in their native regions (Mitchell & Power 2003; Torchin *et al.* 2003). Research demonstrates that enemy release can explain invaders’ success in some systems; plants in their native range often experience more damage and reduced growth as a result of herbivores and fungal pathogens relative to those in novel environments (Wolfe 2002; DeWalt *et al.* 2004). Expanding upon the ERH, the evolution of increased competitive ability (EICA) hypothesis, posits that invaders in a novel environment, freed from their natural enemies, can experience rapid evolution, allowing for allocation of valuable resources toward growth and competition rather than defense (Blossey & Nötzold 1995; Reznick 2001;

Müller-Schärer & Steinger 2004). Research indicates that a genetic shift in fitness traits (e.g., fecundity and biomass) does occur, providing evidence in favor of the EICA hypothesis in many systems (Daehler & Strong 1997; Willis & Blossey 1999; Siemann & Rogers 2003; Leger & Rice 2003).

Invasive plants also may benefit from associations formed with novel symbionts in their invaded ranges. This idea is coined as the enhanced mutualism hypothesis (EMH) (Hoffman & Mitchell 1986; Richardson *et al.* 2000) and may explain some successful invasions. Local soil microbes can promote exotic plant growth; both the invasive Chinese tallow tree (*Triadica sebifera*) in Texas and Canada goldenrod (*Solidago canadensis*) in Asia benefit from these novel associations (Nijjer *et al.* 2008; Sun & He 2010). Likely, other examples exist; however, research to-date is limited. Research presented in Chapter 2 addresses this hypothesis and its potential for explaining, in part, the success of one of the most aggressive invaders within western North America.

1.1.4. *Bromus tectorum*

Bromus tectorum (Poaceae, Bromeae), or cheatgrass, is an invasive winter annual grass native to Eurasia now widespread throughout the U.S. and Canada (U.S. Department of Agriculture 2011). Likely introduced in ship ballast, it was first documented in the mid-1800s in eastern North America (Warg 1938). It was accidentally introduced in the late 1800s to western North America as a grain contaminant (Mack 1981) and at least two purposeful introductions were made near the turn of the century. In 1897, seed was

planted in an experimental farm in Pullman, Washington (Mack 1981) and in 1915, cheatgrass was touted as a “100-day” grass and seed was sold throughout Wallowa County, Oregon (Hedrick 1965). By 1914, it was documented throughout the U.S. (U.S. Forest Service 1914) and it now occupies 40 million ha within the western U.S. (DiTomaso 2000c). Although cheatgrass doesn’t dominate communities in its native range (Hierro *et al* 2005), its invasion in the West threatens the integrity of native ecosystems through the displacement of native species and the interference of natural processes. Pre-adaptation to climate, habitat alteration (Mack 1981) and its ability to compete as a mycorrhizal or non-mycorrhizal plant (Richardson *et al.* 2000) have been suggested as contributors to its success. Because of its early season growth habit, it has become increasingly problematic within the sagebrush steppe because it dries at the onset of summer and produces highly flammable fine fuels. As a result, the natural fire interval of 60-110 years has been reduced to only 3-5 years (Whisenant 1990). The ecological consequences include increased fire size, distribution and frequency (D’Antonio & Vitousek 1992), which further promote its establishment (Mosely *et al.* 1999).

1.2. Fungal endophytes

1.2.1. *Definition and classification*

Fungi are among the most diverse organisms in the world, second only to the insects (Purvis & Hector 2000). The number of fungal species worldwide is estimated at 1.5 million (Hawksworth 2001) and, of that, 1.38×10^6 are posited to be unique endophytic species (Dreyfuss & Chapela 1994). Endophytic fungi are ubiquitous organisms that

invade and colonize living plant tissue for at least part of their life cycle. The term “endophyte” was first coined by DeBary (1866) to describe fungi ranging from virulent foliar pathogens to mycorrhizal root symbionts. More recently, despite their ability to penetrate living tissue, fungi that grow through the rhizosphere and into roots (i.e., mycorrhizal fungi, Rodriguez *et al.* 2009) or that induce observable disease symptoms (i.e., virulent pathogens, Carroll 1986; Wilson 1995) are not typically categorized as endophytes. Endophytes are symbiotic with their host (Petrini 1986; Clay 2004; Schulz & Boyle 2006; Sieber 2007) and although the association is often symptomless, the symbioses range from mutualistic to antagonistic (Wilson 1995; Clay 1996; Saikkonen *et al.* 1998; Clay & Schardl 2002; Schardl *et al.* 2004; Kuldau & Bacon 2008). Latent pathogens are often classified as endophytes (Wilson 1995; Petrini 1986).

Based on phylogeny and life history traits, endophytic fungi were originally organized into two broad groups – clavicipitaceous (C-endophytes) and non-clavicipitaceous (NC-endophytes). More recently, these groups have been divided into four distinct functional classes based on a number of different criteria related to life history, fungal-host interactions and effect on plant ecophysiology (Rodriguez *et al.* 2009). C-endophytes are classified as Class 1 endophytes whereas NC-endophytes have been further divided into three functional groups (Class 2, 3 and 4). Specifically, endophytes are categorized into one of the four classes based on: host range (single species to highly ubiquitous), tissue colonization (localized to general), extent of *in planta* colonization (limited to extensive) and biodiversity (low to high), mode of transmission (vertical to horizontal) and host

fitness benefits (non-habitat or habitat-adapted). (Rodriguez *et al.* 2009).

1.2.2. Community assemblage and diversity

The association between plants and endophytes has been long-established (> 400 million years); Krings *et al.* (2007) found evidence of colonization by three different endophytes in the ancient Rhynie chert plant (*Nothia aphylla*) in Scotland. Extensive research has demonstrated that fungal endophytes are universal throughout all ecosystems and plant species (Petrini 1986; Arnold 2007).

Within a plant host, endophyte diversity can range from limited (Wille *et al.* 1999; Ahlholm *et al.* 2002) to extensive (Carroll & Carroll 1978; Petrini 1986, Arnold 2007; Arnold & Lutzoni 2007; Shipunov *et al.* 2008). Ganley & Newcombe (2006) found a single *Lophodermium* haplotype to be dominant within western white pine (*Pinus monticola*) populations throughout the Rocky Mountain region. Although a specific endophyte may be dominant, overall diversity may still be high. In a survey of coastal redwood (*Sequoia sempervirens*), one endophyte, *Pleuroplacoema* sp., was dominant throughout all populations although 16 different endophytes were documented (Rollinger & Langenheim 1993). Lodge *et al.* (1996) found endophytic diversity to be relatively high in *Manilkara bidentata*; 17 different species were documented within a single leaf and 22 total species were observed on all leaf blades.

Fungal endophytes have been studied extensively, but the mechanisms that guide

community diversity are often unknown. Factors such as biogeography (Arnold & Lutzoni 2007), environmental conditions (Lacey & Magan 1991; Marin *et al.* 1998; Seghers *et al.* 2004; Saunders & Kohn 2009), co-evolution (Carroll 1988; Cheplick & Clay 1988; Clay 1988; Petrini *et al.* 1992; Schulthess & Faeth 1997; Saikkonen *et al.* 1998) and host genotype (Todd 1988; Bailey *et al.* 2005; Pan *et al.* 2008; Shipunov *et al.* 2008) are often important in determining community assemblage. On a large scale, endophytic diversity and occurrence are dependent upon biogeography; Arnold & Lutzoni (2007) not only found diversity to be greater at the equator than at the poles, but also observed occurrence to be distinct among biogeographic regions (i.e., arctic, temperate or tropical). At a more local scale, environmental conditions such as water availability, temperature, agro- and plant chemicals can influence diversity (Lacey & Magan 1991; Marin *et al.* 1998; Seghers *et al.* 2004; Saunders & Kohn 2009).

Co-evolution with a host also may affect endophytic presence and diversity. Over time, some pathogenic fungi evolve to form benign or mutualistic associations with their host (Carroll 1988; Petrini *et al.* 1992; Cheplick & Clay 1988; Clay 1988; Bacon 1993; Elmi & West 1995). Benefits such as herbivory defense (Cheplick & Clay 1988; Clay 1988) or enhanced competitive abilities (Bacon 1993; Elmi & West 1995) afforded to a host may result in increased dominance of a specific endophyte conferring those benefits. Furthermore, some endophytic species (e.g., *Neotyphodium*) that confer benefits to a host can also limit colonization of other endophytes via mechanisms such as chemical production (Schulthess & Faeth 1997; Saikkonen *et al.* 1998).

Host genotype can affect endophyte richness, diversity and composition (Todd 1988; Bailey *et al.* 2005; Pan *et al.* 2008). Shipunov *et al.* (2008) demonstrated that host genotype can influence the endophytic assemblage in spotted knapweed. Endophyte communities within spotted knapweed from its native and introduced ranges differed significantly; a specific haplotype of *Alternaria alternata* was dominant in plants from the native range while *Alternaria tenuissima*, *Cladosporium herbarum* and an *Epicoccum* sp. were the most relatively abundant endophytes.

1.3. Plant-fungal symbioses

1.3.1. *Mutualistic associations*

Endophytic symbionts can be of significant ecological importance. Plant hosts provide endophytes with nutrition, shelter and, for vertically transmitted endophytes, increased dissemination potential (via seed) (Siegel *et al.* 1987; Latch 1993; Saikkonen *et al.* 1998; Schardl *et al.* 2004; Kulda & Bacon 2008). In return, endophytes may confer increased fitness and growth, protection against biotic and abiotic stresses and other advantages to their hosts (Latch *et al.* 1985; Siegel *et al.* 1987; West *et al.* 1988; West *et al.* 1993; West 1994; Clay & Shardl 2002; Lucero *et al.* 2006; Kulda & Bacon 2008; Rodriguez *et al.* 2008; Newcombe *et al.* 2009). Research has demonstrated that endophytes commonly associated with members of the Poaceae family can influence phenotypic traits such as increased vegetative reproduction (Clay & Schardl 2002; Ernst *et al.* 2003), stress tolerance (West *et al.* 1988; West *et al.* 1993; Elmi & West 1995; Clay & Schardl 2002; Redman *et al.* 2002) and improved nutrient uptake (Malinowski *et al.* 2000). Other

endophytes can provide their host protection against pathogenic fungi (Arnold & Herre 2003) and herbivory from insects, nematodes and vertebrates (Prestidge & Gallagher 1988; Clay 1996; Clay & Schardl 2002; Rudgers *et al.* 2007; Kuldau & Bacon 2008).

1.3.2. *Antagonistic associations*

Plant-fungal interactions can negatively affect a host when an endophyte acts pathogenic (Cheplick *et al.* 1989; Paszkowski 2006; Wäli *et al.* 2006; Newcombe *et al.* 2009). As a result, endophytes may adversely influence phenotypic plant traits. Under low nutrient conditions when plants must compete for nutrients, Cheplick *et al.* (1989) demonstrated that biomass of perennial ryegrass (*Lolium perenne*) and tall fescue was negatively affected when plants were infected with *A. coenophialum*. Arnold (2002) and Arnold & Engelbrecht (2007) showed that endophytic infection can reduce drought tolerance within a host. Likewise, photosynthesis can be affected by asymptomatic endophyte infection; Pinto *et al.* (2000) demonstrated that endophytes *Colletotrichum musae* and *Fusarium moniliforme* reduced photosynthetic efficiency in banana (*Musa*) and maize.

1.3.3. *Ecological implications*

Fungal symbionts can enhance native community functionality. van der Heijden *et al.* (1998) demonstrated that increased species richness within the mycorrhizal community results in an increase in biodiversity and ecosystem functioning within its associated plant community. Within a host, the endophyte community can be quite diverse (Carroll & Carroll 1978; Petrini 1986; Sieber-Canavesi & Sieber 1993; Vandenkoornhuyse *et al.*

2002; Arnold 2007; Arnold & Lutzoni 2007; Shipunov *et al.* 2008) and this diversity (or lack thereof) can also contribute to ecological community processes (Leuchtman & Clay 1997; Saikkonen *et al.* 1998; Rudgers & Clay 2007). Specifically, fungal diversity can be an influencing factor on natural communities' composition and stability as well as their resistance to invasion (Clay & Holah 1999; Levine & D'Antonio 1999).

Conversely, plant-fungal interactions can have detrimental consequences for a native community. Fungal symbionts may enhance an invader's competitive abilities and novel associations may facilitate invasions by alien plant species (Hoffman & Mitchell 1986; Richardson *et al.* 2000). Schmidt & Scow (1986) contend that the invasion success of guava (*Psidium guajava*) and quinine (*Cinchona pubescens*) on the Galapagos Islands may be facilitated by native mycorrhizas. Likewise, spotted knapweed in its invaded range has been shown to benefit from the mycorrhizal network of adjacent native plants (Marler *et al.* 1999). Fungal endophytes may also promote knapweed invasion through allelopathic effects on competing native species (Newcombe *et al.* 2009). Some research indicates that specific endophyte infections within a host can facilitate its invasion into habitats where another species is dominant (Lucero *et al.* 2006). In tall fescue, Rudgers *et al.* (2004) demonstrated that a stronger negative correlation between the success of the fescue and plant community diversity existed when fescue plants were infected with vertically transmitted fungal symbionts than when they were not. *Neotyphodium coenophialum* increases the competitive dominance of tall fescue and facilitates its invasion into diverse plant communities (Clay & Holah 1999; Rudgers *et al.* 2005) and

consequently, reduces species richness and diversity within that community (Clay & Holah 1999). Furthermore, tall fescue is afforded increased population stability under high stress conditions when infected with *A. coenophialum* (West *et al.* 1993).

1.4. Research objectives

In recent years, much focus has been directed toward unraveling the mysteries and understanding the mechanisms that drive successful plant invasions. Although numerous hypotheses have been posited, many are insufficient in elucidating the success of certain invaders, such as cheatgrass. This research investigates the association between cheatgrass and its endophytic fungal communities. The objectives of these studies were to determine 1) the role fungal endophytes have in cheatgrass fitness, 2) what influences endophytic community assemblage and 3) whether endophytes contribute to the invasion success of cheatgrass. Chapter 2 presents research focused on a fire-adapted endophyte of cheatgrass. Specifically, we investigated a novel symbiotic association between cheatgrass and the endophytic morel (*Morchella elata*) and addressed whether the EMH could, at least in part, explain the success of cheatgrass as an invader in North America. In Chapter 3, we present our research related to endophyte community assemblage within cheatgrass. The goal was to determine whether a fungivorous nematode, *Paraphelenchus acontoides*, could actively “cultivate” a preferred food source, *Fusarium cf. torulosum*, and influence fungal assemblage *in planta*. Secondly, we investigated whether a shift in the fungal community as a result of the nematode and its cultivar would affect plant fitness and the potential implications for invasion success of cheatgrass.

1.5. Literature cited

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Chapter 2: A novel plant-fungal mutualism associated with fire

2.1. Abstract

Bromus tectorum, or cheatgrass, is native to Eurasia and widely invasive in western North America. By late spring, this annual plant has dispersed its seed and died; its aboveground biomass then becomes fine fuel that burns as frequently as once every three to five years in its invaded range. Cheatgrass has proven to be better adapted to fire there than many competing plants, but the contribution of its fungal symbionts to this adaptation had not previously been studied. In sampling cheatgrass endophytes, many fire-associated fungi were found, including *Morchella* in three western states (New Mexico, Idaho and Washington). In greenhouse experiments, a New Mexico isolate of *Morchella* increased both the biomass and fecundity of its local cheatgrass population, thus simultaneously increasing both the probability of fire and survival of that event, via more fuel and a greater, belowground seed bank, respectively. Reisolation efforts proved that *Morchella* could infect cheatgrass roots in a non-mycorrhizal manner and then grow up into aboveground tissues. The same *Morchella* isolate also increased survival of seed exposed to the heat that develops in the seed bank during a cheatgrass fire. Phylogenetic analysis of Eurasian and North American *Morchella* revealed that this fire-associated mutualism was evolutionarily novel, in that cheatgrass isolates belonged to a clade of North American origin. Mutualisms with fire-associated fungi may be contributing to the cheatgrass invasion of western North America.

2.2. Introduction

Relatively few plants introduced to new regions become invaders. Attempting to predict the invaders, invasion biologists have considered many hypotheses involving biotic interaction (Mitchell *et al.* 2006). Novel mutualisms involving seed dispersal and mycorrhization appear to underlie the success of a few invaders (Richardson *et al.* 2000). However, a general mechanism has proven to be elusive, and even some of the world's most dramatic invasions remain puzzling in key respects.

Introduced late in the nineteenth century from Eurasia, *Bromus tectorum*, or cheatgrass, rapidly became the dominant plant of the treeless steppes of western North America (Mack 1981); in its native range cheatgrass does not dominate plant communities (Hierro *et al.* 2005). Factors contributing to its domination in its invaded range have been identified: climatic pre-adaptation and habitat alteration prominent among them (Mack 1981). Also noted is the ability of cheatgrass to successfully compete in differing ecological scenarios as either a mycorrhizal plant or as a non-mycorrhizal plant (Richardson *et al.* 2000).

However, the ecosystem effects of cheatgrass are tied to its promotion of both the frequency and size of fires (D'Antonio & Vitousek 1992). Cheatgrass-fire feedback is positive in that once cheatgrass density passes a certain threshold, cheatgrass promotes fire, which in turn promotes cheatgrass (Mosley *et al.* 1999). In sagebrush steppe before the cheatgrass invasion, the fire return interval was from 60 to 110 years (Whisenant 1990), but it has now been reduced to three to five years in cheatgrass-dominated

communities (D'Antonio & Vitousek 1992). Domination by cheatgrass makes fire 500 times more likely than it would have been if the community had not been invaded (D'Antonio & Vitousek 1992). As cheatgrass has spread to the edge of forested, higher elevations, its fires have become larger and more damaging (D'Antonio & Vitousek 1992).

By contributing essential thermotolerance to a symbiosis, endophytic fungi can be obligate mutualists of plants (Redman *et al.* 2002). One mechanism of endophyte-mediated thermotolerance involves interaction with the heat shock proteins of the host (McLellan *et al.* 2007). Fungi can also be fire-adapted in their own right, as their fruiting can be more prolific after a fire (Pilz *et al.* 2004), or they can be favoured relative to less tolerant fungi by the soil heating associated with fire (Peay *et al.* 2009). Spores of fire-adapted *Neurospora* may not germinate without heat or chemical conditioning associated with fire (Jacobson *et al.* 2004)

Our objective here was first to determine whether fungi known to be favoured by fire are common in a dominant, fire-adapted invader. Once *Morchella* had been isolated as an endophyte, our corollary objective became the determination of the role of this well-known, fire-adapted fungus in promoting its fire-adapted host.

2.3. Materials and methods

2.3.1. Endophyte sampling and sequencing

Cheatgrass was sampled during 2009 and 2010 in 63 sites in British Columbia, Colorado, Idaho, Illinois, Iowa, Nevada, New Mexico, and Washington. Cheatgrass plants collected in 2008 from two additional sites in Idaho and New Mexico were also included in the sequencing effort. Habitats included coniferous forests, sagebrush-grasslands, desert scrub, agricultural fields and disturbed roadsides. Twenty plants at each site were sampled by removing a single culm (the jointed stem of grasses) from each.

A two-centimetre segment centered on the lowest node of each culm was excised, surface-sterilised in 50% ethanol for five minutes and rinsed in sterile, distilled water for one minute (Luginbuhl & Muller 1980; Schulz *et al.* 1993). Culm segments were plated on potato dextrose agar (PDA) and incubated at ambient room temperature (~20°C).

From the 1,260 culm sections sampled, 1,064 endophytes were isolated. Some endophytes were identified morphologically to genus; the rest were placed into morphotype groups based on all visible macroscopic and microscopic characteristics. The ITS region of representative isolates of 221 morphotype groups was sequenced at the USDA-ARS Systematic Mycology and Microbiology Laboratory in Beltsville, MD, following protocols described below for *Morchella* DNA isolation, amplification and sequencing; the ITS of zygomycetous endophytes was sequenced at the Friedrich Schiller University in Jena, Germany.

2.3.2. Endophyte identifications and associations with thermotolerance

Endophytes were considered matches with recognised species only if the ITS sequences were identical. Endophytes were identified to genus only if their ITS sequence identity was more than 97% but less than 100%. Five of the endophytes were identified as one of two phylotypes of the *Morchella elata* clade (*Mel-6* and *Mel-12*), as described below. These five isolates were characterised further using multilocus DNA sequence data. Associations with thermotolerance were assessed according to the literature associated with those taxa.

2.3.3. *Effects of Morchella on cheatgrass growth and fecundity*

The experiment was conducted as a randomised complete block design with two treatments and 20 replicates of each. Treatments included the control (M-) and inoculation with the NM isolate of the *Mel-6* phylotype (M+). Over the course of the study, pots were randomised bimonthly within each block. The initial experiment was conducted in 2009 and repeated in 2010.

Seeds were collected from northeast of Albuquerque, New Mexico (Zia Indian Reservation) where the NM isolate of the *Mel-6* phylotype had been obtained. Cleaned seeds were surface-sterilised in 50% ethanol for five minutes, and then rinsed for one minute in sterile distilled water (Luginbuhl & Muller 1980; Schulz *et al.* 1993). They were then transferred onto sterile blotter paper within Petri dishes and vernalised in the dark at 2°C for eight weeks. Following vernalization, the Petri dishes were transferred to the greenhouse for germination. Environmental conditions within the greenhouse

included an 18:6 hour photoperiod (day:night) with average temperatures of 25°C (day) and 20°C (night).

M+ seedlings were inoculated through direct contact for 24 hours between seedling roots and mycelium of a PDA culture of the NM isolate of *Mel-6*. M- seedlings were placed into Petri dishes of sterile PDA. Seedlings were planted into a 10 cm² pot filled with a potting mixture (Sunshine Mix #1), which prior to planting, was autoclaved for two hours (121° C and 15 lb/in²) to ensure sterility. To reduce potential phytotoxic effects from the sterilization process, soil was allowed to sit for two weeks before planting (Rovira & Bowen 1966; Koide & Li 1989).

Plants were grown in the greenhouse for four months during which observations were recorded daily with respect to plant health, appearance, flowering and seeding. Seeds were allowed to mature on the plants; once ripened, inflorescences were clipped just below the lowest panicle branch on each culm and were dried in paper bags for 72 hours at 60°C. Once seeds were cast and the plants dried, biomass was harvested. The above-ground biomass was clipped to the soil surface, placed into paper bags and dried for 72 hours at 60°C. Following drying, the above-ground biomass for each individual plant was recorded. Soil was rinsed from the belowground biomass and roots were placed in bags for analysis. In general, Pearson correlations among uncleaned seed weight, cleaned seed weight and seed number were highly positive, ranging from 0.957 to 0.993. Thus, fecundity was determined using the easily determined dry weight of uncleaned seed.

2.3.4. Root colonization

Since some species of *Morchella* have been reported to form mycorrhizal-like associations with plants (Dahlstrom *et al.* 2000, Pilz *et al.* 2004) and mycorrhization could explain effects on growth and fecundity, roots from five M⁺ and five M⁻ plants were cleared, stained, and examined microscopically for structural evidence of mycorrhization. Both M⁺ and M⁻ roots were cut into 2cm segments from three sections of root (near the crown, center and at the tips). Following procedures developed by Brundrett *et al.* (1996), roots were cleared in test tubes in a 10% KOH solution at 85°C for two hours. Once cleared, roots were rinsed with sterile distilled water, stained with 0.03% w/v chlorazol black E in lactoglycerol at 85°C for two hours, and placed in 50% glycerol for two days to remove excess stain. Slide mounts were observed with a Zeiss Axioskop.

2.3.5. Re-isolation of *Morchella* from culms after inoculation of roots

Since *Morchella* has never been reported to grow endophytically in aboveground plant tissues (Taskin *et al.* 2010), colonization of culms from roots by the NM isolate of *Mel-6* was investigated. Segments for re-isolation were centred on surface-sterilised, basal nodes of culms of both inoculated M⁺ and M⁻ cheatgrass plants. Of the 40 plants, five from each treatment type (M⁺ and M⁻) were randomly selected and a single stem from each was selected and clipped at the base. To adjust for the loss in biomass from the clipped stem, fresh weight was recorded for each, and its dry weight estimated on the basis of the ratio of fresh:dry weight.

2.3.6. *Thermotolerance*

During a typical cheatgrass fire, temperatures reach approximately 63°C at depths of 1 to 2 cm below the soil surface (Beckstead *et al. in press*). Buried seeds that survive these temperatures are likely not current-season but seeds from the year before. To determine whether *Morchella* conferred thermotolerance to cheatgrass, 800 seeds were placed in Petri dishes for three days, half on sterile PDA (M-) and half in contact with a culture of the NM isolate of *Mel-6* on PDA (M+). Petri dishes were sealed with parafilm. Seeds were then placed onto sterile filter paper in new Petri dishes and subjected to one of four heat treatments: 20°C (positive control), 55°C, 60°C, or 65°C for one hour. One hundred seeds were in each *Morchella*/temperature combination. Seeds were then imbibed in sterile distilled water for 24 hours and placed in 1% tetrazolium blue for another 24 hours. Seeds with stained and unstained embryos were recorded as viable and non-viable, respectively (Patil & Dadlani 2009).

2.3.7. *Statistical analyses*

Biomass, fecundity and thermotolerance data were analysed with SysStat 12.02.00. In the first two cases, Student's two-sample t-test with separate variances was employed. Thermotolerance data were analysed with a GLM model, nesting temperature within treatment (i.e., M+ vs. M-).

2.3.8. *Morchella* DNA isolation, amplification and sequencing

Total genomic DNA was extracted with the UltraClean Plant DNA Isolation Kit (MoBioLaboratories, Solana Beach, CA, USA). The entire nuclear ribosomal ITS region

and domains D1 and D2 at the 5' end of the nuclear large subunit (LSU) ribosomal RNA, and portions of the *tef1- α* and *rpb2*, genes were amplified (Taskin *et al.* 2010; O'Donnell *et al. in press*). PCR products were cleaned and sequenced using PCR primers.

Sequences were assembled and edited in Sequencher v.4.8 (Gene Codes, Ann Arbor, MI, USA) and deposited in GenBank (accession numbers *tef1- α* : HM756733-HM756737; *rpb2*: HM756738-HM756742; and 28S: HM756728-HM756732).

2.3.9. *Morchella* phylogenetic analysis

Edited sequences from each locus were aligned individually with *Morchella* sequences retrieved from GenBank using ClustalX 2.0.11 and the alignments were adjusted manually in MacClade 4.08 (Maddison & Maddison 2001). The final combined data set used for analysis contained 2622 characters (*tef1- α* :1180 bp, *rpb2*:873 bp, and LSU rDNA:569 bp). Sequences from the ITS rDNA region were not included in this analysis because they cannot be aligned across the breadth of the *M. elata* clade. A partition-homogeneity test (incongruence length-difference test) was implemented to evaluate the homogeneity of different data partition subsets using PAUP* v.4.0d106 (Swofford 2002). The test implemented 1000 replicates (heuristic search; random simple sequence additions; tree-bisection reconnection (TBR); max-trees = 1000). Comparisons were evaluated using a threshold of $P < 0.001$ and were made between all data partitions.

Maximum likelihood (ML) analysis was implemented in PAUP* using the best fitting model for each data partition, as determined using AIC, as implemented by Modeltest 3.7

(Posada & Crandall 1998). The general time reversible model incorporating a proportion of invariable sites and a distribution of rates at variable sites modelled on a discrete gamma distribution was selected by Modeltest for each data partition. Heuristic searches were performed with 1 000 random addition sequence replicates and tree-bisection-reconnection branch swapping, collapse and MulTrees (saving all optimal trees) options in effect. Characters were equally weighted and unordered with gaps treated as missing data. Outgroups were defined as *Disciotis venosa* M504, *Morchella* cf. *esculenta* Mes-17 (M98) and Mes-8 (M218), and *Verpa bohemica* M197 (Taskin *et al.* 2010; O'Donnell *et al. in press*). Maximum parsimony bootstrap support was calculated using the same settings as above with 1 000 replicates, each with 100 random taxon addition replicates (Felsenstein 1985). Bayesian (BI) analyses were conducted with MrBayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using the appropriate model with four incrementally heated Markov chains and two concurrent runs of 10 000 000 generations sampled every 10 000 generations for a total of 1 000 trees saved. The outgroup taxon was defined as *Morchella* cf. *esculenta* Mes-17 (M98). The initial 25% of trees sampled was discarded as burn-in. A majority rule consensus tree was calculated from the remaining pool of trees.

2.4. Results

2.4.1. *Endophyte identifications and associations with thermotolerance*

Many fire-adapted or thermotolerant fungi (39% of 1,064 isolates - Table 1) were isolated as endophytes from cheatgrass. These 419 isolates belonged to 25 taxa that are

associated with fire or heat tolerance (Table 1). Prominent among these was *Morchella*; five endophytic isolates from three states (New Mexico, Washington, and Idaho) were obtained from culm tissue. The remaining 645 isolates belonged to 46 taxa that were not associated with fire or heat tolerance according to the literature: *Allantophomopsis* sp., *Alternaria alternata*, *Alternaria* sp., Apiosporaceae, *Ascochyta* sp., *Aspergillus vadensis*, *Bipolaris spicifera*, Ceratobasidiaceae, *Ceratobasidium* sp., *Chalastospora* sp., *Cochliobolis* sp., *Cytospora* sp., *Didymella fabae*, *Drechslera poae*, *Drechslera* sp., *Epicoccum nigrum*, *Epicoccum* sp., *Fusarium acuminatum*, *F. equiseti*, *F. torulosum*, *F. proliferatum*, *Fusarium* sp., Hypocreales, Leotiomyces, *Leptodontidium* sp., *Lewia infectoria*, *Magnaporthe* sp., *Microdochium nivale*, *Microdochium* sp., *Monographella* sp., *Myrothecium roridum*, *Myrothecium* sp., *Nectria* sp., *Penicillium canescens*, *P. expansum*, *P. olsonii*, *P. raistrickii*, *Pestalotiopsis* sp., Pezizales, Pezizomycete, Pezizomycotina, *Phaeosphaeria nodorum*, *Phaeosphaeria* sp., *Preussia intermedia*, *Preussia* sp., *Pyrenophora lolii*, Pyronemataceae, *Sigmoidea* sp., *Thelavia* sp., *Trichothecium roseum*, *Truncatella* sp., unknown 1 (fungal sp.), unknown 2 (basidiomycete), unknown 3 (basidiomycete), and unknown 4 (ascomycete).

2.4.2. Effects of *Morchella* on cheatgrass growth and fecundity

The effects of the NM isolate of *Mel-6* on fecundity and biomass were measured as dry biomass after the plants had completed their life cycle in each of two, four-month greenhouse studies. Increases in fecundity due to this *Morchella* isolate were 35% ($t=6.80$, $P<0.001$) and 18%, ($t=2.07$, $P=0.05$) in the two experiments (Figure 1). In the

same two experiments, increases in growth due to *Morchella* inoculation were 15% ($t=2.39$, $P=0.03$) and 24% ($t=2.16$, $P=0.04$), respectively (Figure 2).

2.4.3. Root colonization

Mycorrhization with *Morchella* can result in a mantle, and a Hartig net (Dahlstrom *et al.* 2000) but these structures were not observed in cleared and stained roots of M+ plants. However, extensive fungal colonization of M+ roots was evident (Figure 3). Fungal colonization was absent in the M- roots.

2.4.4. Re-isolation of *Morchella* from culms after inoculation of roots

Via re-isolation from five stem nodes, we confirmed that the NM isolate was capable of growing from inoculated roots into aboveground tissue. In contrast, *Morchella* was not re-isolated from five sampled M- stem nodes.

2.4.5. Thermotolerance

At 20°C and 55°C, M+ and M- seed viability did not differ (Figure 4). However, at both 60°C and 65°C, the NM isolate increased seed viability in both the first experiment ($F=5.49$, $P<0.001$) and its repeat ($F=5.74$, $P<0.001$).

2.4.6. *Morchella* phylogenetic analysis

Based on phylogenetic analyses of three nuclear genes (translation elongation factor-1 alpha, DNA-dependent RNA polymerase II, and 28S ribosomal RNA), all five,

endophytic isolates of *Morchella* belonged to one of two *M. elata* phlotypes, *Mel-6* and *Mel-12*; these phlotypes belonged to clades of exclusively North American origin (Figure 5).

2.5. Discussion

Discoveries of symbiont-based thermotolerance in plants (Redman *et al.* 2002; McLellan *et al.* 2007) have potentially far-reaching implications. In this study, we found a diverse guild of fungi associated with fire and heat tolerance in a fire-adapted plant invader that is transforming western North America. To begin the study of the nature of cheatgrass symbioses, *Morchella* was chosen as an initial focus. We determined the effects of *Morchella* on traits that must be central to cheatgrass fitness: fecundity, biomass and seed thermotolerance. Via these traits, we found that *Morchella* can contribute to cheatgrass fitness that in turn should benefit both symbionts due to their adaptations to fire.

However, we have not yet determined whether the other 24 taxa of fire-adapted or thermotolerant fungi function in a similar way. Thus, the other taxa may or may not represent mutualisms that, in turn, may or may not be evolutionarily novel (i.e., native fungi symbiotic with an alien plant) as the *Morchella* isolates proved to be (Figure 5).

Morchella, the genus of true morels, produces highly prized edible fruiting bodies in temperate and boreal forests following fire, and other disturbances (Pilz *et al.* 2007).

Although some species of *Morchella* can form mycorrhizal-like associations (Dahlstrom *et al.* 2000, Pilz *et al.* 2007), we recovered *Morchella* as an endophyte in the

aboveground stem tissue of cheatgrass. Morels are not known to fruit in regions too dry to support forest. This restriction to forest is also thought to be linked to the facultatively mycorrhizal-like nature of tree-morel associations (Dahlstrom *et al.* 2000). Morels are thus never observed fruiting in the vast, treeless parts of intermountain, western North America (Pilz *et al.* 2007), that are now dominated by cheatgrass (Mack 1981). Morels and cheatgrass are among the leading examples of fire-adapted fungi (Pilz *et al.* 2007) and plants (D'Antonio & Vitousek 1992), respectively, yet they have never been ecologically linked due to their presumed lack of distributional overlap.

During the course of this study we did observe that the margins of burned forest are frequently invaded by cheatgrass (Figure 6). Given that *Morchella* commonly fruits in such habitat, it is possible that the novel mutualism developed initially in such an area. Two of our endophytic isolates were obtained from coniferous forest (i.e., Weiser, ID and H99, ID), whereas the other three were from desert scrub (North Zia, NM), and bunchgrass scrub (Hagenah, WA and White Bird, ID). As *Morchella* is not known to sexually reproduce in non-forest communities at lower elevation, it is likely that the North Zia, Hagenah, and White Bird isolates represent asexual populations.

The mechanism by which *Morchella* is dispersed from forest edge to non-forested habitat is unknown, but wind, water and animals are common vectors for fungal spore dispersal (Ingold 1971). Wind dispersal is of particular importance for epigeous fungi like *Morchella* and it can contribute to the distribution of spores over great distances (Allen *et*

al. 1992). For instance, spores of *Thelephora* were observed in unvegetated areas soon after the Mt. St. Helen's eruption (Allen 1987). Conversely, however, researchers in Oregon observed a very low frequency of wind-dispersed basidiospores in sites at forest edges (Ashkannejhad & Horton 2006).

Dispersal of endophytic *Morchella* could also be mediated by its host. In plants producing tillers, rhizomes, tubers and plantlets, endophytes can be dispersed in these host organs (Clay 1986a, Clay 1986b, Rodriguez *et al.* 2009); *Neotyphodium* endophytes are vertically transmitted in seeds of their hosts (Clay & Jones 1984, Clay 1990).

Dispersal of cheatgrass itself is entirely via seed that can be transported long distances in or on fur, hooves, feces, clothing and vehicles (Hulbert 1955, Young *et al.* 1987, Mosely *et al.* 1999, Young 2000). But the ability of *Morchella* to infect cheatgrass seed has not been determined. Nor is it yet known whether herbivores could disperse *Morchella* in feces after consuming infected host tissue, or whether *Morchella* could remain viable in dead, vegetative tissues of cheatgrass that could be then be dispersed by the wind.

According to the enhanced mutualism hypothesis, plant invasions may benefit from novel symbionts (Hoffman & Mitchell 1986, Richardson *et al.* 2000). Nijjer *et al.* (2008) found that the Chinese tallow tree (*Triadica sebifera*) benefited to a greater degree from local soil biota in Texas than did co-occurring native trees. Effects were attributed to arbuscular, mycorrhizal fungi that might have been native to the site. Likewise, a mutually beneficial symbiosis was demonstrated between Canada goldenrod (*Solidago*

canadensis), an invasive forb in Asia, and microbes from soil collected in China that, again, may or may not have been native to the site. Relative to a native Chinese grass (*Stipa bungeana*) grown in the same soil, seedling emergence, growth and competitive ability of the invasive goldenrod were microbially enhanced (Sun & He 2010). Our findings are similar except that our demonstrated mutualism is shown here to be evolutionarily novel.

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Table 2.1. Thermotolerant fungi isolated as endophytes from cheatgrass.

Endophyte ^a	Accession No.	No. ^b	Sites ^c	Association with fire or heat ^d	Reference
<i>Absidia</i> sp. (CID 296, 374)	28S HQ829144, HQ829173, respectively	2 (2)	CO	Identified in the Mt. St. Helen's devasation zone Survival following 2 minutes at 100°C	Carpenter <i>et al.</i> 1982 Warcup 1951
<i>Acremonium</i> sp. (CID 35, 55, 60, 83, 87, 89, 93, 99, 100, 105, 110, 138, 139, 147, 156, 165, 166, 167, 198, 200, 201, 216, 217, 218, 237, 238, 254, 264, 268, 272, 283, 285, 295, 298)	28S HQ829069, HQ829074, HQ829075, HQ829076, HQ829078, HQ829079, HQ829080, HQ829081, HQ829082, HQ829085, HQ829086, HQ829096, HQ829097, HQ829099, HQ829100, HQ829101, HQ829102, HQ829103, HQ829107, HQ829109, HQ829110, HQ829114, HQ829115, HQ829116, HQ829123, HQ829124, HQ829128, HQ829132, HQ829134, HQ829136, HQ829140, HQ829141, HQ829143, HQ829145, respectively	34 (41)	BC, CO, ID, NM, WA	Identified in the Mt. St. Helen's devasation zone Found in recently burned prairie soils	Carpenter <i>et al.</i> 1982 Wicklow 1973
<i>Aspergillus</i> sp. (CID 122, 125, 213, 310, 372, 377)	28S HQ829092, HQ829094, HQ829113, HQ829151, HQ829172, HQ829175, respectively	6 (82)	BC, CO, IA, ID, IL, NM, NV, WA	Identified in the Mt. St. Helen's devasation zone Growth more abundant on heated- than unheated-soil Survival following 6 minutes at 100°C Recolonization successful following steam-treatment of soil Isolated from compost; spores germinated from 25°-50°C Germination and colony growth stimulated by heat treatment	Carpenter <i>et al.</i> 1982 Seaver and Clark 1910 Warcup 1951 Warcup 1951 Fergus 1964 Warcup and Baker 1963
<i>Aureobasidium</i> sp. / <i>A. pullulans</i> (CID 103, 113, 114, 116, 327, 334)	28S HQ829084, HQ829087, HQ829088, HQ829090, HQ829153, HQ829155, respectively	6 (20)	CO, ID, WA	Identified in the Mt. St. Helen's devasation zone	Carpenter <i>et al.</i> 1982
<i>Chaetomium</i> sp. (CID 236, 241, 280)	28S HQ829122, HQ829126, HQ829138, respectively	3 (17)	CO, ID, NM, WA	Higher frequency following exposure to 40°-100°C in soil samples vs. nonsteamed soil Isolated from burned prairie soils Rapidly grew on straw in compost at 50°C	Zak and Wicklow 1978 Wicklow 1975 Fergus 1964
<i>Chromelosporium</i> sp. (CID 312)	n/a ^e	1 (2)	ID	Identified in the Mt. St. Helen's devasation zone	Carpenter <i>et al.</i> 1982

Endophyte ¹	Accession No.	No. ²	Sites ³	Association with fire or heat ⁴	Reference
<i>Cladosporium</i> sp. (CID 28, 29, 32, 54, 228, 281, 308)	28S HQ829063, HQ829064, HQ829067, HQ829073, HQ829120, HQ829139, HQ829150, respectively	7 (19)	CO, ID, NV, WA	Found in recently burned prairie soils	Wicklow 1973
<i>Coniochaeta</i> sp. (CID 36, 170)	28S HQ829070, HQ829104, respectively	2 (6)	ID, NM	Found in recently burned prairie soils Higher frequency following exposure to 70°-85°C in soil samples vs. nonsteamed soil Ascospores germinated after 2 weeks following exposure to aerated steam at 55°C for 60 seconds Isolated from burned prairie soils	Wicklow 1973 Zak and Wicklow 1978 Wicklow and Hirschfield 1979 Wicklow 1975
<i>Curvularia</i> sp. (CID 31)	28S HQ829066, respectively	1 (6)	CO	Thermotolerance conferred to <i>Dichanthelium lanuginosum</i>	Redman <i>et al.</i> 2002
<i>Fusarium oxysporum</i> (CID 84, 199, 207, 208, 220, 261, 270, 375)	28S HQ829077, HQ829108, HQ829111, HQ829112, HQ829117, HQ829131, HQ829135, HQ829174, respectively	8 (84)	BC, CO, ID, NM, NV, WA	Growth more abundant on heated- than unheated-soil Found in recently burned prairie soils Survival following 6 minutes at 100°C Recolonization successful following steam-treatment of soil	Seaver and Clark 1910 Wicklow 1973 Warcup 1951 Warcup 1951
<i>Morchella</i> phylotypes within <i>M. elata</i> complex (CID 2, 5, 16, 40, 121)	28S HM756729, HM756730, HM756728, HM756731, HM756732, respectively efla HM756734, HM756735, HM756733, HM756736, HM756737, respectively RPB2 HM756739, HM756740, HM756738, HM756741, HM756742, respectively	5 (6)	ID, WA, NM	Prolific fruiting after burns Fruiting within first year following postfire/posteruption of Mt. St. Helen's volcano Fruiting within Mt. St. Helen's volcanic devastation zone	Pilz <i>et al.</i> 2004 Claridge <i>et al.</i> 2009 Carpenter <i>et al.</i> 1982
<i>Mucor</i> sp. (CID 351, 352, 357, 358, 359, 360, 361, 362, 364, 365, 367, 368, 369, 371)	28S HQ829158, HQ829159, HQ829160, HQ829161, HQ829162, HQ829163, HQ829164, HQ829165, HQ829166, HQ829167, HQ829168, HQ829169, HQ829170, HQ829171, respectively	14 (20)	BC, CO, ID, WA	Identified in the Mt. St. Helen's devastation zone Growth more abundant on heated- than unheated-soil Survival following 2 minutes at 100°C Isolated from surface sample of compost; grew well at 25-50°C	Carpenter <i>et al.</i> 1982 Seaver and Clark 1910 Warcup 1951 Fergus 1964
<i>Paecilomyces</i> sp. / <i>P. lilacinus</i> (CID 4, 101, 135, 306)	28S HQ829056, HQ829083, HQ829095, HQ829149, respectively	4 (5)	CO, ID, WA	Found in recently burned prairie soils	Wicklow 1973

Endophyte ¹	Accession No.	No. ²	Sites ³	Association with fire or heat ⁴	Reference
<i>Penicillium</i> sp. (CID 6, 42, 184, 195, 221, 240, 259, 260, 265, 299)	28S HQ829057, HQ829071, HQ829105, HQ829106, HQ829118, HQ829125, HQ829129, HQ829130, HQ829133, HQ829146, respectively	10 (21)	BC, CO, ID, NM, WA	Identified in the Mt. St. Helen's devasation zone Found in recently burned prairie soils Survival following 6 minutes at 100°C Recolonization successful following steam-treatment of soil Germination and colony growth stimulated by heat treatment	Carpenter <i>et al.</i> 1982 Wicklow 1973 Warcup 1951 Warcup 1951 Warcup and Baker 1963
<i>Peziza ostracoderma</i> (CID 8, 12)	28S HQ829059, HQ829060, respectively	2 (2)	ID, WA	Found developing most abundantly on steamed soil	Fergus 1960
<i>Phoma</i> sp. (CID 30, 115, 117, 277)	28S HQ829065, HQ829089, HQ829091, HQ829137, respectively	4 (5)	ID, NM, WA	Identified in the Mt. St. Helen's devasation zone Found in recently burned prairie soils Recolonization successful following steam-treatment of soil	Carpenter <i>et al.</i> 1982 Wicklow 1973 Warcup 1951
<i>Podospora</i> sp. (CID 52, 337)	28S HQ829072, HQ829156, respectively	2 (3)	ID, WA	Higher frequency following exposure to 40°C in soil samples vs. nonsteamed soil	Zak and Wicklow 1978
<i>Pyronema</i> sp. (CID 7, 124)	28S HQ829058, HQ829093, respectively	2 (3)	ID, WA	Fruiting within first year following postfire/posteruption of Mt. St. Helen's volcano Collected on fireplaces Spore germination higher at 50°C than 20°C following a 2-minute heat treatment Growth more abundant on heated- than unheated-soil Fruits more abundantly on soil sterilized at 135°-145°C than at 95°-110°C Cultured from bonfire cinders	Claridge <i>et al.</i> 2009 Moore and Korf 1963 El-Abyad and Webser 1968 Seaver and Clark 1910 Seaver 1909 Moore-Landecker 1975
<i>Pyrenophora semeniperda</i> (CID 22, 23)	28S HQ829061, HQ829062, respectively	2 (2)	ID	Higher thermal death point than cheatgrass	Beckstead <i>et al.</i> 2011
<i>Rhizopus</i> sp. (CID 348)	28S HQ829157	1 (15)	BC, CO, ID, NM	Exhibits optimal growth at 50°C	Peixoto <i>et al.</i> 2003

Endophyte ¹	Accession No.	No. ²	Sites ³	Association with fire or heat ⁴	Reference
<i>Sordaria</i> sp. (CID 33)	28S HQ829068	1 (5)	CO, ID, NM	Survival following 4 minutes at 100°C Higher frequency following exposure to 55°-100°C in soil samples vs. nonsteamed soil	Warcup 1951 Zak and Wicklow 1978
<i>Sporormiella</i> sp. (CID 329)	28S HQ829154	1 (1)	NM	Higher frequency following exposure to 35°-85°C in soil samples vs. nonsteamed soil Ascospores germinated after 6 weeks following exposure to aerated steam at 55°C for 60 seconds Isolated from burned prairie soils	Zak and Wicklow 1978 Wicklow and Hirschfield 1979 Wicklow 1975
<i>Trichoderma</i> sp. (CID 143, 229, 286)	28S HQ829098, HQ829121, HQ829142, respectively	3 (44)	CO, ID, WA	Survival following 4 minutes at 100°C Recolonization successful following steam-treatment of soil	Warcup 1951 Warcup 1951
<i>Ulocladium</i> sp. (CID 225, 324)	28S HQ829119, HQ829152, respectively	2 (3)	CO, ID, WA	Identified in the Mt. St. Helen's devasation zone	Carpenter <i>et al.</i> 1982
<i>Verticillium</i> sp. (CID 245, 301, 302)	28S HQ829127, HQ829147, HQ829148, respectively	3 (5)	CO, ID, NM, NV, WA	Growth more abundant on heated- than unheated-soil	Seaver and Clark 1910

^a Endophytes were considered tentative matches with recognised species only if their ITS sequences were identical. From 97% up to but not including 100% ITS sequence identity, endophytes were identified only to genus. Based on the discovery of two phylotypes of *Morchella* as cheatgrass endophytes (i.e., *Mel-6* and *Mel-12*), five isolates were characterised further using multilocus DNA sequence data (Figure S1). CID refers to culture identification numbers at the University of Idaho.

^b Number of morphotypes sequenced as this taxon (total number of isolates of this taxon). Thus, of 1,064 isolates, 419 (39%) were equal to, or closely related to, taxa that are known as fire followers and/or thermotolerant fungi.

^c Sites where endophyte was isolated from cheatgrass (BC=British Columbia, CO=Colorado, IA=Iowa, ID=Idaho, IL=Illinois, NM=New Mexico, NV=Nevada and WA=Washington).

^d Association with fire and/or heat, as reported for this taxon in the linked reference.

^e Endophyte identified based on morphological characteristics rather than sequence data.

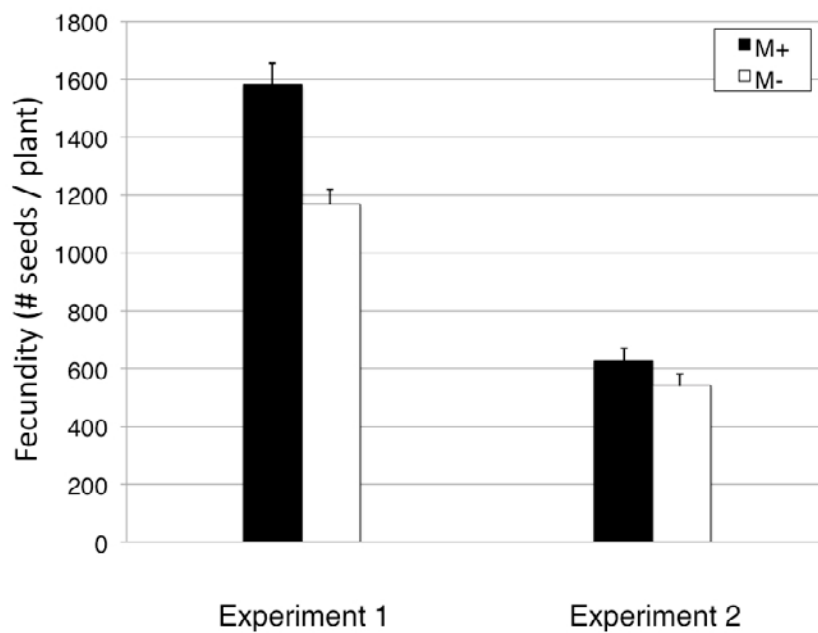


Figure 2.1. *Morchella* increased cheatgrass fecundity in both experiments ($t=6.80$, $P<0.001$ and $t=2.07$, $P=0.05$, respectively).

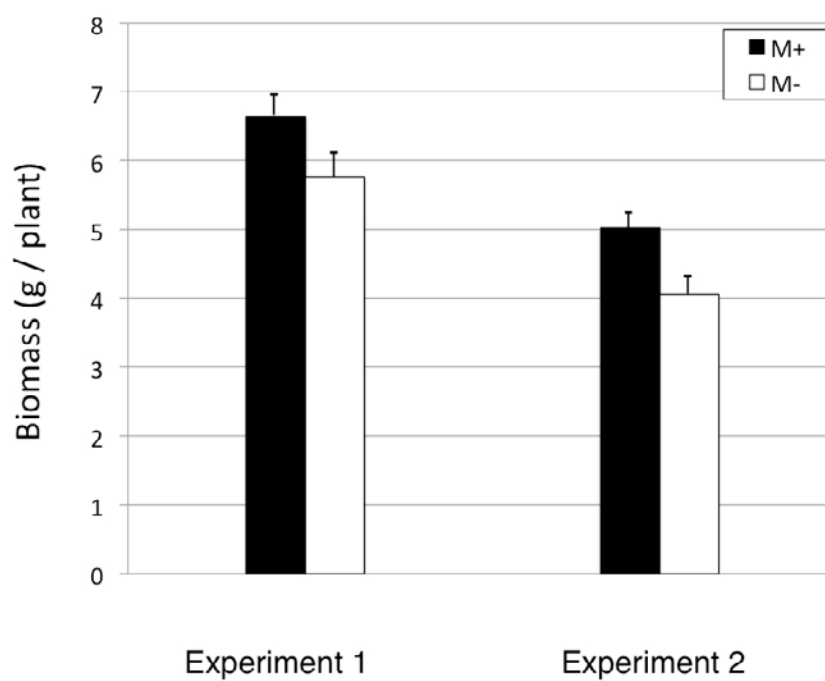


Figure 2.2. *Morchella* increased cheatgrass biomass in both experiments ($t=2.39$, $P=0.03$ and $t=2.16$, $P=0.04$, respectively).

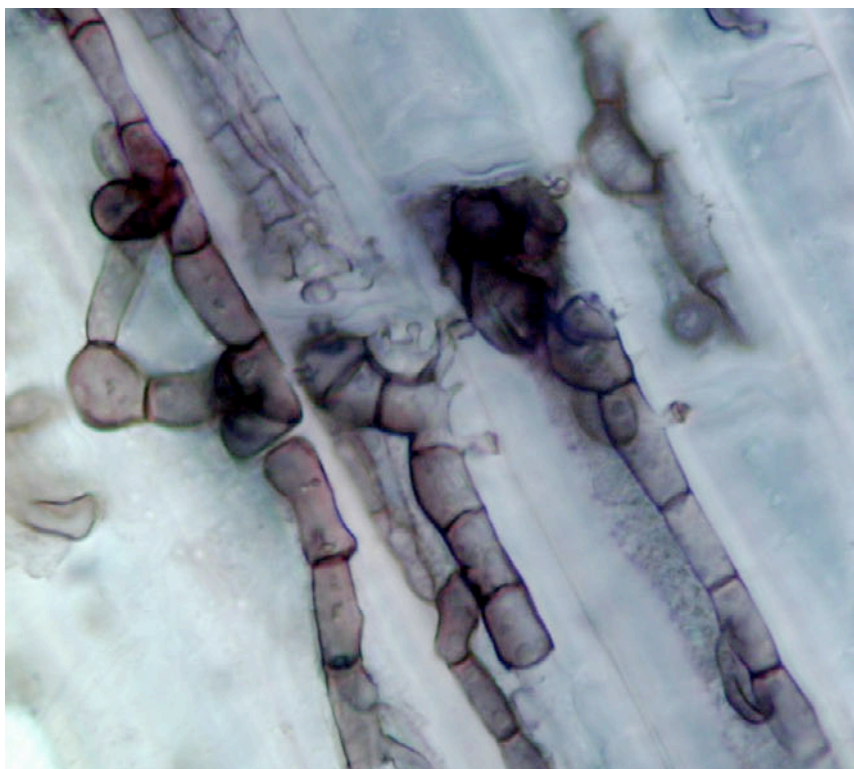


Figure 2.3. Non-mycorrhizal colonization of M+ plants.

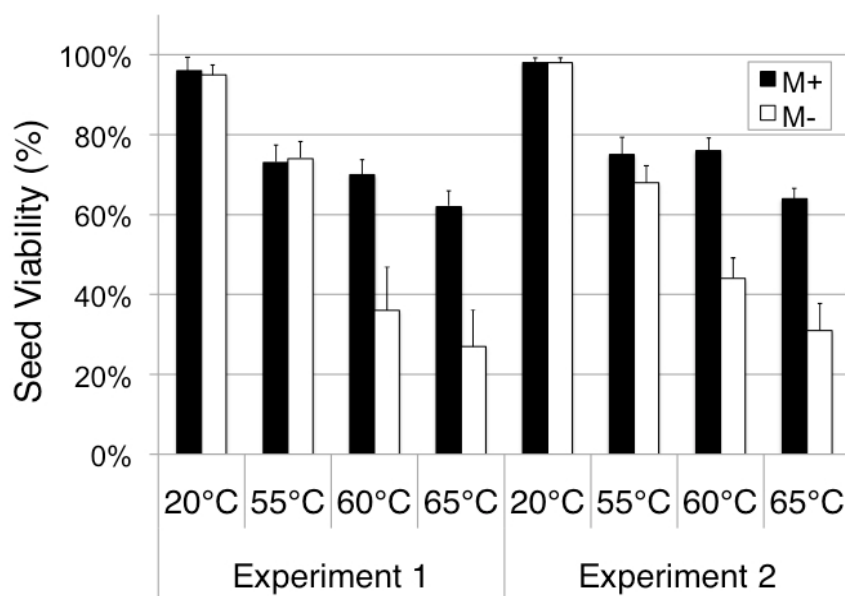


Figure 2.4. Temperatures reached during typical cheatgrass fires were more lethal to M- than M+ seed at 60°C and 65°C (Experiment 1, $F=5.49$, $P<0.001$); Experiment 2, $F=5.74$, $P<0.001$).

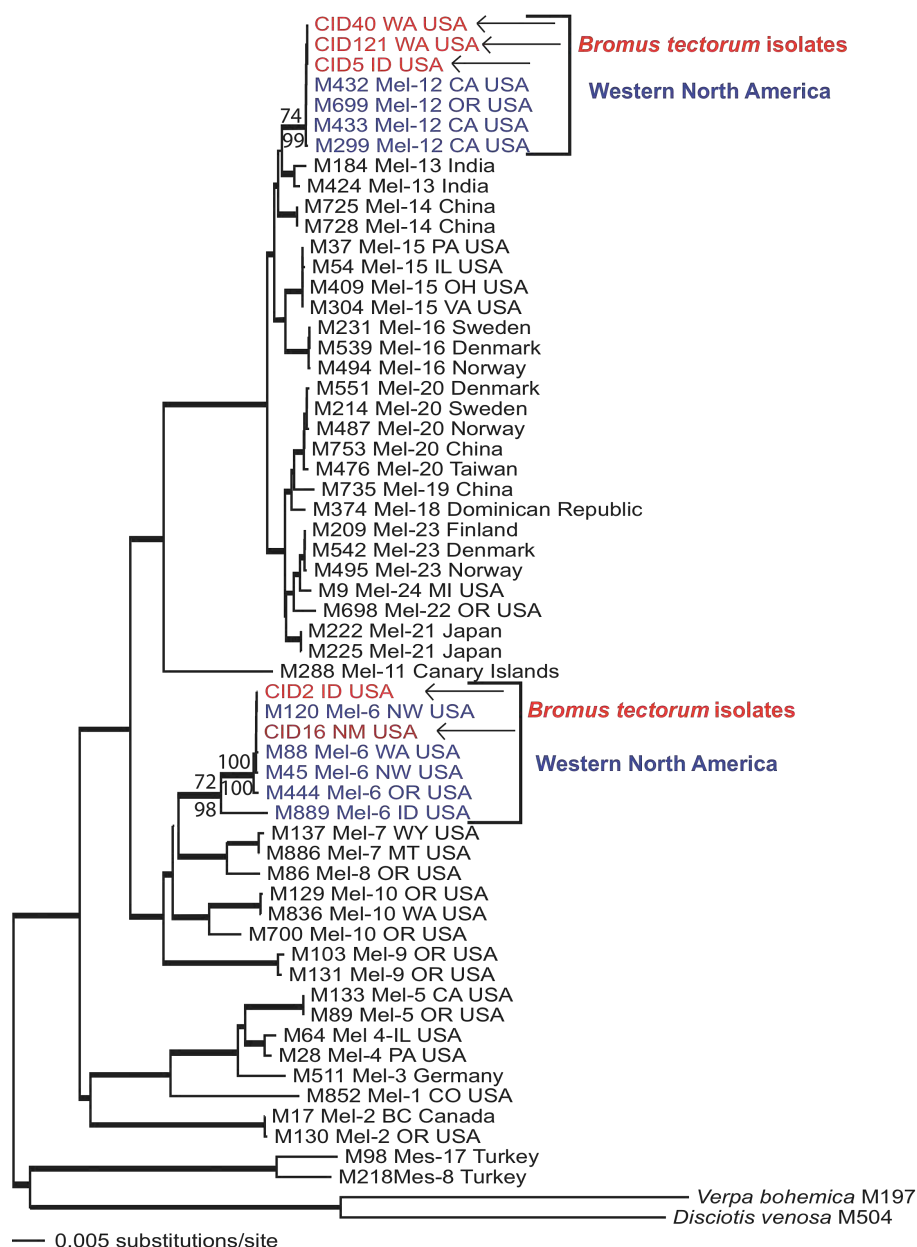


Figure 2.5. Maximum likelihood phylogenetic tree inferred from *tef1- α* , *rpb2*, and 28S nrLSU genes for phylogenetic species in the *Morchella elata* clade (black morel mushrooms). *Bromus tectorum* isolates are highlighted in red and identified with arrows. MP bootstraps are shown above and Bayesian PP are shown below the branches for nodes of interest. Thickened branches indicate support >95 % PP and >70 % MP bootstraps.



Figure 2.6. Cheatgrass in morel habitat above the Weiser River of ID in July 2008.

Chapter 3: A fungivorous nematode and fungal cultivar alter the endophyte community of *Bromus tectorum*

3.1. Abstract

In its invaded range in western North America, *Bromus tectorum* (cheatgrass) can host more than 100 sequence-based phylotypes of endophytic fungi, of which an individual cheatgrass plant hosts a subset. In general, research suggests that recruitment of a particular subset of endophytes by an individual plant will be determined by plant genotype, the environment, and dispersal of locally available endophytes. Discovery of a strong association between a fungivorous nematode, *Paraphelenchus acontoides*, and an endophytic fungus, *Fusarium* cf. *torulosum*, in *B. tectorum* led to an investigation of the role of the nematode in structuring endophyte communities in British Columbia and Colorado. In greenhouse and laboratory experiments, we determined that *P. acontoides* preferred *F. cf. torulosum* to other endophytic fungi from the Colorado site, and that it increased the relative abundance of *F. cf. torulosum* within the endophyte community. The ‘cultivation hypothesis’ (i.e., that the fungivorous nematode was using living cheatgrass plants to ‘cultivate’ its preferred fungus) was thus supported. Host plant growth was unaffected by this cultivation of *F. cf. torulosum* by *P. acontoides* within the tissues of the plant host.

3.2. Introduction

Endophytic fungi are ubiquitous in nature (Petrini 1986; Clay 2004; Schulz & Boyle 2006; Sieber 2007) and although infection is typically asymptomatic (Wilson 1995),

symbioses with a plant host can range from mutualistic to antagonistic (Clay 1996; Clay & Schardl 2002; Schardl *et al.*; Spiering 2004; Kuldau & Bacon 2008; Saikkonen *et al.* 1998). A few endophytic species or haplotypes often dominate within a host (Ahlholm *et al.* 2002; Shipunov *et al.* 2008). For instance, research investigating the endophytic community of western white pine (*Pinus monticola*) from multiple populations throughout the Rocky Mountains revealed that a *Lophodermium* haplotype was the dominant endophyte (Ganley & Newcombe 2006). Although few species are often dominant, endophytic fungi can form diverse community assemblages (Carroll & Carroll 1978; Petrini 1986; Arnold 2007; Arnold & Lutzoni 2007; Shipunov *et al.* 2008). Within a single leaf of a single host diversity has been documented as high as 17 different species (Lodge *et al.* 1996; Gamboa & Bayman 2001) and, even at a small spatial scale, singletons can often comprise a significant portion of the endophytic community (Arnold *et al.* 2000; Arnold & Lutzoni 2007). Although considerable research has investigated diversity within specific hosts, much is still unknown about what influences endophyte community structure.

Arnold & Lutzoni (2007) found biogeography to be an important factor in the incidence and diversity of endophytes. Their research demonstrated that the diversity of endophytes at both the individual and plant community levels increased with decreasing latitude (i.e., from poles to equator). Furthermore, endophytes isolated within a specific biogeographic zone (i.e., arctic, temperate or tropical) were often absent from other zones (Arnold & Lutzoni 2007).

At the local level, other factors are operative. Water availability and temperature as well as agro- and plant chemicals can affect the endophytic community in maize (*Zea mays*) (Lacey & Magan 1991; Marin *et al.* 1998; Seghers *et al.* 2004; Saunders & Kohn 2009). For example, Marin *et al.* (1998) demonstrated that inter- and intraspecific endophytic interactions were altered, allowing different fungi to dominate at different temperatures and water availabilities. Saunders & Kohn (2009) demonstrated that production of plant defense compounds helped to shape the endophyte community within maize, and variable leaf chemistry may, in general, explain differences in endophyte communities among host species (Arnold & Herre 2003).

The presence of certain endophytes has been demonstrated to depress colonization by other endophytes. Schulthess & Faeth (1997) found that when *Neotyphodium* was present in Arizona fescue (*Festuca arizonica*), the frequency of other endophytes declined. Specific endophytes may be competitively superior because of mycotoxin production or stimulation of host plant defenses (e.g., premature leaf abscission and chemical toxin production) that limit colonization and growth of other endophytes (Schulthess & Faeth 1997; Saikkonen *et al.* 1998). Therefore, the presence of one dominant or beneficial endophyte may influence the presence and diversity of other potential endophytes within a host.

A living plant can serve as a significant filter for diversity since it controls initial endophyte entry into its tissues. Thus, it is not surprising that host genotype affects the

structure of mycorrhizal communities (Mummey & Rillig 2006; Korkama *et al.* 2006), as well as richness, diversity and composition of endophytes within plants (Todd 1988; Bailey *et al.* 2005; Pan *et al.* 2008). In western North America, for example, the endophyte community of *B. tectorum* (Baynes *et al.* in review) differs substantially from that of *Centaurea stoebe* (Shipunov *et al.* 2008), another common plant invader of the region. Although both species are native to Eurasia and both were sampled within similar habitat types within their invaded range, little overlap was observed between their endophyte communities.

In addition to these above-mentioned, community-structuring factors, members of endophytic communities could also directly affect the relative abundance of one another. Although endophyte-endophyte interactions have been little studied, they may be similar in importance to microbial interactions within soil communities. For example, microarthropods are selective feeders (Maraun *et al.* 1998) with a preference for conidial fungi over arbuscular mycorrhizal fungi (Klironomos & Kendrick 1996). Nematodes, common in soil communities (Bongers & Bongers 1998; Newsham *et al.* 2004), can also influence growth of fungi (Riffle 1967; Sutherland & Fortin 1968; Shafer *et al.* 1981; Ingham 1988; Giannakis & Sanders 1989), and species composition (Newsham *et al.* 2004). Interactions between endophytic nematodes and fungi can have consequences for host plant health (Powell 1971; Pitcher 1978; Nordmeyer & Sikora 1983a,b; Sikora & Carter 1987), contributing to diseases like vascular wilt and root-rot in banana (Powell 1971; Sikora & Carter 1987; Sikora & Schlösser 1973; Gowen *et al.* 2005). Conversely,

Stewart *et al.* (1993) found that endophytic fungi could inhibit gall-forming nematodes, improving plant health.

Fungivorous nematodes are sometimes isolated as endophytes along with fungi. These nematodes could change the relative abundance of endophytic fungi that they selectively or preferentially consume within plant tissue. Because we initially isolated a fungivorous nematode along with an endophytic *Fusarium* sp. that seemed unusually abundant relative to other endophyte samples within *B. tectorum*, we hypothesized that the nematode was using living *B. tectorum* plants to ‘cultivate’, or increase the relative abundance of, the *Fusarium* sp. that it preferred to consume.

The objectives of our research were to test this cultivation hypothesis, via preference and suitability assays directed at the nematode, and secondarily, via inoculations of *B. tectorum* with the nematode and/or its putative fungal cultivar. Finally, we determined whether this nematode-fungus interaction affected host plant fitness (i.e., height and biomass).

3.3. Materials and methods

3.3.1. Sampling and isolation of endophytes in *Bromus tectorum*

Bromus tectorum was collected from 63 sites throughout the United States and Canada (i.e., British Columbia, Colorado, Idaho, Illinois, Iowa, Nevada, New Mexico and

Washington – Table 1) during 2009 and 2010. Collections were made from a variety of habitats including coniferous forest, sagebrush-grassland, desert scrub, agricultural fields and disturbed roadside. At each site 20 green stems were collected (Seabloom *et al.* 2009). Sampling was conducted twice at one site; Piney River, CO was sampled in 2009 (Piney River) and again in 2010 (Piney River '10).

A two-centimeter segment centered on the lowest culm node was removed from each plant. Culm segments were surface-sterilized in 50% ethanol (EtOH) for five minutes and rinsed with sterile, deionized (DI) water for one minute (*Schulz et al. 1993 in* Luginbuhl & Muller 1980). For each population, imprint plates were made to ensure efficacy of sterilization. Culm segments were placed on potato dextrose agar (PDA) in Petri dishes and sealed with parafilm. Endophytic fungi and nematodes emerging from segments were isolated and cultured; nematodes were co-cultured with their fungal symbionts. All cultures were stored on laboratory benches at ambient room conditions (20°C with a 10:14 hour photoperiod, light:dark) to allow for fungal growth.

3.3.2. Identification of endophytic fungi and nematodes

Endophytes isolated from all 63 *B. tectorum* populations were morphotyped based on culture and microscopic characteristics. A subset of these isolates, including two morphologically similar *Fusarium* cultures from Piney River (CID 018) and Nelson (CID

273), was sent to the Systematic Mycology and Microbiology Lab for sequence identification (Baynes *et al.* in review).

Two additional cultures of *Fusarium* isolated from Piney River (CID 314 and CID 383), morphologically identical to CID 018, were also identified using morphological and molecular approaches. For the morphological identification, cultures were grown on PDA for two weeks to measure colony diameter and allow for the production of sporulating structures. The identification was confirmed using Leslie & Summerell (2006).

For sequencing and phylogenetic analysis, isolates of *Fusarium* were grown in 5 mL of potato dextrose broth in 15×60 mm Petri dishes incubated at 25°C for 3 days. Mycelium from the cultures was separated from the media and pressed between paper towels to remove excess media and used for DNA extraction. DNA was extracted using ArchivePure DNA cell/tissue kit from 5 PRIME, Inc. (Gaithersburg, MD) following protocol provided by the manufacturer. The DNA were used as templates in polymerase chain reactions. A section of translation elongation factor 1 alpha (TEF) was amplified using primers Ef-700f (Samuels & Ismaiel 2011) and Ef2 (O'Donnell *et al.* 2000). Internal transcribed spacer (ITS) was amplified using primers ITS5 and ITS4 (White *et al.* 1990). The PCR mixture and the thermal-cycler program for amplification of both loci were the same as described previously (Samuels & Ismaiel 2009). Approximately 0.5kb and 0.6 kb products of TEF and ITS were amplified, respectively. The amplicons were cleaned enzymatically using Exosap-IT (USB Corporation, Cleveland, OH). The

purified products were directly sequenced using BigDye Terminator v3.1 chemistry on an automated 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Both strands of each amplicon were sequenced using the primers used in generating them.

The sequences were assembled and edited to construct a consensus sequence using Sequencher 4.9 (Gene Codes, Madison, WI). The sequences of the two isolates had identity of 100%. One of the two sequences was subjected to basic local alignment search tool (BLAST) using BLSTN program available @ <http://www.ncbi.nlm.nih.gov>. The search indicated that several species of *Fusarium* in the study of Kristensen *et al.* (2005) and few isolates in the study of O'Donnell *et al.* (2009) had high sequence similarity to the two isolates under study. The nexus alignment file from Kristensen *et al.* (2005) was retrieved from Treebase home page (<http://www.treebase.org>). The sequences of our isolates plus the few isolates of O'Donnell *et al.* (2009) were added to the alignment file. We also reduced the number of haplotypes in each clade in the tree. The additional sequences were realigned manually.

A phylogenetic tree was obtained under parsimony criterion using PAUP 4.0b10 (Swofford 2002) with a heuristic search, 1000 random stepwise addition, tree bisection reconnection (TBR) as branch swapping algorithm and MULTREES on. All characters were equally weighted and gaps were treated as missing. The tree was rooted using *Fusarium equiseti* as the outgroup based on the study of Kristensen *et al.* (2005). Support for the branches was performed with bootstrap using 1000 pseudo-replicates of the data,

100 random additions per replicate and TBR branch swapping. Bootstrap values greater or equal to 70% were considered significant (Hillis & Bull 1993).

The two *Fusarium* isolates (CID 314 and CID 383) were deposited in CBS and sequences were deposited in GenBank as CID 314 (ITS JN133579, TEF JN133577) and CID 383 (ITS JN133580, TEF JN133578).

The USDA-ARS Nematology Laboratory identified endophytic nematodes that were isolated with, and subsequently co-cultured with, two *Fusarium* isolates (CID 314 and CID 383). Nematodes sent for morphological identification were rinsed from the Petri plates, placed in 4% formalin for 24 hours and then rinsed in sterile DI water. Nematodes were then placed in 1.5 mL plastic tubes with sterile DI water for shipment. Nematodes sent for sequence identification were rinsed from the plates and placed in 70% alcohol in 1.5 mL plastic tubes for shipment.

3.3.3. Effects of a fungivorous nematode and a putative fungal cultivar on the endophyte community

3.3.3.1. Field surveys

Prior to endophyte and nematode isolation, individual *B. tectorum* plant weight (aboveground fresh weight) and height (from base to first inflorescence branch) were input into an Excel database; endophyte isolation and identification results were also compiled into the database. These data allowed for analyses of plant height and weight

as well as endophyte frequency, richness, evenness and diversity within and by population.

3.3.3.2. Experiment 1

Experimental design included two treatments: *F. cf. torulosum* inoculum with (N+) or without (N-) nematodes. One local population of *B. tectorum* was employed with 15 replicates (i.e., plants) per treatment. The two fungal inoculant solutions were prepared by removing a 12cm² section of mycelium from a *F. cf. torulosum* culture with nematodes (N+) and thoroughly mixing into 150mL of sterile DI water. The same procedure was employed for the second inoculation solution from a *F. cf. torulosum* culture without nematodes (N-).

Seed were harvested from a *B. tectorum* population on Hog Island along the Clearwater River near Lewiston, ID in 2009 [46°26'52.77"N 116°51'42.42"W]. Seed were surface sterilized in 50% EtOH for five minutes and rinsed with sterile DI water for one minute (Schulz *et al.* 1993 in Luginbuhl & Muller 1980). The seeds were placed in UV-sterilized, covered Petri plates and allowed to germinate at ambient room temperature and light.

Bromus tectorum seedlings were transplanted into autoclaved potting soil (Sunshine Mix #2) and UV-sterilized trays (20x25x8 cm). For each treatment, three seedlings were planted into five trays. Seedlings were planted at an equal distance from each other and

the inoculant was immediately pipetted into shallow holes in the soil, equidistant to each plant (3mL of inoculant per hole for a total of 9mL per tray). Roots, fungi and nematodes were allowed to freely interact within the soil environment.

Bromus tectorum plants were harvested after four weeks. Excess soil was rinsed from each plant and aboveground and belowground fresh weights were recorded. Three random 3cm sections were clipped from both the root and leaf tissue of the harvested plants. Fresh weight of the clippings and remaining plants (aboveground and belowground biomass) were recorded. After weighing, the plants were placed into separate paper bags and dried for 72 hours at 60°C. Following the drying period, plant dry weight biomass was recorded for each plant. These results along with the fresh weight results were used to calculate total dry weight biomass for each individual plant.

Plant tissue was surface sterilized using the same procedure as used to sterilize seed. Sterilized plant tissue was plated onto PDA; Petri plates were sealed with parafilm and stored on laboratory benches at ambient room conditions. Cultures were observed daily and fungal isolates were identified morphologically to genus based on macroscopic and microscopic morphology.

3.3.3.3. Experiment 2

Using the same seed source, the experimental design from Experiment 1 was repeated for Experiment 2 but with additional replication for each treatment (n=50). Five seedlings were planted into each tray, equidistant from one another. The inoculant was pipetted into shallow holes in the soil at an equal distance from each plant (3mL of inoculant per hole for a total of 15mL per tray). The solution, plant ratios, and proportions were equivalent to those in Experiment 1. Trays containing plants and fungi were covered in Experiment 2 to minimize contamination. *Bromus tectorum* plants were harvested after four weeks and the same procedures were followed as in Experiment 1.

3.3.3.4. Experiment 3

Experimental design for Experiment 3 was similar to the first two experiments, but inoculum comprised all four of the endophytes isolated from the Piney River *B. tectorum* population rather than just *F. cf. torulosum*. Specifically, *F. cf. torulosum*, *Curvularia* sp., *Penicillium olsonii* and an unidentified endophyte (B115) were used to make the inoculum, both with (N+) and without (N-) nematodes. A 3cm² section of mycelium from each fungal culture was removed and mixed together thoroughly into 150mL of sterile DI water. Inoculum:plant ratios were equivalent to those in the first experiments; each treatment was replicated (n=50) and the same seed source was utilized. *Bromus tectorum* plants were harvested after four weeks and the same procedures were followed as in Experiments 1 and 2.

3.3.4. *Fungal preference and suitability assays*

3.3.4.1. Preference assays

Nematodes were offered a choice of fungi in a 'cafeteria' design. Fungi isolated from Piney River *B. tectorum* were cultured on PDA in Petri dishes (8.5cm diameter). Small plugs (0.5cm²) of two inoculants, *F. cf. torulosum* and *P. olsonii*, were placed on opposite sides of the plate. The plates were sealed with parafilm and the fungi were allowed to grow for three days. On the third day, a diameter line was drawn on the back of the plate, halfway between the mycelium of each fungus. Approximately 50 nematodes (*P. acantioides*) were pipetted along the line onto the agar. The plates were resealed and left for three additional days under ambient laboratory conditions. On the third day after the nematodes addition, Petri dishes were placed under a dissecting microscope for observation. Nematodes were counted in each of the sectors delineated by the diameter line. Counts were repeated three times and averaged. A second assay with a different fungal combination (i.e., *F. cf. torulosum* and *Curvularia* sp.) was conducted following the same methods. Each assay was replicated four times.

3.3.4.2. Suitability assays

Nematodes (~75) were placed into additional Petri dishes containing only *F. cf. torulosum*, *P. olsonii* and *Curvularia* sp. to test whether *P. acantioides* would graze, survive and reproduce on fungi other than *F. cf. torulosum*. For each fungus, two plates were prepared (N+). Six additional Petri dishes were prepared as control plates (N-), two for each fungus. These plates were left undisturbed for two weeks under ambient laboratory conditions. Six 0.5cm² plugs were randomly removed from each culture and

placed into a sterile dish for observation. Using a dissecting microscope, nematodes (alive, eggs and dead) were counted from each of the six plugs. Counts for each plug were repeated three times and averaged.

Because nematodes are often concealed within the mycelium and agar, plug data were supplemented by a secondary method for determining density (number of individuals per 0.5cm^2). Once plug counts were completed, six plugs of each endophyte type were placed into a small glass bottle with 6mL of sterile DI water and vigorously shaken for one minute. From the solution, 1mL was pipetted into a 0.5cm^2 gridded Petri plate. Nematodes (alive, eggs and dead) were counted three times and averaged. This process was repeated for all 6mL of solution for each of the cultures.

Additional observations related to grazing suitability were made using *Agaricus bisporus*. *Agaricus bisporus* was grown in culture but was not isolated as an endophyte from cheatgrass. Four plates (two N+, two N-) were observed over the same time period as the other fungi. Density data were not recorded for *A. bisporus*; only observational data was recorded.

3.3.5. Statistical methods

Data were analyzed with Systat 12.02.00 (Systat Software, Inc. 2007) and online computer software (Preacher 2001; University of Manitoba 2010). For field-collected samples, chi-square analyses were used to compare the frequency of the putative fungal

cultivar when nematodes were present relative to when nematodes were absent (Preacher 2001). Richness, diversity (Simpson's) and evenness (Shannon's) analyses were conducted for each of the 63 populations (University of Manitoba 2010). For the three greenhouse experiments, chi-square analyses were conducted to compare the re-isolation frequency of the putative fungal cultivar when nematodes were included (N+) versus excluded (N-) in the inoculum (Preacher 2001). To determine endophyte preference, chi-square analyses were conducted (Preacher 2001) and density data from the suitability assays was analyzed using ANOVA with Bonferroni pairwise comparisons (Systat Software, Inc. 2007). Field and greenhouse biomass data were analyzed using Student's two-sample t-test with separate variances (Systat Software, Inc. 2007).

3.4. Results

3.4.1. *Sampling and isolation of endophytes in *Bromus tectorum**

From the 63 populations sampled, 1,064 fungal endophytes were isolated with more than 100 sequence-based identifications made. Of the 63 sites sampled, only two yielded co-occurring endophytic nematodes and fungi (i.e., Nelson, BC [49°29'9.43"N, 117°18'7.21"W] and Piney River (2009), CO [39°50'24.99"N, 106°38'26.85"W]).

3.4.2. *Identification of endophytic fungi and nematodes*

Sequence-based identifications were made for three of the *Fusarium* isolates (CID 018, CID 314 and CID 383) from Piney River. Isolates CID 314 and CID 383 were identified as *F. cf. torulosum*; CID 018 was identified as a *Fusarium* sp. Isolate CID 018 as well as all other *Fusarium* cultures from Piney River were morphologically identical to CID 314

and CID 383 and were classified as *F. cf. torulosum*. A sequence-based identification was also made for the morphologically similar isolate CID 273 from Nelson. Results from a BLAST search identified this isolate as *Fusarium* species. With the exception of one isolate, CID 207 that was identified as *Fusarium oxysporum*, all other *Fusarium* cultures from Nelson were morphologically identical to CID 314 and CID 383 and thus were classified as *F. cf. torulosum*.

With the additional sequences from GenBank added to the tree of Kristensen *et al.* (2005), the final sequence data had 27 taxa and 723 characters of which 545 were constant, 58 parsimony-uninformative, and 120 (17%) were parsimony-informative characters. The two isolates under study along with an isolate deposited as *Fusarium* sp. (GenBank accession number GQ505419) formed a highly supported subclade as shown in Figure 1. This subclade had strong sister-relationship with *F. torulosum* clade. In Kristensen *et al.* (2005), all the species in Figure 1 were included in the monophyletic group M that included all the species that produced moniliformin but not trichothecene. Even though moniliformin production has not been reported for *F. torulosum*, inclusion of the species within the group suggests the potential for such an activity.

The internal transcribed spacer regions (ITS) of the two isolates were also identical suggesting that these two isolates represent one haplotype. When ITS sequence of one of the two isolates was used in BLAST search many identical or highly homologous hits deposited under different species names or as *Fusarium* sp. were available suggesting

inability of the locus to distinguish between closely related species in *Fusarium*; therefore, we did not use the ITS in any phylogeny analysis.

Endophytic nematodes were only isolated with the *Fusarium* morphotype identified as *F. cf. torulosum*. Two species of nematodes were co-isolated with the *F. cf. torulosum* and identified as the polyphagous *Panagrolaimus artyukhovskii* and the fungivorous *Paraphelenchus acontioides* (Hunt 1993). All greenhouse and laboratory experiments were conducted using *P. acontioides* isolated with one of the *F. cf. torulosum* cultures from Piney River.

3.4.3. *Effects of a fungivorous nematode and its fungal cultivar on the endophyte community*

3.4.3.1. Field surveys

Host plant fitness in the Piney River and Nelson sites was unaffected by *F. cf. torulosum* and the nematodes. Plant height did not differ significantly between *B. tectorum* with *F. cf. torulosum* and nematodes (N+) and those without nematodes (N-) (Piney River, $t=1.467$, $df=2.486$, $p=0.256$; Nelson, $t=-1.253$, $df=7.724$, $p=0.247$). Likewise, for both sites, there was no significant difference in fresh weight between N+ and N- plants (Piney River, $t=2.050$, $df=11.875$, $p=0.063$; Nelson, $t=-0.490$, $df=2.541$, $p=0.663$).

Relative frequency of *F. cf. torulosum* at Piney River and Nelson sites was high: 73% and 69%, respectively. Likewise, nematode isolation was high at both sites; nematodes

were observed in 84% and 89% of the *F. cf. torulosum* isolates at Piney River and Nelson, respectively. From these two sites, frequency of *Fusarium* spp. was relatively

high (i.e., a near 3:1 ratio) in comparison to the other 61 sites (i.e., a near 1:9 ratio). Not surprisingly, a chi-square analysis of the 63 sites demonstrated that *Fusarium* spp. frequency was significantly higher in N+ and N- *B. tectorum* (chi-square=159.427, df=1, $p<0.001$) (Table 2). *Re*-sampling at Piney River in 2010 yielded a low isolation frequency of *Fusarium* sp. (20%) relative to 2009 efforts; nematodes were absent from all Piney River 2010 isolates.

Endophytic *F. cf. torulosum* and its co-occurring nematodes influenced indices of richness, diversity and evenness of the endophytic communities at these two sites (Table 1). For the 63 populations sampled, fungal phylotype richness varied from 0 to 21 with a mean of 7.18. Both Piney River and Nelson sites were below the mean with values of 4 and 6, respectively. With respect to evenness, values ranged from 0.000 to 1.000 among the 63 populations sampled; Piney River and Nelson values were 0.548 and 0.601, respectively. Only three populations had lower values; one of these, St. Maries, produced no endophytes and in another, Mississippi, *Aspergillus niger* was the dominant endophyte. Endophytic diversity values ranged from 0.000 to 1.000 among all 63 populations. Diversity at Piney River and Nelson were comparatively low to the other populations (0.394 and 0.497, respectively); only three populations had lower values.

One population (Nisqually John) had a very low isolation rate (one endophyte) and the two other populations had a high isolation rate of a single endophyte (*A. niger* at Mississippi and *Fusarium* sp. at Dillon Lake) that reduced their respective diversities.

3.4.3.2. Experiment 1

The relative re-isolation frequency of *F. cf. torulosum* was significantly higher in N+ plants relative to N- plants, 38% and 14%, respectively (chi-square=4.406, df= 1, p=0.036) (Table 3). Other endophytes isolated included *Alternaria* sp., *Penicillium* sp., *Fusarium oxysporum* (N+ only), *Rhizopus* sp. and several unidentified bacterial endophytes, all which were considered common airborne contaminants in the greenhouse. *Fusarium oxysporum* was distinguished from *F. cf. torulosum* by comparing both culture morphology and micromorphic features (Nelson *et al.* 1983). The presence of nematodes did not affect host plant biomass (t=-1.401, df=20.410, p=0.176).

3.4.3.3. Experiment 2

Fusarium. cf. torulosum re-isolation frequency was significantly higher in N+ plants relative to N- plants, 27% and 20%, respectively (chi-square=4.480, df=1, p=0.034) (Table 3). Other endophytes again isolated included airborne contaminants such as *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., *Trichoderma* sp., *Ulocladium* sp. (N-only), *F. oxysporum* and *Rhizopus* sp. Again, nematodes were re-isolated from plants inoculated with *F. cf. torulosum* and always in association with this fungus and no other. Nematode presence did not affect host plant biomass (t=0.145, df=82.918, p=0.885).

3.4.3.4. Experiment 3

Once again, re-isolation frequency of *F. cf. torulosum* was significantly higher in N+ versus N- plants, 17% and 6%, respectively (chi-square=7.922, df=1, p=0.005) (Table 3).

Other inoculants (i.e., *Curvularia* sp. and *P. olsonii*) were also re-isolated from both treatments although the unidentified endophyte (B115) was not. Other endophytes isolated included airborne contaminants *Aspergillus* sp., *Alternaria* sp., *Trichoderma* sp., *Chaetomium* sp., *Acremonium* sp., *F. oxysporum*, *Rhizopus* sp. and a second species of *Penicillium*. Nematodes were re-isolated exclusively in association with *F. cf. torulosum*. Biomass was not analyzed.

3.4.4. *Fungal preference and suitability assays*

3.4.4.1. Preference assays

Three endophytes from the Piney River site (i.e., *F. cf. torulosum*, *P. olsonii* and *Curvularia* sp.) were employed in assays to determine whether the nematode, *P. acontioides*, preferred *F. cf. torulosum*. In all four plates of the *F. cf. torulosum*-*P. olsonii* preference assay, more nematodes were observed within the mycelial sector of *F. cf. torulosum* (chi-square=12.875, df=3, p=0.005) than in the sector of *P. olsonii* (Table 4). Likewise in the *F. cf. torulosum*- *Curvularia* sp. preference assay, the nematodes preferred *F. cf. torulosum* to *Curvularia* sp. (chi-square=7.883, df=3, p=0.049) (Table 4).

3.4.4.2. Suitability assays

Paraphelenchus acontioides is a fungivorous nematode that can consume several different fungal species (Hunt 1993). In the experiments reported here, *P. acontioides* grazed and reproduced upon the *F. cf. torulosum* cultures but also upon the *Curvularia* sp. and *A. bisporus* cultures (Figure 2a-b, 2d). In contrast, nematode survival and reproduction was limited in the *P. olsonii* cultures (Figure 2c). *Fusarium cf. torulosum* aerial and radial mycelial growth was significantly impacted by nematode presence (Figure 2a). Nematode density averaged 54 (alive), 5 (eggs) and 0 (dead) from the plug counts and 133 (alive), 17 (eggs) and 1 (dead) from the solution counts.

Nematode grazing also reduced aerial and radial growth of the *Curvularia* species. In the N- plates, *Curvularia* sp. filled the plate within the two-week period but was completely grazed in the N+ plates (Figure 2b). From the plug counts, nematode density averaged 66 (alive), 5 (eggs) and 0 (dead). Nematode counts from solution averaged 159 (alive), 5 (eggs) and <1 (dead). Compared to the N+ *F. cf. torulosum* cultures, living (alive + eggs) nematode counts did not differ significantly in either the plug ($p=0.289$) or solution counts ($p=0.138$) (Figure 3).

Nematode grazing was evident, although limited, at the end of the two-week period in the N+ *P. olsonii* cultures. Hyphae appeared to be partially grazed although radial growth was not suppressed; the fungus grew rapidly and filled the entire plate (Figure 2c). While nematodes survived initially within the *P. olsonii* cultures, their activity and mobility were diminished compared to the *Curvularia* sp. and *F. cf. torulosum* cultures.

Nematodes did not reproduce within the *P. olsonii* cultures. From both the plug and solution counts, nematode density averaged 0 (alive), 0 (eggs) and <1 (dead). Compared to the N+ *F. cf. torulosum* and *Curvularia* sp. cultures, living (alive + egg) nematode

counts were significantly lower for both the plug ($p \leq 0.001$) and solution counts ($p \leq 0.001$) (Figure 3).

Aerial and radial growth were completely suppressed in both sets of *A. bisporus* N+ plates; the fungus was entirely grazed within the two-week period (Figure 2d). In the control plates, the mycelium grew and filled approximately half of the plate in the two-week period.

3.5. Discussion

This research provides evidence that a fungivorous nematode can exploit a living host plant to cultivate a preferred endophyte, thereby influencing the relative abundance of it and other fungi within the endophytic community. Although *P. acontioides* readily consumed other fungi, it preferentially cultivated *F. cf. torulosum* within *B. tectorum*. As a result, relative abundance of this cultivar, *F. cf. torulosum*, increased when *P. acontioides* was present.

The living host plant often serves a primary filter of microbial diversity and relative abundance (Todd 1988; Bailey *et al.* 2005; Pan *et al.* 2008). However, as our data

indicates, particular members of the endophyte community also may influence the relative abundance of other members. When nematodes were present (i.e., Piney River and Nelson), *F. cf. torulosum* frequency increased. This relationship held even when another *B. tectorum* population was employed; the greenhouse experiments utilized seedlings from a *B. tectorum* population from the Clearwater River, ID. Likewise, our field data demonstrated that nematodes and *F. cf. torulosum* influenced endophytic richness, evenness and diversity. Not only were the number and relative abundance of other fungal phylotypes generally lower at the Piney River and Nelson sites relative to that of other populations lacking nematodes and *F. cf. torulosum* but also overall fungal diversity was reduced. In some ecological systems (e.g., where resources may be relatively unlimited), an increase in abundance of a particular species may not always affect overall diversity within that system. However, competition for limited resources can create a more closed system; a community may become “saturated” with a few species exerting dominance (Mouquet *et al.* 2003). The endophytic community within *B. tectorum* seemingly is such a system as evidenced by the reduction in diversity when a specific endophyte (i.e., *F. cf. torulosum*) is present. Because *in planta* diversity is apparently finite in *B. tectorum*, presence of nematodes and their cultivar, *F. cf. torulosum*, serve an important role in reducing endophytic diversity in *B. tectorum*.

Fungivorous nematodes feed on a variety of fungi (Giannakis & Sanders 1989; Ruess & Dighton 1996; Hasna *et al.* 2007), but many show a preference for particular fungi (Ruess *et al.* 2000; Hasna *et al.* 2007). Results from our field, greenhouse and laboratory

experiments provided evidence that *P. acontioides* prefer *F. cf. torulosum* as a food source despite its ability to consume other fungi. All field-collected plant tissue that yielded nematodes also yielded *F. cf. torulosum*. Furthermore, nematodes were absent from all other endophyte cultures (e.g., *Curvularia* sp. and *P. olsonii*) isolated from the

Piney River and Nelson populations, and from the other 61 populations. Likewise, in the three greenhouse experiments, nematodes were only re-isolated with the *F. cf. torulosum* cultures. In particular, Experiment 3 served as an *in planta* preference test where *Curvularia* sp., *P. olsonii* and B115 were co-inoculated with *F. cf. torulosum* into *B. tectorum*. Significantly, *P. acontioides* was re-isolated only in *F. cf. torulosum* cultures despite its ability to consume other Piney River-isolated endophytes (e.g., *Curvularia* sp.) if given no choice.

Suitability and preference assays further confirmed that *P. acontioides* prefers *F. cf. torulosum*. Although living nematode densities were equivalent for *F. cf. torulosum* and *Curvularia* sp. in the suitability assays, *P. acontioides* preferred *F. cf. torulosum* in the preference assay. Evidently, it will consume *Curvularia* sp. if provided no choice, but it preferred *F. cf. torulosum*.

Preference may also be based on avoidance of fungi toxic to the nematode (e.g., *P. olsonii*). Although grazing was initially evident in the *P. olsonii* cultures, nematode activity diminished over the two-week period with no surviving nematodes remaining at the end of the suitability assay. Likewise, in the preference assay (*P. olsonii*-*F. cf.*

torulosum), nematode activity was initially observed within the *P. olsonii* colony, although it was quite limited relative to that within the *F. cf. torulosum* colony. Previous research has demonstrated that nematodes may find a fungus initially favorable but once

toxic compounds are produced by the colony, the nematode is negatively affected (Mankau 1969; Ciancio 1995; Hasna *et al.* 2007).

The cultivation mechanism by which nematodes increase the relative abundance of *F. cf. torulosum* in *B. tectorum* was not definitively determined here. Previous research with plant parasitic nematodes has shown that nematodes graze more efficiently when a chemical attractant is detected (Perry 1996) and *F. cf. torulosum* may produce a signal that attracts *P. acntioides* and stimulates the nematode to cultivate it. Nematodes can promote fungal growth through hyphal grazing (Ingham *et al.* 1985) and fungi can compensate for this grazing pressure (Mikola & Setälä 1998). Positive correlations between fungivorous nematodes and fungal biomass have previously been documented (Ekschmitt & Griffiths 1998). Nematodes may carry bacteria or hyphal fragments and spores on their surfaces and within their digestive systems, dispersing the microbes as they migrate (Bird & McKay 1987; Fu *et al.* 2005). Fungus-dispersing nematodes can migrate through plant tissue (Neher 2010), and this alone may have allowed *F. cf. torulosum* to dominate the endophytic community of *B. tectorum*.

Our results indicate a mutualistic relationship between *P. acontoides* and *F. cf. torulosum* in that the former cultivates the latter for food. However, the association of each with the host plant appears commensalistic, inasmuch as their symbiosis benefits from the endophytic niche provided by the host without effect on the latter (i.e., no effect on biomass of field-collected and greenhouse plants). It is possible that an increase in relative abundance of *F. cf. torulosum* could cause secondary shifts in the abundance of parasitic or mutualistic members of the community that would result in effects on the host plant.

Bromus tectorum is an aggressive invader within western North America and it has the capacity to dominate landscapes upon introduction into new habitats (Stewart & Hull 1949; Mack 1981). Invasive species are more abundant in their invaded than native ranges (Broennimann *et al.* 2007), and interaction with novel endophytes may be one of the contributing factors to an invader's success (Baynes *et al.* in review). It is not clear, however, whether *P. acontoides* and *F. cf. torulosum* are novel symbionts for *B. tectorum*. The native range of *P. acontoides* is unknown, although many species of *Paraphelenchus* described to-date have been from Asia or Europe (Carta *et al.* 2011). Only one record of *P. acontoides* has been documented within the United States (Illinois) prior to the collection made from *B. tectorum* in Piney River. This sole discovery was from the rhizosphere of Kentucky bluegrass (*Agrostis stolonifera*) (Carta *et al.* 2011), another introduced grass to North America (USDA-ARS 2011).

The native range of *F. cf. torulosum* may be like that of the fungus that it most closely resembles, namely *F. torulosum*, a synonym of *F. sambucinum* var. *coeruleum* among others (Nirenberg 1995) and confirmed by Logrieco *et al.* (1995). *Fusarium torulosum* is reported primarily from in post-harvest studies of cereals including *Avena*, *Hordeum*, and

Triticum but has been reported from *Betula*, *Buxus*, *Humulus*, *Juniperus*, *Quercus*, soil, and *Solanum* and roots of various plants in temperate regions (Benyon *et al.* 2000; Desjardins *et al.* 2000; Kristensen *et al.* 2005; Logrieco *et al.* 1995; Nirenberg 1995) along with the human isolate in GenBank and as an endophyte of *Pennisetum clandestinum* in Australia (Ryley *et al.* 2007). This latter report suggests that *F. torulosum* is the cause of kikuyu poisoning of livestock due to the production of mycotoxins. Kristensen *et al.* (2005) state that *F. torulosum* and the related species *F. flocciferum* and *F. tricinctum*, are not known to produce trichothecenes but predict that both *F. torulosum* and *F. flocciferum* may possess the ability to produce monilioformin. They also cite Langseth *et al.* (1999) who found that “a single strain of *F. torulosum* has produced monilioformin in one out of two experiments” (p. 182). Ryley *et al.* (2007) cite literature in which a number of mycotoxins are produced by *F. torulosum*. The presence/absence of toxins produced by this fungus would certainly have an influence on the nematode, host plant, and/or competition as an endophyte. At present, we do not know whether the interaction of *P. acontioides* and *F. cf. torulosum* is restricted to *B. tectorum*.

Although associated with a number of plant hosts, the literature is unclear about whether *F. torulosum* causes plant diseases. Reasons for the absence of disease in *B. tectorum* in this study may include the following: 1) *B. tectorum* is resistant to this fungus-nematode association; 2) *F. cf. torulosum* is functionally distinct from *F. torulosum*; 3) *P.*

acantioides reduces the pathogenicity of *F. cf. torulosum* whereas other nematodes increase it.

Based on work with five *Fusarium* toxins against two plant-parasitic nematodes, antagonism or synergism could occur depending on relative concentrations in the rhizosphere (Ciancio 1995). At least one *Fusarium* toxin, beauveracin from *F. bulbicola*, was lethal to a fungal-feeding plant parasitic nematode, *Bursaphelenchus xylophilus* and to the bacterial-feeding *Caenorhabditis elegans* (Shimada *et al.* 2010).

An old monograph on grass endophytes did not list *Fusarium* as an endophyte (Bacon & Fahey 1994). However more recent studies on endophytic *Fusaria* and nematodes have appeared in the literature. Endophytic *F. oxysporum* suppressed the plant parasitic nematodes *Radopholus similis* (Vu *et al.* 2004), *Meloidogyne incognita* (Dabat & Sikora 2007), and *Pratylenchus goodeyi* (Mwaura *et al.* 2010). An endophytic non-pathogenic *Fusarium solani* suppressed plant parasitic root knot nematode in tomato. This stylet-bearing nematode promoted inner root colonization of the fungus (Siddiqi *et al.* 2002), so this distantly related stylet-bearing *Paraphelenchus* nematode's ability to promote *Fusarium* colonization here has some precedent.

Whereas host plant genotype and environmental conditions can often be determining factors in endophyte community structure (Todd 1988; Lacey & Magan 1991; Marin *et al.* 1998; Seghers *et al.* 2004; Bailey *et al.* 2005; Pan *et al.* 2008; Saunders & Kohn 2009), our research indicates that a fungivorous nematode and its fungal cultivar also can serve as a significant factor. We are unaware of any other research demonstrating a nematode's cultivation of one member of a fungal endophyte community. Future studies investigating the role of microfauna in cultivating specific endophytes *in planta* would be valuable to enhance our understanding of how endophyte communities are assembled.

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Table 3.1. Richness, evenness and diversity for 63 sampled *B. tectorum* populations. Highlighted rows indicate sites from which nematodes were isolated.

Population		Richness	Evenness	Diversity
Aztec	NM	11	0.913	0.861
Bandelier	NM	4	0.876	0.667
Benewah	ID	3	0.999	0.667
Berthoud Falls	CO	10	0.959	0.880
Big Meadow	ID	5	0.885	0.722
Bruno Gulch	CO	14	0.952	0.910
Camp Grizzly	ID	8	0.971	0.860
Colorado Springs	CO	5	0.847	0.698
Coyote Run	WA	10	0.978	0.891
Crow Hill	CO	8	0.918	0.831
Dillon Lake	CO	3	0.625	0.379
Dworshak	ID	6	0.915	0.782
East Suzie	NV	4	0.874	0.654
Elk Meadow	CO	6	0.808	0.717
Fall Creek	ID	18	0.918	0.913
Freeman	ID	5	0.894	0.734
Georgetown	CO	5	0.881	0.720
Granite Point	ID	3	0.960	0.640
Guanella Pass	CO	5	0.935	0.760
Hagenah	WA	10	0.912	0.858
Heyburn Lake	ID	9	0.973	0.875
Heyburn State Park	ID	7	0.941	0.815
Highway 95	ID	9	0.931	0.848
Highway 99	ID	11	0.961	0.889
Hubert Gulch	WA	6	0.993	0.828
Johnson	WA	10	0.959	0.881
Kendrick	ID	5	0.916	0.750
Kootenay	BC	6	0.885	0.765
Little Boulder	ID	2	1.000	0.500
Little Salmon River	ID	7	0.969	0.840
Lower Deadman	WA	5	0.946	0.765
Lower Moscow Mt.	ID	6	0.931	0.781
Mississippi	IL	4	0.409	0.259
Moose Creek	ID	5	0.963	0.776
Moscow Mt. South	ID	4	0.959	0.722
Nelson	BC	6	0.601	0.497
Nisqually John	ID	1	n/a	0.000
Parker Farm	ID	6	0.999	0.833
Pearl Button	IA	3	0.790	0.500
Piney River '09	CO	4	0.548	0.394
Piney River '10	CO	8	0.956	0.854
Potlatch	ID	7	0.935	0.819
Puffer Butte	WA	14	0.895	0.878
Rest Area	WA	21	0.947	0.934
Rock Creek	ID	5	0.928	0.750
Ryegrass	WA	3	0.870	0.571
San Ysidro	NM	7	0.250	0.815
Sandia Mt.	NM	12	0.827	0.800
Santa Fe	NM	8	0.901	0.821
SJNF	ID	8	0.974	0.860
Sky City	NM	8	0.801	0.734
Skyline	ID	4	0.882	0.680
Sperry Grade	ID	11	0.962	0.891
St. Maries	ID	0	0.000	1.000
Steptoe Canyon	WA	8	0.834	0.771
Strychnine	ID	6	0.936	0.790
Tent Rock	NM	4	0.726	0.533
UI Exp. Station	ID	8	0.885	0.793
Wawawai	WA	7	0.866	0.776
Weiser	ID	20	0.937	0.926
WERC	ID	7	0.946	0.827
White Bird	ID	11	0.954	0.886
Winchester	ID	6	0.796	0.671

Table 3.2. In field-collected *B. tectorum*, relative isolation frequency of *Fusarium* spp. was significantly higher when nematodes present (N+): n=63, chi-square=159.427, df=1, $p \leq 0.001$.

Field-collected <i>B. tectorum</i>				
	<i>Fusarium</i> <i>spp.</i>	Other endophytes	Total	Relative frequency
N+ plants	37	14	51	0.73
N- plants	107	906	1013	0.11
Total	144	920	1064	0.14

Table 3.3. In greenhouse experimental *B. tectorum*, relative re-isolation frequency of *F. cf. torulosum* was significantly higher when nematodes were present (N+). Experiment 1: chi-square=4.406, df=1, $p=0.036$, Experiment 2: chi-square=4.480, df=1, $p=0.034$, and Experiment 3: chi-square=7.922, df=1, $p=0.005$.

Greenhouse experimental <i>B. tectorum</i>				
	<i>F. cf.</i> <i>torulosum</i>	Other endophytes	Total	Relative frequency
Experiment 1				
<i>F. cf. torulosum</i> (N+)	11	18	29	0.38
<i>F. cf. torulosum</i> (N-)	4	25	29	0.14
Total	15	43	58	0.26
Experiment 2				
<i>F. cf. torulosum</i> (N+)	35	93	128	0.27
<i>F. cf. torulosum</i> (N-)	21	107	128	0.20
Total	56	200	256	0.22
Experiment 3				
<i>F. cf. torulosum</i> (N+)	23	109	132	0.17
<i>F. cf. torulosum</i> (N-)	7	112	119	0.06
Total	30	221	251	0.12

Table 3.4. In preference assays, three days post-inoculation with ~50 living nematodes in each plate, nematode abundance was significantly greater in *F. cf. torulosum* relative to *P. olsonii* (chi-square=12.875, df=3, p=0.005) and *Curvularia* sp. (chi-square=7.883, df=3, p=0.049) cultures.

	Nematodes Plate 1	Nematodes Plate 2	Nematodes Plate 3	Nematodes Plate 4	Total
<i>F. cf. torulosum</i>	42	61	56	103	262
<i>P. olsonii</i>	6	10	1	3	20
Total	48	71	57	106	282
<i>F. cf. torulosum</i>	46	51	60	44	201
<i>Curvularia</i> sp.	7	10	1	7	25
Total	53	61	61	51	226

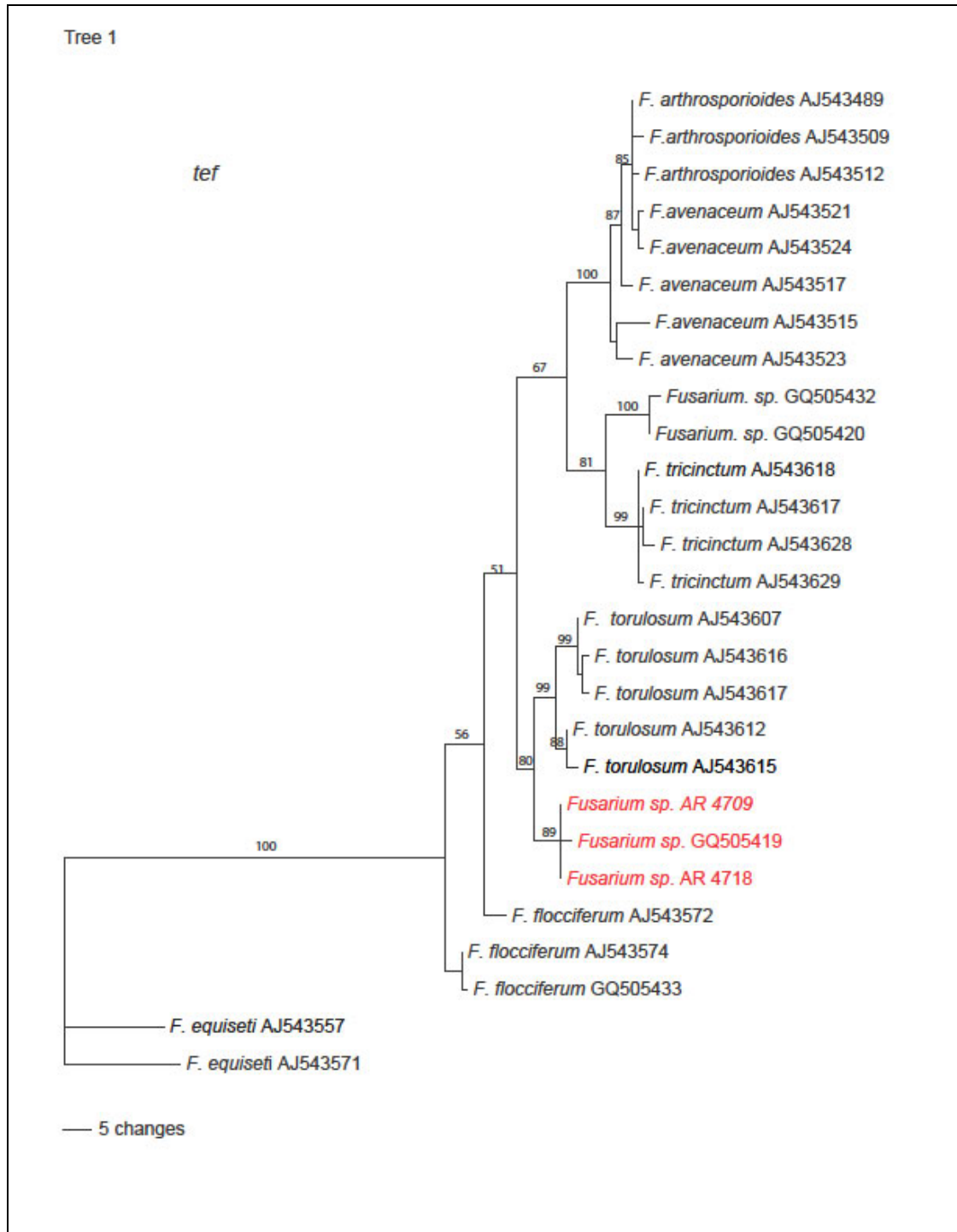


Figure 3.1. One of 3 most parsimonious trees tree showing position of *Fusarium* cf. *torulosum* (AR 4709: *tef* JN133577, ITS JN133579 and AR 4718: *tef* JN133578, ITS JN133580) within phylogeny of related *Fusarium* species. The tree was based on translation elongation factor 1 alpha (TEF) sequence data. Tree had 220 steps, consistency index 0.87, Homoplasy index 0.13. Numbers on the branches represent bootstrap values greater than 50% obtained via 1000 replicates. Two isolates of *F. equiseti* were used as outgroup taxa.

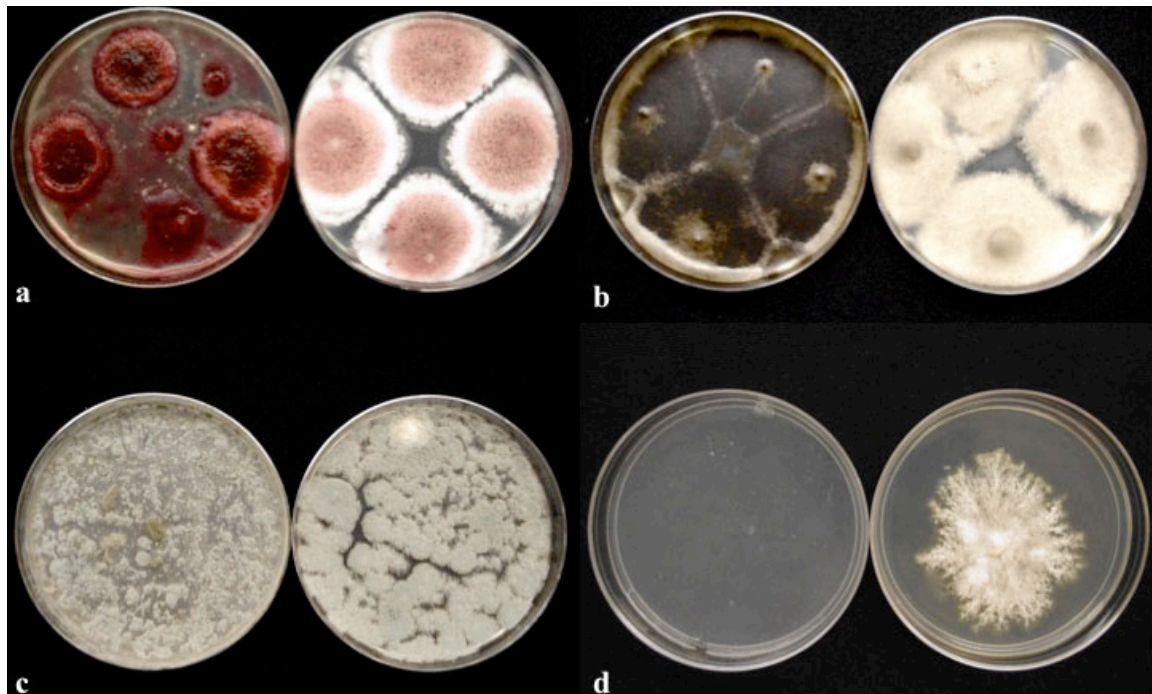


Figure 3.2. Growth suppression by *P. acontoides* in (a) *F. cf. torulosum*, (b) *Curvularia* sp., (c) *P. olsonii*, and (d) *A. bisporus* cultures two weeks post-inoculation with ~75 living nematodes. For each set, left image (N+) and right image (N-). Nematodes affected culture morphology of *P. olsonii* the least.

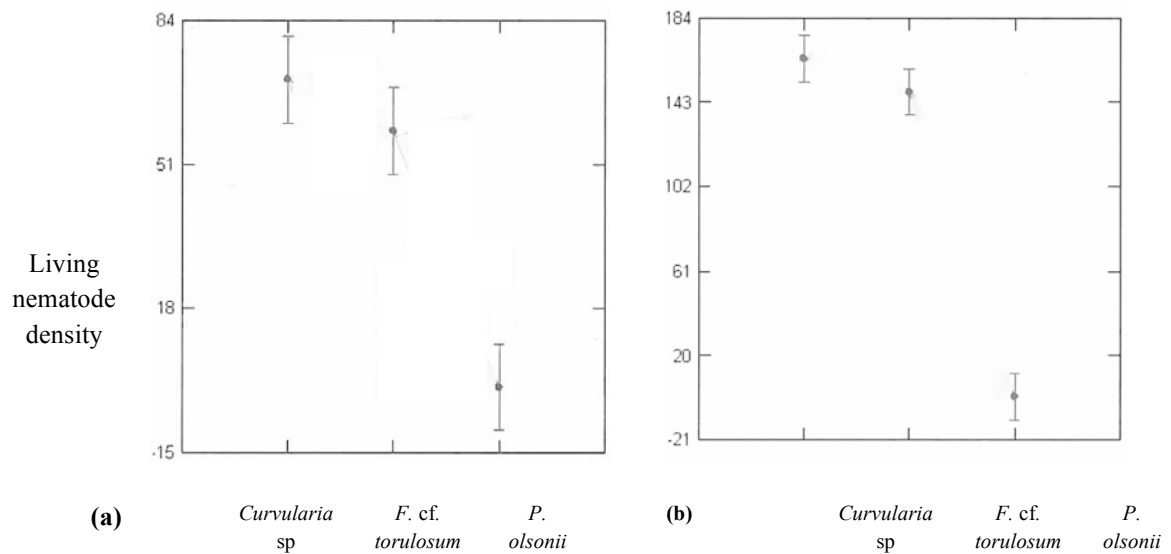


Figure 3.3. Suitability assays (plug (a) and solution (b) densities for living nematodes in *Curvularia* sp., *F. cf. torulosum* and *P. olsonii* cultures) two weeks post-inoculation with ~75 living nematodes. Because plug densities were relatively low, supplemental solution densities were analyzed. Analyses for plug and solution counts were conducted using ANOVA ($F=65.754$, $p \leq 0.001$ and $F=296.257$, $p \leq 0.001$, respectively). Results from a pairwise comparison (using Bonferroni test) indicate that *Curvularia* sp. and *F. cf. torulosum* are significantly more suitable for nematode survival and reproduction relative to *P. olsonii*. Significant differences in number of living nematodes were observed between *Curvularia* sp. and *P. olsonii* plug ($p \leq 0.001$) and solution ($p \leq 0.001$) densities and between *F. cf. torulosum* and *P. olsonii* plug ($p \leq 0.001$) and solution ($p \leq 0.001$) densities. No significant differences were detected between *F. cf. torulosum* and *Curvularia* sp. plug ($p=0.289$) and solution ($p=0.138$) densities.