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Using direct amplification and next-generation sequencing technology to explore foliar endophyte communities in experimentally inoculated western white pines

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ABSTRACT

Fungal endophytes can influence survivability and disease severity of trees. Here we characterized the endophyte community in *Pinus monticola* (western white pine), an important species in the northwest USA, largely decimated by pathogenic fungi. We also assessed the ability to successfully inoculate seedlings with desirable endophytes, with the long-term goal of providing a protective microbiome and added defense from pathogens. *P. monticola* seedlings were inoculated in the field with potential pathogen antagonists and fungi isolated from healthy mature trees. Following inoculations direct amplification and next generation sequencing were used to characterize fungal endophyte communities, and explore interspecific competition, diversity, and co-occurrence patterns in needle tissues. Negative co-occurrence patterns between inoculated fungi and potential pathogens, as well as many other species, suggest early competitive interactions. Our study explores early endophyte community assemblage and shows that fungal inoculations may influence tree growth.

1. Introduction

Endophytes, defined here as microorganisms that inhabit healthy, asymptomatic plant tissues, represent a species-rich body of organisms with diverse ecological functions (Petrini, 1991; Wilson, 1995). Endophytes inhabit all plant species studied (Arnold et al., 2000) and can enhance plant resistance to herbivory (Cheplick and Clay, 1988, Zhang et al., 2011), increase plant growth rate and drought resistance (Rodriguez et al., 2009), enhance host resistance to pathogens (Arnold et al., 2003; Ganley et al., 2008) and increase invasibility of exotic plant species (Aschehoug et al., 2012; Rout et al., 2013). Foliar fungal endophytes that colonize above ground plant tissue (Class 3 endophytes) are especially diverse (Arnold et al., 2003; Rodriguez et al., 2009). Class 3 fungal endophytes form variable, localized infections through horizontal transmission of environmental propagules (Rodriguez et al., 2009). They show higher abundances in evergreens than in deciduous plants (Rodriguez et al., 2009; Lau et al., 2013) as they accumulate over a longer period of time. However, far less is known about the functional role of fungal endophyte communities in evergreen systems, in part, due to long life expectancies of these hosts and the large sampling effort necessary to characterize the hyperdiverse fungal community present within each individual organism. Still, studies have shown that Class 3 endophytes play an important ecological role within these systems. For example, endophytes in conifer systems produce toxins that impede development of herbivorous insect larvae (Clark et al., 1989; Miller et al., 2002), improve growth of pine seedlings *in vitro* (Pohjanen et al., 2014), can extend survival time of trees infected with pathogens (Ganley et al., 2008) and modify the severity of pathogen infection (Berube et al., 1998; Ridout and Newcombe, 2015).

The spatial and temporal dynamics of endophyte community assembly and species interactions within hosts are relatively unknown. The effects of established species on the colonization probability of subsequent fungi (priority effects), along with variation in the time of arrival of different species, influence

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fungal community assembly (Fukami et al., 2010). Many fungal endophytes produce antimicrobial metabolites that can inhibit the colonization of secondary species (Soman et al., 1999; Yu et al., 2010; Wang et al., 2013), which may provide protection and increase the life span of their host plant (Berube et al., 1998; Ganley et al., 2008). The factors that shape initial fungal assemblages in conifers also play a role in subsequent ecosystem functions. Endophyte species present in living tissue can act as pioneer decomposers when needles senesce (Müller et al., 2001), and influence the rate of nutrient cycling (Fukami et al., 2010). However, previous research has concentrated on culturable endophytes and has not included the vast number of species that may reside within a host species, but remain unobserved due to biases inherent to culture-based assessments. Relying exclusively on culture-based methods has lead to incorrect estimates of community composition and richness as some species go undetected due to differences in growth rates of endophytic fungi and the inability of some fungi to grow in culture (Hyde and Soytong, 2008). Without a more complete understanding of the unculturable endophyte community present within trees, conclusions about community assemblage, species richness, and the ecological consequences of endophytic community structure remain elusive.

Next generation sequencing (NGS) methods enable us to gain a more comprehensive understanding of fungal richness than allowed through culture-based methods alone. Recent NGS technology has enabled researchers to perform large-scale metagenomic studies on almost any system, and has been used extensively to study the microbial diversity of soils (Delmont et al., 2012), fungal root symbionts (Lekberg et al., 2011) and foliar endophytes of deciduous trees (Jumpponen and Jones, 2009). In the current study, NGS was used to explore the establishment of experimentally inoculated endophyte communities in 60 western white pine (*Pinus monticola*) seedlings. Western white pines have declined to less than 10% of their natural habitat in the last century, largely due to damage from fungal pathogens such as blister rust (*Cronartium ribicola*) (Fins et al., 2002), which can cause up to 100% mortality in some populations (Sniezko et al., 2014). Foliar endophytes co-inhabit hosts along with pathogens, such as white pine blister rust, and have ample opportunity to influence disease. Here, white pine seedlings in the field were inoculated with fungal endophytes known to inhabit healthy, mature, nearby trees (Larkin et al., 2012). NGS was employed to characterize early community assemblage, priority effects and persistence of inoculated fungi. Inoculant treatments were chosen based on their potential for pathogen antagonism (Ganley et al., 2008; Wang et al., 2013), and seedling growth was used as a response variable to assess seedling health over 2 yr. To our knowledge, this is the first attempt to use NGS to explore the effects of experimentally inoculated endophyte communities with any conifer species. With a broader, more inclusive perspective on fungal endophyte biodiversity, we will better understand the interspecific interactions that drive community assemblage and subsequent ecological processes.

2. Materials and methods

2.1. Study site description/field sampling

In spring of 2011 we obtained approximately 400 F2 generation 'Bingham' hybrid *P. monticola* seedlings from the Franklin H. Pitkin Forest Nursery in Moscow, Idaho, 60 of which were randomly selected for this experiment. Before planting, we performed a soft screening for fungal endophyte colonization that may have occurred inside the controlled growth chamber. A small subset of needles was collected from 10 randomly selected seedlings. All needles were washed within 48 hr of collection and surface-sterilized in 70% ethanol for 1 min, 6% sodium hypochlorite for 5 min, and then washed again in 70% ethanol for 1 min (Larkin et al., 2012). Surface-sterilized needles were sectioned and placed on 90 mm plates containing 2% malt extract agar (MEA). To confirm efficiency of the sterilization procedure, a subset of surface-sterilized needles was imprinted onto MEA plates and monitored for fungal growth. No fungal growth from the needles or on imprinted control plates was seen after more than 30 d incubation. Based on these observations we concluded that endophyte colonization of seedlings was, at most, low at time of planting due to limited exposure to environmental propagules. Low infection rates in greenhouse-propagated seedlings have been reported before (Arnold and Herre, 2003; <1% of foliar tissue), and it is possible that our sampling efforts were simply not robust enough to capture such low levels of colonization. The 2 yr old seedlings were planted on 80 ha at the base of the Swan Range on the east side of the Swan Valley in Western Montana (centered at 47.523°N −113.671°W). The study area receives 610–710 mm of precipitation per year. Minimal western white pine regeneration has occurred here in recent years, as browsing pressure, exotic grasses, soil compaction, lack of disturbance and blister rust disease now limit natural recruitment of this species. To protect from deer-browse, each tree was immediately enclosed in plastic fencing. Seedlings were divided into three plots ranging from 0.4 to 0.8 ha each. All three plots had similar plant species composition and were within 0.6 km of each other.

2.2. Endophyte inoculation

Each treatment consisted of an experimental community of endophytes previously isolated from surface-sterilized needles of *P. monticola* trees in the northwestern United States (Larkin et al., 2012; Ganley and Newcombe, 2006, Table 1). Treatment one (established) included endophytic species isolated from a single healthy, mature western white pine tree found at the study site. Treatment two (pezizales) contained fungal isolates in the order Pezizales, thought to help mediate resistance against fungal pathogens (Ganley et al., 2008). Treatment three (antagonists) consisted of potential antagonists of fungal pathogens (Ganley et al., 2008) and those that produced visible secondary metabolites in culture. Treatment four contained sterile water (control). Briefly, endophyte suspensions were prepared in sterile malt

Table 1
Table of inoculum groups. Lowest available taxonomic designation is given.

Treatment	Taxa	Accession numbers
1. Established	*Davidiella, Mycosphaerella*, Helotiaciae, Rhytismataceae, Sordariomycete, *Pachnocybe*	HQ845749, HQ845750, HQ540686, HQ535880, HQ535867, HQ823754, JF705944
2. Pezizales	Pezizales, *Chromelosporium, Pseudoplectania*, Pezizomycotina	HQ602679.1, HQ602669, HQ602673
3. Antagonists	*Davidiella, Mycosphaerella, Epicoccum, Nemania, Lophodermium, Coniochaeta, Xylaria, Phoma*	HQ602657, HQ540677, HQ540678, HQ602660, HQ823748, HQ535945, JF749176, JF764806.1, HQ823755, HQ823758, HQ823761,HQ823762, JF705939
4. Control	NA	NA