

Common foliar fungi of *Populus trichocarpa* modify *Melampsora* rust disease severity

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Summary

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- Nonpathogenic foliar fungi (i.e. endophytes and epiphytes) can modify plant disease severity in controlled experiments. However, experiments have not been combined with ecological studies in wild plant pathosystems to determine whether disease-modifying fungi are common enough to be ecologically important.
- We used culture-based methods and DNA sequencing to characterize the abundance and distribution of foliar fungi of *Populus trichocarpa* in wild populations across its native range (Pacific Northwest, USA). We conducted complementary, manipulative experiments to test how foliar fungi commonly isolated from those populations influence the severity of *Melampsora* leaf rust disease. Finally, we examined correlative relationships between the abundance of disease-modifying foliar fungi and disease severity in wild trees.
- A taxonomically and geographically diverse group of common foliar fungi significantly modified disease severity in experiments, either increasing or decreasing disease severity. Spatial patterns in the abundance of some of these foliar fungi were significantly correlated (in predicted directions) with disease severity in wild trees.
- Our study reveals that disease modification is an ecological function shared by common foliar fungal symbionts of *P. trichocarpa*. This finding raises new questions about plant disease ecology and plant biodiversity, and has applied potential for disease management.

Introduction

Plant disease plays a key role in the ecology and evolution of plants (Thrall *et al.*, 2007), and in the efficiency of plant production for food and bioenergy (Stokstad, 2007; Rubin, 2008). Understanding the factors controlling disease severity is therefore fundamental to both science and society. Plant genetic resistance, pathogen virulence, and the environment have long been known to collectively determine disease severity (McNew, 1960). A potentially fundamental, though poorly understood, factor also influencing plant disease severity is the community of non-pathogenic microbes living in and on the leaves of most of the world's land plants (Rodríguez *et al.*, 2009; Vorholt, 2012).

Microfungi establish locally in and on leaves and form diverse communities (Arnold *et al.*, 2002). These fungi are horizontally transmitted, so they must renew their association with a deciduous host each year (Stone *et al.*, 2000). Although foliar fungal communities can vary among hosts and along environmental gradients (U'Ren *et al.*, 2012; Zimmerman & Vitousek, 2012), several taxa are common in temperate, angiosperm hosts: for example, species of *Alternaria*, *Cladosporium*, *Epicoccum* and *Trichoderma* (Vázquez de Aldana *et al.*, 2013). It is often assumed that these and other foliar fungal taxa are latent saprotrophs or secondary pathogens (Ellis, 1972; Funk & Centre, 1985; Schulz *et al.*, 1999). While

this is true in some cases (Müller *et al.*, 2001), the ecological roles of some of the most common foliar fungi are not known.

Some foliar fungi have been shown to modify disease severity in their host plants by interacting directly with pathogens (e.g. competition and mycoparasitism; Sharma & Heather, 1983; Woo *et al.*, 2006) and/or by modulating plant defense responses (Mejía *et al.*, 2014). Through such interactions, foliar fungi can either decrease or increase plant disease severity, acting as 'pathogen antagonists' or 'pathogen facilitators', respectively (Arnold *et al.*, 2003; Saunders & Kohn, 2008; Kurose *et al.*, 2012; Busby *et al.*, 2013; Raghavendra & Newcombe, 2013; Ridout & Newcombe, 2015). Pathogen antagonists are similar to more widely recognized plant defensive mutualists, such as ants (Davidson & McKey, 1993) and grass endophytes (Clay, 1988). Studies evaluating spatial and temporal variation in defensive mutualisms are needed to improve our understanding of their ecology and evolution (Bronstein, 2009; Clay, 2014).

Pathogen antagonists have long been known in agricultural systems where they are used as biocontrols (Larkin & Fravel, 1998; Lee *et al.*, 2009); likewise, pathogen facilitators have been used to increase the severity of diseases of invasive plants (Morin *et al.*, 1993). By contrast, our understanding of disease modification in wild plant pathosystems is limited because the foliar fungal taxa studied have been undetermined, relatively unusual

(Busby *et al.*, 2013; Adame-Álvarez *et al.*, 2014), or associated primarily with temperate gymnosperms (Ridout & Newcombe, 2015). To our knowledge, no previous study has combined experimental tests of disease modification with ecological studies to determine the abundance and distribution of pathogen antagonists and pathogen facilitators in a wild plant pathosystem.

Here, we tested the hypothesis that common foliar fungi of the model tree *Populus trichocarpa* influence the severity of disease caused by the tree's major rust pathogen, *Melampsora × columbiana*. We did this by coupling an ecological study of foliar fungal communities in wild tree populations with *in planta* experimental manipulations using fungal isolates collected from those same populations. First, we used traditional culture-based approaches to identify and isolate common taxa for experimentation. Second, we conducted inoculation experiments to test whether common fungal taxa modify rust disease severity. Third, we conducted a molecular field survey to evaluate the relationship between the abundance and distribution of experimentally identified disease-modifying fungi and rust disease severity in wild trees. Finally, we conducted an independent analysis of the

molecular data set to identify fungi in the community most strongly correlated with rust disease severity, that is, candidate disease-modifying fungi. Together, our field-based, experimental and modeling results support the hypothesis that disease modification is an ecological function shared among common foliar fungi of *P. trichocarpa*.

Materials and Methods

Culture-based characterization of foliar fungal communities

In October 2012, we used culture-based methods to characterize foliar fungal communities of wild *Populus trichocarpa* Torr. & A. Gray from 10 wild tree populations located across the core of the tree's geographic range in the Pacific northwest of the USA (Fig. 1a,b; Supporting Information Table S1). The range is bisected by the Cascade Mountains, which create a strong east–west moisture gradient (Dunlap & Stettler, 1996, 2001). Five of our study populations are located on the moist, western side of the Cascade Mountains; the other five are located on the dry, eastern

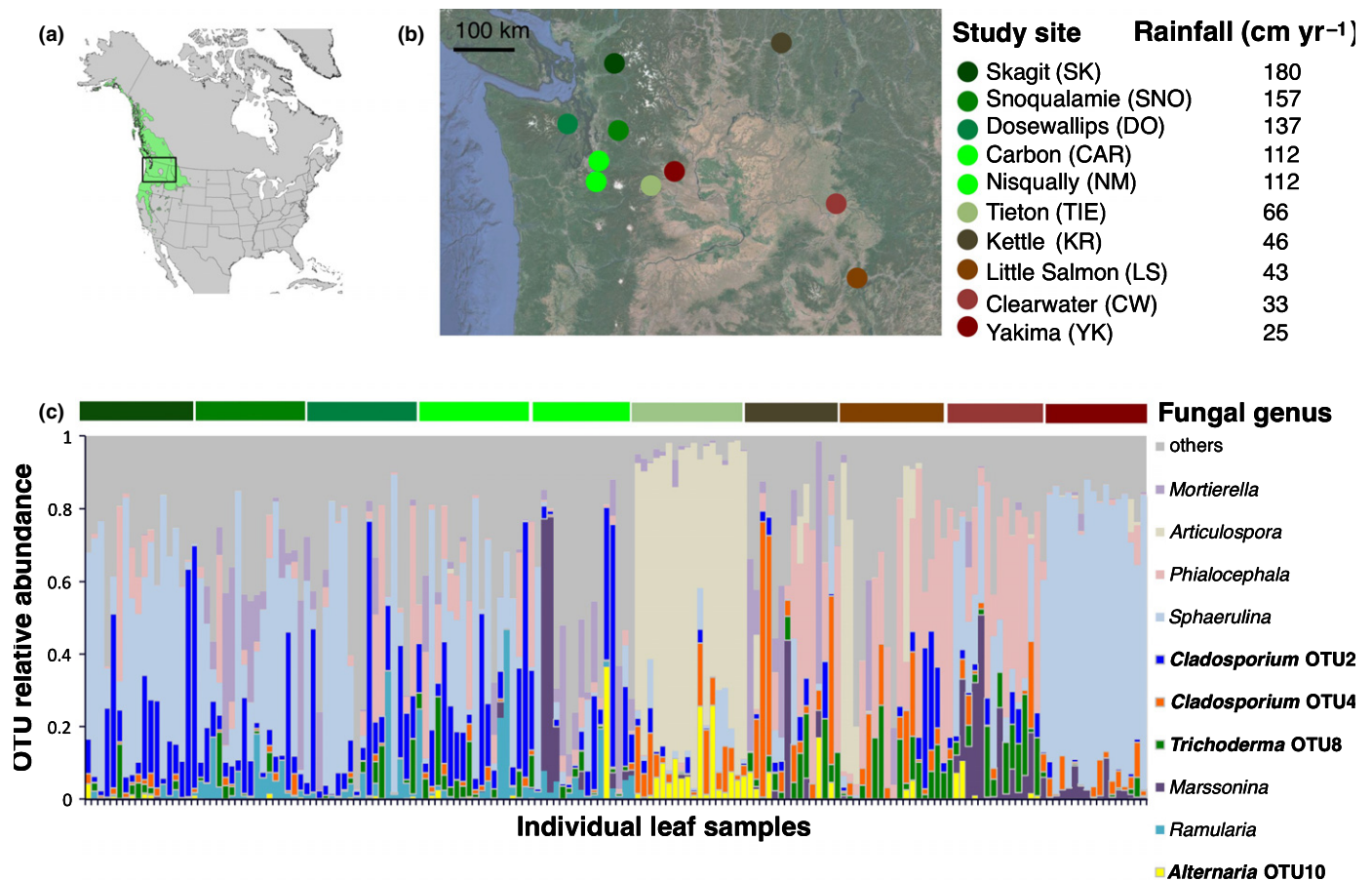


Fig. 1 Foliar fungal communities across the study area are compositionally variable, yet *Cladosporium* (operational taxonomic unit 2 (OTU2) and OTU4), *Alternaria* (OTU10) and *Trichoderma* (OTU8) are common in many sites. The geographic range of *Populus trichocarpa* is shown with the study area boxed (a, from USGS Geosciences and Environmental Change Science Center: Digital Representations of Tree Species Range Maps from 'Atlas of United States Trees' by Elbert L. Little, Jr (US Geological Survey, 1999) and study site locations color-coded by the rainfall gradient (b). OTU relative abundance within leaves is shown for taxa with > 1% mean relative abundance across all sites; less abundant taxa are shown collectively in gray (c). Leaves are grouped along the x-axis by study site, with color-coded site label located above the bars. The four genera matching to experimental isolates are listed by their OTU number.

side. Each tree population is associated with an independent river valley, with all eastern rivers draining into the Columbia River and all western rivers draining into Puget Sound.

In each population we collected nine leaves per tree (targeting leaf positions 3–5 on different lower canopy branches) from six haphazardly selected trees. We surface-sterilized leaves in a laminar flow hood by soaking in a 1% sodium hypochlorite (NaOCl) solution for 2 min followed by two rinses (1 min each) in sterile deionized water (Raghavendra & Newcombe, 2013). While this protocol is designed to isolate endophytes (i.e. fungi living within leaves), it could potentially capture fungi on the leaf surface that survive surface-sterilization (Schulz *et al.*, 1993). We confirmed the efficacy of our surface sterilization method by imprinting a subset of surface-sterilized leaves on both potato dextrose agar (PDA) and paper towels, removing the leaves, then monitoring the media for fungal growth.

Six leaves per tree were placed on moistened, sterile paper towels within sealed, sterile plastic bags. The three remaining leaves were cut to fit Petri plates containing PDA medium. Approximately 2 months later, fungi emerging from leaves on sterile paper were systematically surveyed with the aid of dissecting and compound light microscopes. We used morphological features to identify and record the presence of individual fungal genera emerging from leaf replicates. This community data set was used to evaluate the frequency of fungal genera within leaves, and specifically to identify common taxa for experimental manipulations.

We collected fungal isolates from both paper and PDA leaf cultures and maintained them in pure culture for identification based on DNA sequence data, and for experimental use. For each fungal genus encountered in our surveys, we attempted to collect replicate isolates from several tree populations. However, exhaustive isolation of all taxa encountered in our surveys was not feasible given the diversity encountered.

We sequenced the internal transcribed spacer (ITS) region of each fungal isolate's nuclear ribosomal DNA using ITS1F and ITS4 primers to determine taxonomic identity (White *et al.*, 1990; Gardes & Bruns, 1993). DNA was extracted from cultured fungal material using Life Technologies' PrepMan[®] Ultra Sample Preparation Reagent following OEM instructions (Applied Biosystems[®], Foster City, CA, USA). Our PCR reaction volume was 20 µl and included 4 µl of genomic DNA; the cycle sequencing reaction volume was 10 µl and included 1 µl of the PCR product. Forward and reverse sequencing was carried out on an ABI PRISM 3730 Genetic Analyzer. Regions with more than a 5% chance of an error per base were trimmed in GENEIOUS 6.1.7 (<http://www.geneious.com>, Kears *et al.*, 2012). Each isolate's forward and reverse reads were paired to generate a consensus sequence, which was used in BLAST queries to determine taxonomic identity (Altschul *et al.*, 1990). All sequences have been deposited in the National Center for Biotechnology Information (NCBI) GenBank accession nos. KM520336–KM520368 (Table S2).

Experimental fungal isolates

We selected fungal isolates that occurred frequently in the culture-based field survey for controlled, *in planta* experimental

manipulations. In total, we tested 34 experimental isolates representing approximately three isolates from each of the 10 study sites, and encompassing seven fungal genera that are among the most common in both molecular and culture-based studies of foliar fungi (Vázquez de Aldana *et al.*, 2013) and air spora (Adams *et al.*, 2013): *Alternaria*, *Arthrinium*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Penicillium*, and *Trichoderma* (Table S2). A single *Trichoderma* isolate from the Clearwater site was collected before the 2012 collection (Raghavendra & Newcombe, 2013).

None of the selected fungi are known pathogens of *P. trichocarpa*, which have been studied extensively (Newcombe, 1996). Moreover, we inoculated *P. trichocarpa* leaves with each isolate individually and thereafter successfully reisolated each isolate from asymptomatic surface-sterilized leaves. Thus, each isolate is endophytic in *P. trichocarpa* according to standard definitions of endophytism, for example, '...infections are inconspicuous, the infected host tissues are at least transiently symptomless, and the microbial colonization can be demonstrated to be internal...' (Stone *et al.*, 2000).

Isolates were maintained as pure cultures on Petri plates containing PDA before experiments. We prepared fungal inoculum for experiments in a laminar flow hood by: (1) flooding a 2- to 3-wk-old plate(s) with sterile deionized water; (2) passing a sterile glass rod along the surface to dislodge spores; (3) suspending spores in sterile deionized water, and (4) standardizing each inoculum solution to 1 million spores ml⁻¹ by using a hemocytometer to estimate spore concentration. A few isolates did not sporulate profusely in culture (e.g. *Alternaria*), so we did not reach the desired inoculum spore concentration. For these isolates, we scraped mycelium from cultures, homogenized mycelium using a Tissue Tearor[™] (Biospec, Bartlesville, OK, USA), and included the mycelium along with spores in the inoculum solution for those particular isolates. We confirmed the viability of each inoculum by plating on PDA.

Experimental rust isolate

Our experimental isolate of *Melampsora × columbiana* was collected from the aecia of infected leaves of the pathogen's alternate host, *Pseudotsuga menziesii*, at the Clearwater study site. The isolate was maintained on glasshouse plants by serial inoculations of asexually produced urediniospores onto fresh leaves. Our method for inoculating leaves with rust follows Newcombe (1998).

Plant material

We utilized replicate cuttings of a single genotype of *P. trichocarpa* for experiments. This genotype is a wild tree in the Clearwater population, the same site where we collected our experimental rust isolate. Cuttings with dormant buds were collected in winter 2013. We planted two-bud cuttings (c. 10 cm in length) in 1-gallon pots containing Sunshine Mix 1[®] (SunGro Horticulture Vancouver, Canada) and a tablespoon of Osmocote[®] (Scotts, Morrisville, NC, USA). Plants were c. 4–6 months old at the time of the experiment.

Before and during experiments, all plants were watered daily and fertilized as needed with a 20-20-20 NPK mix supplemented occasionally with Alaska Fish Fertilizer® (Central Garden and Pet, Walnut Creek, CA, USA) with 5-1-1 NPK. Supplemental lighting in the glasshouse was activated when light intensities fell below 300 W m^{-2} from 06:00 to 21:00 h. Temperature was maintained at $18^\circ\text{C} : 15^\circ\text{C}$, day : night.

In planta inoculation experiments

Given the large number of plants required to conduct this experiment ($n=155$), we ran two separate experiments: one including 10 *Epicoccum* isolates and a control group ($n=4$ replicate cuttings per group), and the second including the remaining 24 isolates and a control group ($n=5$ replicate cuttings per group). In both experiments, we inoculated leaves of a treatment group with a single fungal isolate (or sterile water for control). We inoculated three to 10 leaves per plant (leaf positions 3–5; multiple ramets on some plants enabled a larger sample size). On each plant, we inoculated a single, additional leaf with the fungus to confirm that it was nonpathogenic under the experimental conditions.

We inoculated leaves by applying inoculum on the abaxial surface of misted leaves using cotton swabs. Plants were kept overnight in an enclosed chamber to maintain moisture on the leaf surface. Approximately 24 h later, we inoculated rust onto the same leaves (except the additional leaf used to confirm that the fungus was nonpathogenic). We used cotton swabs to evenly spread urediniospores onto the abaxial surfaces of the selected, misted leaves (Newcombe, 1998). Again, we kept plants overnight in an enclosed chamber. Two weeks later, we detached and photographed rust-inoculated leaves. We used automatic thresholding in ASSESS 2.0 (American Phytopathological Society, St Paul, MN, USA) to quantify rust disease severity as the percentage of the abaxial leaf surface covered by uredinia.

We used restricted maximum likelihood (REML) based linear mixed-effects models to compare rust disease severity between each fungal treatment group and the control group. Because foliar fungi infect leaves locally (Stone *et al.*, 2000) and have local effects on disease severity (Arnold *et al.*, 2003; Raghavendra & Newcombe, 2013), we treated each individual leaf as an experimental replicate. Our models included foliar fungus as a fixed effect, and cutting replicate as a random effect to account for local environmental effects on rust disease severity (e.g. variation within the glasshouse and inoculation chamber). We used an *F*-test to evaluate the statistical significance of the foliar fungus factor, and a restricted likelihood ratio test to evaluate the significance of the cutting replicate factor. We additionally calculated marginal and conditional R^2 values which describe the proportion of variance explained by the fixed factor, and both fixed and random factors, respectively (Nakagawa & Schielzeth, 2013). We used planned contrasts to evaluate differences between each foliar fungus treatment group and the control group (i.e. not all pair-wise comparisons were made). With planned contrasts, correcting for multiple hypothesis tests is debated (Saville, 1990), so we

report *P* values both with and without a correction. For these analyses we used the *lmer*, *step* and *glht* functions in the *lme4*, *lmerTEST* and *multcomp* packages in R version 3.0.2 (R Core Team 2013).

Molecular field survey of foliar fungal communities

We conducted a molecular field survey to evaluate the relationship between the abundance and distribution of experimentally identified disease-modifying fungi and rust disease severity in wild trees. In October 2013, we sampled three leaves per tree (targeting leaf positions 3–5 on three different lower canopy branches) from six *P. trichocarpa* trees in the same 10 river valley populations described above ($n=60$ trees; $n=180$ leaves). Leaves were transported in coolers, and processed within 24 h. Rust disease severity was measured on each leaf by counting the number of uredinia in a 1-cm^2 panel dropped from 10 cm above five locations on the leaf (the tip and two spots on each side of the midrib) and taking the mean (Raghavendra & Newcombe, 2013). Leaf surface sterilization protocols were the same as those described above. Leaves were air-dried in a laminar flow hood, frozen, and lyophilized.

We used Qiagen DNeasy 96 Plant Kits (Qiagen, Valencia, CA, USA) and standard protocols for DNA extractions on freeze-dried leaf material. Approximately 10 mg of dried, crushed material per leaf was used in each DNA extraction. We used an ethanol precipitation on all extracted DNA to remove impurities. DNA was then re-suspended in $200 \mu\text{l}$ of elution buffer.

We used a modified version of the primer set ITS1F and ITS2 for parallel sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) (Smith & Peay, 2014). Each $25\text{-}\mu\text{l}$ PCR reaction included: $6 \mu\text{l}$ of genomic DNA, $0.5 \mu\text{l}$ of each $10 \mu\text{M}$ primer, $5 \mu\text{l}$ of OneTaq Standard Reaction Buffer (New England BioLabs, Ipswich, MA, USA), $0.5 \mu\text{l}$ of 10 mM dNTPs (New England BioLabs), and 0.63 units of OneTaq Hot Start DNA polymerase (New England Biolabs) to minimize non-specific amplification and primer dimerization. PCR conditions were: denaturation at 94°C for 1 min; 35 amplification cycles of 30 s at 94°C , 30 s at 52°C and 30 s at 68°C ; followed by a 7-min final extension at 68°C (30 cycles was found to be insufficient for amplification because of low starting DNA template). We visualized PCR products using gel electrophoresis, and cleaned samples using the Agencourt Ampure XP kit (Beckman Coulter, Brea, CA, USA).

We quantified PCR products using the Qubit hs-DS-DNA kit (Invitrogen, Carlsbad, CA, USA) on a Tecan Infinite F200 Pro plate reader (Tecan, Morrisville, NC, USA) (285 nm excitation and 530 nm emission). PCR products were then pooled at equimolar concentrations. The library was processed at the Stanford Functional Genomics Facility for 250-bp paired-end sequencing on an Illumina MiSeq. A spike of 30% PhiX was added to the library to increase sample heterogeneity. Three samples yielded no sequence data; raw sequence data for the remaining 177 samples are deposited in NCBI's Short Read Archive (accession no. SRP064132, <http://www.ncbi.nlm.nih.gov/books/NBK47529/>).

We used both QIIME (Caporaso *et al.*, 2010) and UPARSE (Edgar, 2013) to process the Illumina data set. The prefiltered forward and reverse reads were 238 bp long. The distal priming site was trimmed using CUTADAPT (Martin, 2011); low-quality regions were then trimmed using TRIMMOMATIC (Lohse *et al.*, 2012). Forward and reverse reads were paired with USEARCH v.7.0.1001, using a minimum Phred score sequence cut-off threshold of 3 and a minimum sequence length of 75 bp. Paired reads were discarded if they contained > 0.25 expected errors.

High-quality sequences were grouped into operational taxonomic units (OTUs) in USEARCH using UPARSE-OTU and UPARSE-OTUref algorithms at 97% similarity. This algorithm included both reference-based and *de novo* chimera detection. OTU taxonomy was assigned with the BLAST algorithm in QIIME which utilizes the UNITE fungal ITS database, then checked manually for the 500 most abundant OTUs using BLAST searches against the NCBI GenBank. In total, we identified 968 OTUs after removing singletons and obvious laboratory contaminants. We removed the *Melampsora* OTU from the data set because a primer mismatch with ITS2 prevented its nonbiased amplification.

We used ITS sequence data to match OTUs from the molecular data set to our experimental isolates, considering a match to occur at > 97% sequence similarity. Four of the most common (top 15 of 968) OTUs – *Cladosporium* OTU2, *Trichoderma* OTU8, *Alternaria* OTU10 and *Cladosporium* OTU4 – matched to experimental isolates that either significantly decreased rust disease severity (OTU2 and OTU8) or showed a trend toward disease facilitation (OTU10 and OTU4). Given the abundance of these particular OTUs in the molecular data set, we were able to reliably model the relationship between each OTU's abundance and disease severity. We excluded sparsely represented OTUs from this analysis (i.e. mean relative abundance < 1%), and OTUs that were < 97% similar to experimental isolates (i.e. *Penicillium* and *Chaetomium*).

We evaluated rust disease severity models for the four tree populations where rust was recorded on the majority of trees (Table S1), and excluded samples with exceptionally few sequences from our analyses (< 1000 total; $n = 7$). First, we transformed absolute OTU abundance (number of ITS sequences) to relative OTU abundance by dividing the number of ITS sequences for each OTU in a leaf by the total number of ITS sequences in that leaf. Second, we visually inspected the relationship between OTU relative abundance and rust disease severity to select appropriate functions. For OTU8 and OTU2, we fitted exponential decay functions for rust disease severity, $f(X) = a \times \exp(-s \times X)$, where X was OTU relative abundance and a and s were estimated parameters. For OTU10 and OTU4, we fitted inverse exponential functions for rust disease severity, $f(X) = a \times (1 - \exp(-s \times X))$, where X was OTU relative abundance and a and s were estimated parameters.

To evaluate model fits, we compared the OTU model (model 0) to three additional models: model 1, a null model in which predicted disease severity was equal to mean observed rust disease severity; model 2, a site-null model in which predicted disease severity was equal to mean observed disease severity within each

site (i.e. a varied as a function of site); and model 3, a site model including the OTU in which a also varied as a function of site. Incorporating the site effect into models enabled us to disentangle potential confounding between OTU and environmental effects. A significant effect of OTU relative abundance on disease severity would be supported if either or both the OTU-only model (model 0) and the OTU-site model (model 3) were more parsimonious than the null model (model 1) and the site-null model (model 2). For all models, we solved for maximum likelihood parameter values using simulated annealing (*likelihood* package in R) (Goffe *et al.*, 1994). We calculated asymptotic two-unit support intervals to evaluate uncertainty in the parameter estimates, and used Akaike information criterion (AIC; corrected for small sample size) to compare and rank models. The model with the minimum AIC value was selected as the most parsimonious. We explored models incorporating the effects of individual trees; however, AIC values were strongly penalized for the additional parameters (data not shown).

Finally, we used the molecular data set to evaluate the abundance and distribution of OTU2, OTU4, OTU8 and OTU10 across all study sites (i.e. including the additional six sites where rust was not found on the majority of trees). We calculated the Pearson's product moment correlation between site rust disease severity and the balance of the common pathogen antagonists and pathogen facilitators within a site, $(\text{OTU8} + \text{OTU2}) - (\text{OTU10} + \text{OTU4}) / (\text{OTU8} + \text{OTU2} + \text{OTU10} + \text{OTU4})$.

Independent analysis of foliar fungal community contribution to rust disease severity

We followed the methods of Wagner *et al.* (2014) to independently identify candidate OTUs within the molecular data set influencing rust disease severity. Again, we included data from the four sites where rust was recorded on the majority of trees and excluded samples with exceptionally few sequences (< 1000 total; $n = 7$). We compressed the OTU table into a single axis of variation by conducting a principal coordinate analysis using a Bray–Curtis distance matrix based on OTU relative abundance data. We then used the first principal coordinate (PCo1, explaining 29% of the variation in foliar fungal communities) as a proxy for the foliar fungal community in models of rust disease severity.

First, we visually inspected the relationship between PCo1 and rust disease severity to select an appropriate function. To make the model more intuitive, PCo1 was transformed to a positive scale by adding a constant and multiplying by -1 . We fitted an exponential decay function for disease severity, $f(X) = a \times \exp(-s \times X)$, where X was PCo1 score and a and s were estimated parameters. We followed the same model comparison criteria described above for OTU-rust models to evaluate the model fit (i.e. comparing the PCo1 model (model 0) to a null model (model 1), a site-null model (model 2), and a site model including PCo1 (model 3). A significant PCo1 effect on rust disease severity would be supported if either or both the PCo1 model (model 0) and PCo1-site model (model 3) were more

parsimonious than the null model (model 1) and the site-null model (model 2). Again, we used the same methods described above to solve for maximum likelihood parameter values and to compare model fits.

After finding a significant relationship between PCo1 and rust disease severity, we next identified OTUs in the molecular data set driving this pattern by calculating Pearson's product moment correlations between PCo1 and OTU relative abundance (Wagner *et al.*, 2014). OTUs either strongly negatively or positively correlated with PCo1 were identified as candidate pathogen antagonists or pathogen facilitators, respectively. We determined the statistical significance of correlations for a subset of OTUs that were both among the 20 most strongly correlated with PCo1 and among the 15 most abundant overall ($n=9$ OTUs). We used a Holm correction for multiple hypothesis tests.

Results

Culture-based and molecular surveys of foliar fungal communities of *Populus trichocarpa*

Species of *Epicoccum*, *Cladosporium* and *Alternaria* were most frequently isolated from cultured leaves across the study area (39%, 30%, and 14% of leaves, respectively) (Fig. S1). Other common taxa found in our culture-based survey included species of *Trichoderma*, *Chaetomium*, *Penicillium*, *Arthrinium*, *Phoma*, *Cytospora* and *Ulocladium* (23 genera total).

Our molecular data set yielded 968 OTUs. Despite the disparity in taxonomic diversity between the culture-based and molecular data sets, both methods identified species of *Cladosporium*, *Alternaria* and *Trichoderma* as common (Figs 1c, S1). However, the two data sets did not always agree: some taxa were less common in the molecular data set than in the culture-based data set (e.g. *Penicillium* and *Chaetomium*), while others were more common in the molecular data set (e.g. *Phialocephala* and *Mortierella*).

Inoculation experiments

Foliar fungal isolates from five of seven genera and from five of 10 tree populations significantly modified rust disease severity in the inoculation trials, with effects ranging from modest (i.e. 2 times controls) to profound (i.e. 40 times) (Fig. 2a; Table 1). Pathogen antagonists included species of *Cladosporium*, *Trichoderma*, *Chaetomium* and *Penicillium*. An isolate of *Epicoccum* was identified as a pathogen facilitator; species of *Alternaria* and *Cladosporium* showed a trend toward facilitation (i.e. statistical significance was found before but not after correcting for multiple comparisons). Overall, foliar fungi explained 16% of the variation in rust disease severity in the experiment that included the 10 *Epicoccum* isolates and a control group, and 53% of the variation in rust disease severity in the experiment including the remaining 24 foliar fungal isolates and a control group (Table 1). We observed no signs or symptoms of disease on the foliar fungus-only inoculated leaves, confirming that the 34 isolates are not pathogenic on *P. trichocarpa*.

Rust disease severity models

The molecular survey showed patterns in the abundance and distribution of common foliar fungi consistent with our experimental results. In particular, several of our experimental isolates matched to common OTUs in the molecular data set, and some of those OTUs correlated with rust disease severity in wild trees in the directions predicted by our experimental manipulations. Isolates of *Trichoderma*, *Cladosporium* and *Alternaria* that modified rust disease severity experimentally (note in some cases significant only before correcting for multiple comparisons; Table 1) matched to some of the most abundant OTUs in the entire data set: OTU8 (*Trichoderma*), OTU2 (*Cladosporium*), OTU4 (*Cladosporium*) and OTU10 (*Alternaria*) (Fig. 1c). Consistent with experimentally confirmed antagonism, *Trichoderma* OTU8 and *Cladosporium* OTU2 exhibited negative relationships with rust disease severity in leaves of wild trees; consistent with the trend toward facilitation, *Alternaria* OTU10 and *Cladosporium* OTU4 exhibited positive relationships with disease severity (Fig. 2b–e; Table 2). Statistical models supported the inclusion of OTU abundance as a predictor of field observations of disease severity for OTU8 and OTU10 (model 3 $AIC_{\text{site-OTU8}}=566$; model 3 $AIC_{\text{site-OTU10}}=555$; model 1 $AIC_{\text{site-null}}=568$; Table 2). By contrast, site-only models were more parsimonious for OTU2 and OTU4 (model 3 $AIC_{\text{site-OTU2}}=570$; model 3 $AIC_{\text{site-OTU4}}=572$; model 1 $AIC_{\text{site-null}}=568$; Table 2), indicating that environmental effects could not be teased apart from OTU effects.

In addition to the significant relationships between individual OTUs and rust disease severity in the four tree populations affected by rust disease, we also found evidence that common disease-modifying OTUs correlated with rust disease severity across the entire study area. Patterns in the balance between the two common antagonists (OTU2 and OTU8) and two pathogen facilitators (OTU4 and OTU10) within foliar fungal communities correlated with rust disease severity in the predicted directions across all 10 study populations ($r=-0.54$; $P<0.001$; Figs 2f, S2).

Independent analysis of foliar fungal contribution to rust disease severity in wild trees

Likelihood models supported a significant relationship between PCo1 and rust disease severity, consistent with the foliar fungal community influencing patterns in disease severity in wild trees (Fig. 3; Table 2). More specifically, PCo1 explained 29% of the variation in fungal community structure, and the most parsimonious, AIC-selected disease severity model supported the inclusion of PCo1 (model 3 $AIC_{\text{site-PCo1}}=562$; model 1 $AIC_{\text{site-null}}=568$). Finally, correlative analysis of PCo1 and OTU relative abundances revealed OTUs in the community driving this pattern. In particular, we identified six common, candidate disease-modifying OTUs, including OTU8 *Trichoderma* ($P<0.001$), OTU10 *Alternaria* ($P=0.048$), and OTU2 *Cladosporium* ($P=0.07$) (Table S3). The other three candidate disease-modifying OTUs were not isolated in culture and thus

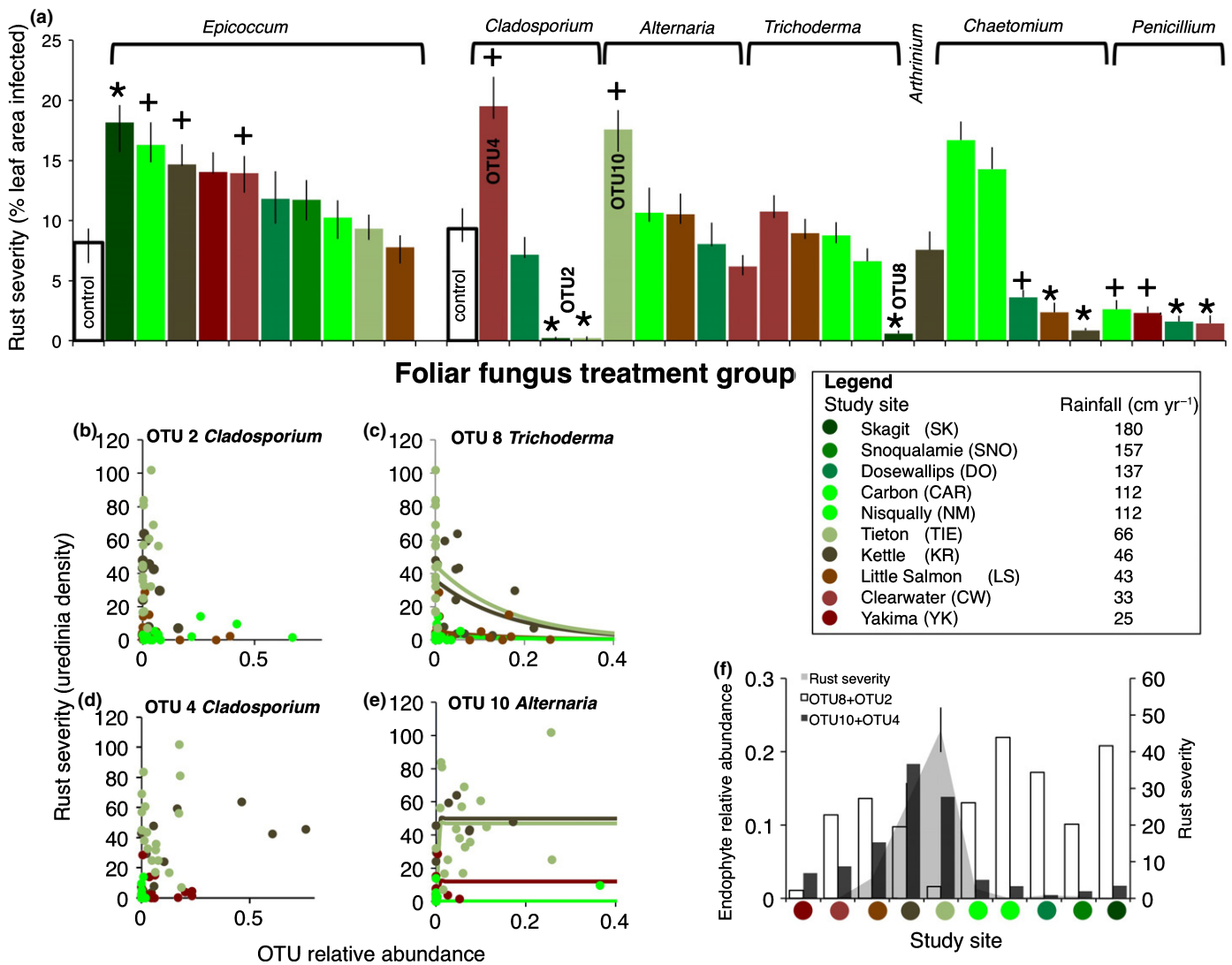


Fig. 2 Common foliar fungi alter rust disease severity experimentally, and some correlate with rust disease severity in wild trees in the predicted directions. (a) Mean disease severity is shown for leaves inoculated with individual fungi before inoculation with *Melampsora* rust. Foliar fungi are color-coded by site of origin; open bars show control data. Statistical significance ($P < 0.05$) for univariate planned contrast tests is indicated by +, and * for those comparisons that remained significant ($P < 0.05$) after correcting for multiple tests (Table 1). Error bars, \pm SE ($n = 155$). (b–e) Relationship between disease severity in wild trees and the relative abundance of four common OTUs that match to experimental isolates. Observed data are color-coded by site rainfall gradient ($n = 65$). Model fits supporting operational taxonomic unit (OTU) effects on disease severity are shown by color-coded lines; plots without lines indicate nonsignificant relationships (Table 2). (f) The abundance and distribution of the same four OTUs also correlated with site disease severity (see also Supporting Information Fig. S2). Error bars are standard errors.

were not tested experimentally (e.g. *Articulospora* and *Phialocephala*).

Synthesis

Our experimental, modeling, and correlative analyses offer partial to complete support for pathogen antagonists and facilitators of *Melampsora* rust disease. All lines of evidence supported *Trichoderma* (OTU8) as a rust antagonist. Experimental evidence strongly supported *Cladosporium* (OTU2) as a rust antagonist, and OTU2 was also independently selected among all OTUs as a candidate rust antagonist. Support for pathogen facilitation was less robust. While *Epicoccum* (OTU24) facilitated rust disease

severity experimentally, it was not independently identified as a pathogen facilitator (and its abundance was insufficient for modeling). We observed an experimental trend toward facilitation for *Alternaria* (OTU10); modeling results bolstered support for rust disease facilitation, and OTU10 was independently selected among all OTUs as a candidate rust facilitator. We found only a trend toward facilitation for *Cladosporium* (OTU4) in experiments. Finally, we found correlative support that the four most common, disease-modifying fungi (Figs 2f, S2), and the fungal community overall (Fig. 3), influenced disease severity in wild trees. Whether these patterns reflect the contribution of individual taxa within communities, or additive/synergistic effects of foliar fungi on rust disease severity is not known.

Table 1 Restricted maximum likelihood (REML) based linear-mixed effects model results for glasshouse inoculation experiments; *P* values for planned contrasts are shown for univariate tests and for tests corrected for multiple comparisons (ns, *P* values > 0.05)

First experiment				
Factor	df	Test statistic	<i>R</i> ²	<i>P</i>
Foliar fungus	10	2.4	0.16	0.021
Cutting	1	9.9	0.18	0.0017
Planned contrasts			<i>P</i>	<i>P</i> corrected
OTU1431/24 <i>Epicoccum</i> , TIE			ns	ns
OTU24 <i>Epicoccum</i> , KR			0.0205	ns
OTU1431/24 <i>Epicoccum</i> , YK			ns	ns
OTU24 <i>Epicoccum</i> , LS			ns	ns
OTU1431/24 <i>Epicoccum</i> , SK			0.0011	0.0096
OTU1431/24 <i>Epicoccum</i> , DO			ns	ns
OTU1431/24 <i>Epicoccum</i> , CAR			ns	ns
OTU24 <i>Epicoccum</i> , CW			0.049	ns
OTU1431/24 <i>Epicoccum</i> , SNO			ns	ns
OTU1431/24 <i>Epicoccum</i> , NM			0.013	ns
Second experiment				
Factor	df	Test statistic	<i>R</i> ²	<i>P</i>
Foliar fungus	24	9.9	0.53	<0.001
Cutting	1	55	0.17	<0.001
Planned contrasts			<i>P</i>	<i>P</i> corrected
OTU8/794 <i>Trichoderma</i> , SK			<0.001	<0.01
OTU8/794 <i>Trichoderma</i> , NM			ns	ns
OTU8/794 <i>Trichoderma</i> , CW			ns	ns
OTU26 <i>Trichoderma</i> , CAR			ns	ns
OTU26 <i>Trichoderma</i> , LS			ns	ns
OTU2 <i>Cladosporium</i> , SK			<0.001	<0.01
OTU2 <i>Cladosporium</i> , TIE			<0.001	<0.01
OTU2 <i>Cladosporium</i> , DO			ns	ns
OTU4 <i>Cladosporium</i> , CW			0.039	ns
OTU10 <i>Alternaria</i> , TIE			0.044	ns
OTU10 <i>Alternaria</i> , CW			ns	ns
OTU10 <i>Alternaria</i> , LS			ns	ns
OTU10 <i>Alternaria</i> , NM			ns	ns
OTU10 <i>Alternaria</i> , DO			ns	ns
<i>Chaetomium</i> , KR			<0.001	<0.01
<i>Chaetomium</i> , DO			0.022	ns
<i>Chaetomium</i> , LS			0.0019	0.033
<i>Chaetomium</i> , CAR			ns	ns
<i>Chaetomium</i> , NM			ns	ns
<i>Penicillium</i> , NM			0.0062	ns
<i>Penicillium</i> , YK			0.0046	ns
<i>Penicillium</i> , CW			<0.001	<0.01
<i>Penicillium</i> , DO			<0.001	<0.01
OTU170 <i>Arthrinium</i> , YK			ns	ns

Cutting was treated as a random effect. Codes following operational taxonomic unit (OTU) names indicate the site from which the fungus was isolated (see Supporting Information Table S1 for names).

Discussion

Our experimental and field-based study revealed that disease modification is an ecological function shared by common foliar

fungal symbionts of *P. trichocarpa*. First, *in planta* inoculation experiments demonstrated that a diverse group of common foliar fungi of *P. trichocarpa* (e.g. species of *Cladosporium*, *Trichoderma* and *Epicoccum*) can alter the severity of the tree's major foliar fungal disease, caused by the rust pathogen *M. × columbiana*. Second, a molecular field study showed that spatial patterns in the abundance and distribution of some of the most common disease-modifying fungi significantly correlated with rust disease severity within leaves and across the study area, consistent with foliar fungi contributing to disease severity. Finally, model results supported fungal communities influencing rust disease severity in wild trees, and independently identified *Alternaria*, *Cladosporium* and *Trichoderma* as candidate pathogen antagonists or facilitators within those communities.

Our results shed light on a long-standing question regarding the ecological importance of foliar fungi. In contrast to mycorrhizal, pathogenic, and saprotrophic fungi, whose ecological roles have long been recognized in plant ecology, we learned only recently that foliar fungi are capable of altering plant disease severity (Arnold *et al.*, 2003). A growing body of evidence supports foliar fungal disease modification in different plant species (Arnold *et al.*, 2003; Ganley *et al.*, 2008; Adame-Álvarez *et al.*, 2014; Ridout & Newcombe, 2015), plant genotypes (Busby *et al.*, 2013; Raghavendra & Newcombe, 2013), and pathogen species (e.g. biotrophs and fungal and bacterial necrotrophs and hemibiotrophs; Arnold *et al.*, 2003; Ganley & Newcombe, 2006; Adame-Álvarez *et al.*, 2014). Our study expands on these findings by showing that disease-modifying fungi are common enough in nature to be ecologically important. In particular, Raghavendra & Newcombe (2013) previously showed that *Stachybotrys* sp., *Trichoderma atroviride*, *Ulocladium atrum* and *Truncatella angustata* are antagonists of *Melampsora* rust disease in *P. trichocarpa* under controlled experimental conditions. Both our culture-based and molecular field surveys spanning the core of the tree's geographic range reveal that three of those four endophytes are rare in wild *P. trichocarpa*, and thus may be less likely to influence rust disease severity in wild plants than the common pathogen antagonists and facilitators identified in the present study. Future research testing this hypothesis will be poised to elucidate the role of foliar fungi in this wild plant pathosystem.

Demonstrating that foliar fungi modify plant disease in controlled experiments is certainly not the same as demonstrating that disease modification occurs in nature. If fact, when endophytes are tested in nature their effects on plant disease can sometimes be inconsistent with *in vitro* assays (Martin *et al.*, 2015). However, our study does provide correlative evidence to suggest that foliar fungi influenced rust disease severity in wild trees, despite expected sources of variation in the relationship between foliar fungi and disease severity in nature. In our modeling analysis of wild tree populations, the effect of an OTU on disease severity is the weighted sum of the effects of all strains/species that were grouped into that OTU using a 97% sequence similarity cut-off. Because fungal strains within a molecular OTU may sometimes vary in their functionality (Köljalg *et al.*, 2013), this relationship is not expected to be perfect. For example, only one of the three *Trichoderma* isolates matching to OTU8 (> 97%

Table 2 Likelihood models support the association between rust disease severity and operational taxonomic unit (OTU) relative abundance (OTU2 and OTU8), and between rust disease severity and the first principal coordinate (PCo1), a proxy for foliar fungal community

Model	No. of leaves	No. of parms	AIC*	Log-likelihood	R^2	Parameter a (KR)			Parameter a (LS)			
						Mean	Lower SI	Upper SI	Mean	Lower SI	Upper SI	
Null	Mean	65	2	607.1	-301.4	0	20.7	14.5	26.7			
Site null	Mean	65	5	567.9	-278.5	0.51	25	16.5	34.5	4.14	0	14.4
PCo1	Exponential decay	65	3	578.9	-286.2	0.37	51	41.3	60			
PCo1-site	Exponential decay	65	6	562.3	-274.4	0.57	176	121	224	22.5	0	48.7
OTU8	Exponential decay	65	3	603.4	-298.7	0.08	26.4	19.3	33.3			
OTU8-site	Exponential decay	65	6	566.5	-276.5	0.54	36	24.5	48.1	5	0	19
OTU10	Inverse exponential	65	3	570.4	-282	0.45	42.3	35.1	49.6			
OTU10-site	Inverse exponential	65	6	555.6	-271.1	0.61	49.9	36.9	63	12.1	0	27.2
OTU2	Exponential decay	65	3	606.2	-300	0.046	24.7	17.6	30.9			
OTU2-site	Exponential decay	65	6	570.3	-278.4	0.51	25.7	16.4	34.7	5.37	0	14.4
OTU4	Inverse exponential	65	3	602.5	-298.1	0.1	25.9	18.7	32.1			
OTU4-site	Inverse exponential	65	6	572.5	-279.5	0.49	27.1	17.6	37.8	7.5	0	15.5

Model	Parameter a (NM)			Parameter a (TIE)			Parameter s			SD		
	Mean	Lower SI	Upper SI	Mean	Lower SI	Upper SI	Mean	Lower SI	Upper SI	Mean	Lower SI	Upper SI
Null										25	21.3	30
Site null	0	0	10.2	46.8	39.3	52.2				17.6	15.7	20.5
PCo1							2	1.5	2.9	19.7	16.8	23.7
PCo1-site	9.56	0	43.8	54.9	53.3	59.9	2.55	2.44	2.7	17.2	16.1	17.9
OTU8							11.4	20.4	28.8	24	20.4	28.8
OTU8-site	2.8	0	9.9	44.8	40.3	52.1	6.07	2.56	11.8	17.3	15.4	19.6
OTU10							356.8	128.5	1000	18.5	15.8	22.2
OTU10-site	0.001	0	15.2	46.7	41.6	52.6	8553	2309	10000	15.8	14.1	18.2
OTU2							4.3	0.26	14.4	24.4	20.7	29.2
OTU2-site	0	0	10.2	45.6	39.6	52.4	0.0002	0	3.09	17.8	15.7	20.4
OTU4							3013	301	23440	23.8	20.3	28.4
OTU4-site	0.99	0	13.7	45.5	39.6	52.4	9465	1609	10000	17.8	15.8	20.9

KR, Kettle River; LS, Little Salmon; NM, Nisqually; TIE, Tieton; AIC, Akaike information criterion; SI, support interval.

*Corrected for small sample size.

similarity) reduced disease severity experimentally, suggesting that not all isolates in nature are antagonists (alternatively, with a larger sample size we would have had more power to detect a smaller effect size). In addition, the ability of foliar fungi to modify disease severity should be constrained by the level of genetic resistance in the host tree (e.g. antagonists cannot reduce disease severity below zero in the leaves of resistant individuals). Variation in *Melampsora* pathogenicity among sites too could affect the relationship between foliar fungi and disease severity. Despite such sources of variation, we still found evidence that common fungi experimentally identified as either antagonists or facilitators correlated with rust disease severity in wild trees. Future research efforts that sequence additional loci will enable greater taxonomic resolution of foliar fungi, thus improving our ability to detect their presence and function in the wild, as well as permitting explicit comparisons across studies.

Unlike the habitat-adapted-symbiosis hypothesis whereby fungal endophytes confer plant tolerance to abiotic stress only under stressful conditions (Rodriguez *et al.*, 2008), our results suggest that the potential for foliar fungi to alter disease severity is not restricted to a particular habitat. Fungi found to modify disease originated from highly variable environments, ranging from

near-desert conditions of the Columbia plateau east of the Cascade mountains to temperate rain forests west of the Cascade mountains (i.e. rainfall gradient 25–180 cm rain yr⁻¹). Despite differences in fungal community composition across the study area, and differences in fungal infection opportunities and disease pressure that probably exist across the moisture gradient (Agrios, 2005), disease-modifying fungi were isolated from five of our 10 study populations. Moreover, patterns in rust-modifying OTUs correlated with rust disease severity across the entire study area. Thus, disease-modifying foliar fungi may contribute to plant defense across a broad range of environments. Based on these findings, we urge future research to test predictive models for host fitness regimes favoring foliar fungal antagonists versus facilitators.

While our study focused on a single plant pathosystem, considering the impacts of disease-modifying foliar fungi on multiple plants within a community will be necessary for elucidating their broader role in plant ecology. For example, the 'same' foliar fungus (e.g. *Penicillium raistrickii*) can occur in two different plant species in the same community, acting as a pathogen antagonist in one plant (*P. trichocarpa*; this study) while a pathogen facilitator in the other (*Pinus ponderosa*; Ridout & Newcombe, 2015).

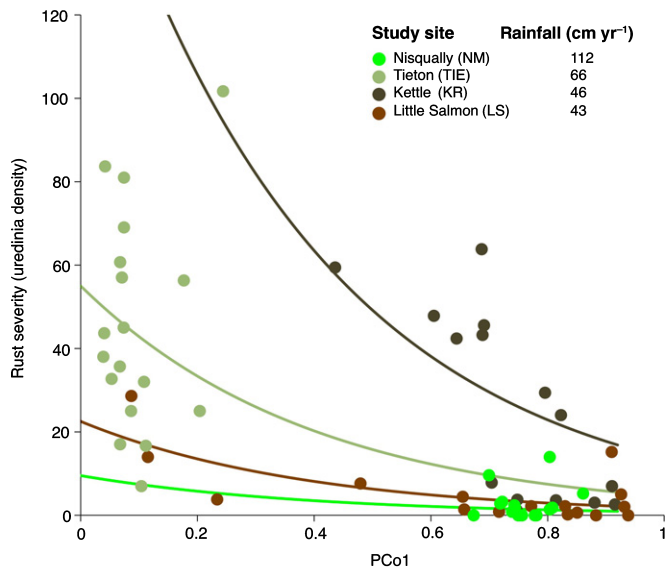


Fig. 3 Independent analysis of foliar fungal communities confirms their association with rust disease severity in wild trees. The relationship between the first principal coordinate (PCo1), a proxy for the foliar fungal community, and disease severity in wild populations ($n = 65$) is shown. Observed data are color-coded by site rainfall gradient; model fits supporting PCo1 effects on disease severity are shown by color-coded lines (Table 2).

Alternatively, disease-modifying fungi may perform the same ecological function in different pathosystems (e.g. species of *Trichoderma* that are bioactive against many pathogens; Woo *et al.*, 2006). Plants may also harbor pathogens of competing plant species (P. E. Busby, pers. obs.). Finally, fungi inhabiting stems and roots can also modify the expression of plant disease (Martin *et al.*, 2015). These observations collectively raise questions about the net effects of foliar fungal disease modification on plant diversity. Disease webs involving plants, foliar fungi and pathogens will be challenging to construct, but given the importance of disease in plant ecology they are a necessary next step.

Results of our study are instructive for disease management in wild and agricultural plant systems. Fortunately, this comes at a time when industry is receptive to the idea of managing the plant microbiome to enhance growth and suppress disease (Jones, 2013; Ledford, 2015). Rust pathogens in particular are among the most devastating to crop plants world-wide, and novel pathogenic virulence often outpaces our ability to develop and deploy genetically resistant plants (Newcombe *et al.*, 2001; Stokstad, 2007). Our results suggest that foliar fungal antagonists could be used to bolster resistance to rust disease in *Populus* plantations. More specifically, because antagonists are common in the leaves of wild trees, they should persist as inoculants into the foliar fungal microbiome of plantation trees and suppress rust disease. Moreover, because the taxa identified as antagonists in our study are common across many host plants, they may be effective in other crop plants as well. Our pilot field trials testing antagonists against *Melampsora* rust are producing promising results (P. E. Busby, unpublished). However, large-scale implementation of pathogen antagonists faces a suite of additional

challenges from cost-effective production and application of inoculants to environmental impact assessment and public approval.

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Author contributions

P.E.B. conceived the project and obtained funding. P.E.B. and G.N. established the study design and carried out the fieldwork and glasshouse experiments. P.E.B. and K.G.P. conducted the DNA sequencing and analysis. P.E.B. wrote the manuscript with contributions from G.N. and K.G.P.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The frequency of foliar fungi isolated from cultured leaves collected from the 10 wild tree populations.

Fig. S2 Correlation between the frequency of foliar fungal pathogen antagonists and pathogen facilitators and site rust disease severity.

Table S1 Study site information

Table S2 Foliar fungal isolates used in glasshouse inoculation experiments and matching OTUs in the molecular data set

Table S3 Correlation between OTU relative abundance and PCo1 for the 15 most common OTUs in the Miseq data set

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