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The influence of genetics, defensive chemistry and the fungal microbiome on disease outcome in whitebark pine trees

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SUMMARY

The invasive fungal pathogen *Cronartium ribicola* infects and kills whitebark pine (*Pinus albicaulis*) throughout western North America. Whitebark pine has been proposed for listing under the Endangered Species Act in the USA, and the loss of this species is predicted to have severe impacts on ecosystem composition and function in high-elevation forests. Numerous fungal endophytes live inside whitebark pine tissues and may influence the severity of *C. ribicola* infection, either directly by inhibition of pathogen growth or indirectly by the induction of chemical defensive pathways in the tree. Terpenes, a form of chemical defence in pine trees, can also influence disease. In this study, we characterized fungal endophyte communities in whitebark pine seedlings before and after experimental inoculation with *C. ribicola*, monitored disease progression and compared fungal community composition in susceptible vs. resistant seedlings in a common garden. We analysed the terpene composition of these same seedlings. Seed family identity or maternal genetics influenced both terpenes and endophyte communities. Terpene and endophyte composition correlated with disease severity, and terpene concentrations differed in resistant vs. susceptible seedlings. These results suggest that the resistance to *C. ribicola* observed in natural whitebark pine populations is caused by the combined effects of genetics, endophytes and terpenes within needle tissue, in which initial interactions between microbes and hosts take place. Tree genotype, terpene and microbiome combinations associated with healthy trees could help to predict or reduce disease severity and improve outcomes of future tree breeding programmes.

Keywords: fungal endophytes, fungal pathogens, Illumina sequencing, microbial community ecology, QIIME, terpenes, whitebark pine.

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INTRODUCTION

Cronartium ribicola J.C. Fisch is a non-native fungal pathogen which causes the disease white pine blister rust in five-needle pine trees native to North America. *Cronartium ribicola* spores colonize five-needle pine trees through needle stomata (Liu *et al.*, 2015). *Cronartium ribicola* mycelium then grows through the needles and branches of susceptible trees and into the main stem, where it eventually girdles and kills the tree (Campbell and Antos, 2000; Patton and Johnson, 1970). *Cronartium ribicola* has spread to nearly the entire range of whitebark pine in western North America (Schwandt *et al.*, 2010) and, in some areas, has reduced whitebark pine to less than 10% of its natural population (Keane and Arno, 1993; Kendall and Arno, 1990). The loss of whitebark pine is predicted to have severe impacts on forest composition and its distribution in high-elevation ecosystems (Hoff *et al.*, 2001), but some genetic resistance to *C. ribicola* has been documented (Sniezko *et al.*, 2011).

To defend against diseases such as white pine blister rust, conifers produce organic defensive compounds, such as terpenes, which can directly inhibit pathogen infection and spread within the host (Karst *et al.*, 2015; Trapp and Croteau, 2001). Conifers continuously produce some terpenes as a form of constitutive resistance to kill or contain pathogens, or to repel herbivores (Bonello *et al.*, 2006; Keeling and Bohlmann, 2006). Terpenes in trees may also be produced as a form of induced resistance, in which compounds are synthesized in response to stressors. Terpenes are an important first line of defence to inhibit the initial growth of pathogenic fungi that infect conifer species (Bridges, 1987; Evensen *et al.*, 2000; Lombardero *et al.*, 2006; Michelozzi *et al.*, 1990), and higher concentrations of terpenes have been linked to increased disease resistance (Michelozzi *et al.*, 1990).

In addition to terpenes, organisms that colonize conifers may also inhibit pathogen infection. Referred to as endophytes, many microorganisms live symbiotically within plant tissues without showing any visible signs or symptoms of infection (Carroll, 1988, Rodriguez *et al.*, 2009). Endophytes can facilitate plant defences by direct interaction with pathogens inside host tissues, decreasing pathogen damage through mycoparasitism or the production of toxic compounds (Atanasova *et al.*, 2013; Evans *et al.*, 2003; Stierle and Stierle, 2015). By the inhibition of pathogens,

endophytes act as a complementary or even substitutive layer of resistance for plants, allowing plants to invest less in their own defensive mechanisms. For example, Arnold *et al.* (2003) found that fungal endophytes decreased pathogen damage in a tropical tree and that endophyte-mediated protection from pathogens was significantly greater in mature leaves, which were less equipped with endemic defences.

Endophytes and pathogens co-occur in trees and endophytes have ample opportunity to influence the initiation and progression of disease. Numerous studies have already demonstrated the potential of fungal endophytes as biocontrols of disease in forest ecosystems (Berube *et al.*, 1998; Ganley *et al.*, 2008; Ridout and Newcombe, 2015). Foliar fungal endophytes isolated from eastern white pine (*Pinus strobus*) have produced compounds toxic to at least two rust pathogens (Sumarah *et al.*, 2011) and some toxic to *C. ribicola* specifically (Sumarah *et al.*, 2015). Hundreds of fungal endophyte species have been recovered in white pines (Bullington and Larkin, 2015; Larkin *et al.*, 2012), and some have already shown the potential to decrease the severity of white pine blister rust disease when inoculated into individual white pine trees (Berube *et al.*, 1998; Ganley *et al.*, 2008).

Terpenes and other chemical compounds in host plants filter the available pool of microbial colonizers from the surrounding environment, both pathogens and endophytes, and also affect the outcome of their interactions (Fig. S1, see Supporting Information). For example, Arnold *et al* (2003) observed that endophytes in tropical forests were host specific, and that outcomes of fungal–fungal interactions were dependent on specific host leaf chemistry. Endophytes in that study also reduced pathogen damage in trees. These findings suggest that host chemistry influences microbial community composition by promoting the colonization of some species whilst inhibiting that of others. Pathogens that can tolerate initial host defensive chemistry and are able to colonize the plant must then compete with co-occurring endophytic colonizers, highlighting the dual importance of host chemistry and endophytic community composition in understanding host plant pathology.

Despite the growing body of evidence on endophytes and their influence on conifers and disease, little is known about how host chemistry and pathogen infection shape the microbial community composition within trees, or vice versa. In order to understand the function of the plant fungal microbiome in these systems, we must first better understand the dynamics of fungal community composition. In this study, we investigated the relationship between *C. ribicola*, (the pathogen causing white pine blister rust), fungal endophytes and terpenes in 20 whitebark pine seedling families (half-siblings or seeds sourced from the same maternal parent) that were experimentally infected with *C. ribicola* in a common garden. We characterized the foliar fungal microbiome both before and after inoculation with blister rust to determine the effect of the pathogen on the endophyte community composition. We looked for fungal endophytes that occurred more often in disease-resistant seedlings to find those species most likely to aid in blister rust resistance. We also analysed terpene composition in the needle tissue of these same seedlings and investigated the relationships between terpene concentration and disease severity characteristics. In addition, we compared terpene concentrations in resistant and susceptible seedlings, whilst accounting for differences caused by genetic variation of the hosts. We also explored the relationships between individual terpenoid compounds and high-abundant endophytic taxa. We hypothesized that exposure to *C. ribicola* would alter endophyte communities in inoculated seedlings, possibly as a result of an induced response of the host as reflected in terpene concentrations. We also predicted that, after inoculation with *C. ribicola*, terpene concentrations and abundances of some endophytes would be higher in resistant than in susceptible seedlings. With this study, our aim was to elucidate the complex relationships between host defensive chemistry, fungal endophyte communities and disease in whitebark pine to assist future restoration efforts. Terpenes and endophyte communities associated with healthy trees in these studies will greatly contribute to our knowledge of the mechanisms underlying disease resistance in natural whitebark pine populations.

RESULTS

Disease development

For this experiment, 141 seeds belonging to 20 whitebark pine seed families (half-siblings or seedlings with the same maternal parent) were inoculated with *C. ribicola* in a common garden setting. We analysed the foliar endophyte communities prior to inoculation and both foliar endophyte communities and terpene concentrations in each seedling approximately 1 year after inoculation. We also assessed the phenotypic characteristics associated with resistance to blister rust of each seedling at 8 months (inspection 1) and 14 months (inspection 2) after inoculation. All 131 inoculated seedlings showed needle spots at inspections 1 and 2. Of the 131 inoculated seedlings, 100 developed cankers on stems or branches by inspection 2, and were labelled as 'susceptible' to *C. ribicola* infection (Table S1, see Supporting Information) for all subsequent analyses. Susceptible seedlings occurred in all half-sibling families. Susceptible seedlings developed 5.4 total cankers on average, ranging from 1 to 13 cankers per seedling. The remaining 31 seedlings from 11 families appeared to be resistant to blister rust, developing zero cankers on stems or branches at the time of needle collection. These seedlings were labelled as 'resistant' for all subsequent analyses. Seed family influenced disease resistance ($\chi^2 = 45.7$, $P < 0.001$) in inoculated seedlings. The most resistant seed families were CA-62 collected

from North Cascades National Park in Region 1, CO-121 from Colville National Forest in Region 1 and CL-72 from Crater Lake National Park in Region 4 (Table S1). The ten seedlings that were never inoculated and served as controls did not present any symptoms of blister rust infection at any point during this study.

Terpenes and disease in whitebark pine

In the needles sampled 1 year after inoculation with *C. ribicola*, we detected 23 total terpenes, including 17 monoterpenes and six sesquiterpenes. The monoterpenes were β-phellandrene, ρ-cymene, γ-terpinene, terpinolene, sabinene hydrate, 4-allylanisole, bornyl acetate, borneol, 3-carene, myrcene, (–)-α-pinene, (+)-α-pinene, (–)-β-pinene, (+)-β-pinene, (–)-limonene, (+)-limonene and terpineol acetate, and the sesquiterpenes were β-caryophyllene, humelene, germacrene, γ-cadinene, cubebene and γ-elemene. Considering only those terpenes whose concentrations could be calculated from available standards, the most abundant terpenes in whitebark pine were 3-carene, followed by (–)-α-pinene and β-phellandrene.

The overall terpene composition showed significant genetic variation amongst families (*Adonis*, $R^2 = 0.332$, $P < 0.001$), indicating the heritability of the foliar terpene profiles of whitebark pine. The terpene profiles also correlated with seedling group (resistant, susceptible and control seedlings) (*Adonis*, $R^2 = 0.047$, $P = 0.01$). Individual terpene compounds and total terpene concentrations varied with overall disease severity when genetic variation amongst seedlings was accounted for systematically (Table 1). As predicted, resistant seedlings contained higher concentrations of multiple terpenes, including (+)-limonene, (–)-α-pinene, total monoterpenes and total terpenoid compounds recovered from needle tissues (Fig. 1). Pearson's correlations between phenotypic characteristics of blister rust infection and these terpenes revealed that (+)-limonene showed a negative correlation with disease severity at the time of collection ($R^2 = -0.171$, $P = 0.049$), but a positive correlation with the number of needle spots at inspection 1 ($R^2 = 0.248$, $P = 0.004$).

Fungal endophytes

After quality filtering and demultiplexing, 1 631 451 total sequences remained, representing 1348 operational taxonomic units (OTUs) in seedlings sampled at inspection 2. The most abundant OTUs were found in at least 75% of seedlings and most closely matched *Cladosporium*, unidentified *Ascomycota*, *Paraphoma*, *Gibberella* and *Pleosporacea* spp. No single OTU was recovered from all trees. We did not detect fungal DNA matching the ITS2 region of *C. ribicola* in needle tissue of any seedlings sampled in this study, despite detecting it in other seedlings at the Dorena Genetic Resource Center (DGRC) that were inoculated and sampled at the same time.

Table 1 Summary of generalized linear mixed models (GLLMs) for white pine blister rust disease severity, showing the relationship between terpenes and disease resistance in 123 *Pinus albicaulis* seedlings inoculated with *Cronartium ribicola*.

Compound		χ^2	*P*
Montoterpenes	(–)-Limonene	1.12	0.29
	(–)-α-Pinene	**5.83**	**0.01**
	(–)-β-Pinene	0.16	0.71
	(+)-Limonene	**4.48**	**0.04**
	(+)-α-Pinene	0.11	0.75
	3-Carene	3.00	0.08
	4-Allylanisole	0.10	0.75
	Borneol	2.06	0.16
	Bornyl acetate	0.21	0.66
	Myrcene	0.25	0.64
	Terpineol acetate	1.66	0.19
	Terpinolene	1.07	0.33
	β-Phellandrene	1.95	0.17
	ρ-Cymene	0.03	0.85
Total monoterpenes		**5.45**	**0.02**
Sesquiterpenes	Germacrene	0.01	0.93
	Humulene	0.30	0.60
	β-Caryophyllene	0.77	0.39
	γ-Cadinene	0.43	0.54
	γ-Elemene	0.74	0.41
	γ-Terpinene	2.21	0.16
Total terpenoids		**5.43**	**0.02**

Bold *P* values are significant.

Of all the variables tested, endophyte communities showed the highest correlation with seedling family (*Adonis*, $R^2 = 0.160$, $P = 0.038$). The geographical regions in which seeds were collected (Fig. 2a) also influenced fungal endophyte composition (Fig. 2b; *envfit*, $R^2 = 0.070$, $P = 0.030$; *Adonis*, $R^2 = 0.101$, $P = 0.054$). Seedlings exhibited a north to south trend in endophyte community variability. Endophyte communities from the most northern seedling populations in Washington and British Columbia differed from communities in seedlings sourced from the most southern populations in southern Oregon. However, the effects of latitude and longitude of the source populations were not significant.

We observed little difference in endophyte community composition between resistant and susceptible seedlings when using a nested design to account for the influence of seed family, after inoculation with *C. ribicola* (*Adonis*, $R^2 = 0.009$, $P = 0.082$); however, differences were observed between all inoculated and control seedlings. Using a nested model to account for the influence of seed family, seedling group (resistant, susceptible and controls) explained most of the variation in endophyte community composition after inoculation (*Adonis*, $R^2 = 0.025$, $P = 0.025$). Endophyte communities in susceptible and resistant seedlings were more similar to each other than to those in control seedlings that were never inoculated (Fig. 3). Control seedlings most closely resembled endophyte communities in seedlings before inoculation, indicating

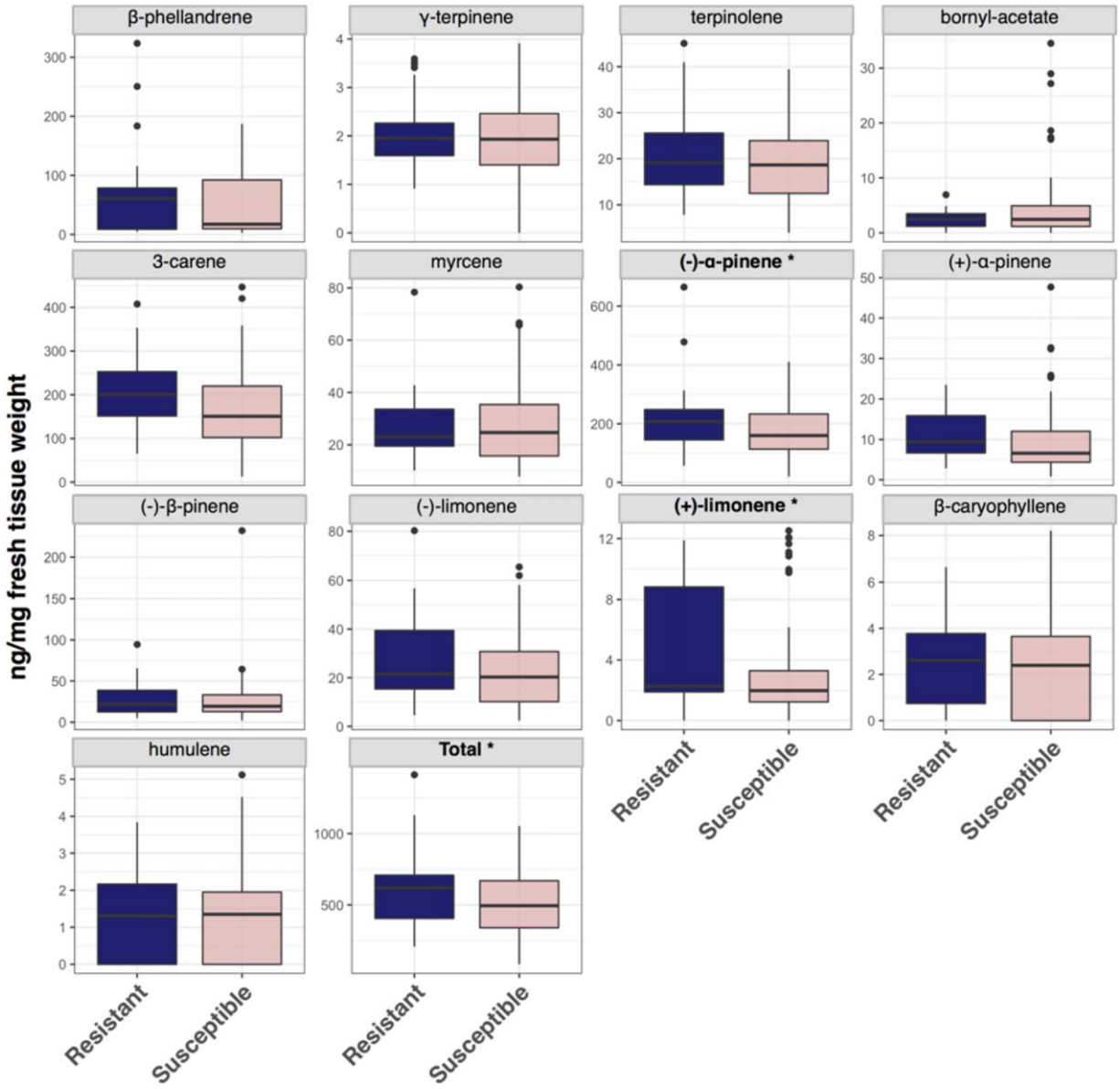

Fig. 1 Concentrations of individual terpenes in resistant and susceptible *Pinus albicaulis* seedlings after infection with *Cronartium ribicola*. *Significant differences.

a direct effect of inoculation on the endophyte community. The experimental design already underway at DGRC did not allow us to implement a full factorial design including all seed families, and so, to confirm our results, we also tested only control and inoculated seedlings from the same families. With this reduced dataset, endophyte communities also differed between control and inoculated seedlings (*Adonis*, $R^2 = 0.081$, $P < 0.020$; Fig. S2, see Supporting Information). Of the more abundant endophytes recovered in the complete dataset, six individual OTUs and three taxonomic groups varied amongst resistant, susceptible and control seedlings (Table S2, see Supporting Information). All of these endophytes decreased in abundance after seedlings were inoculated with blister rust, but many maintained high abundances in control seedlings that were never inoculated.

Looking only at resistant and susceptible seedlings inoculated with white pine blister rust, we saw correlations between abundant endophytes and blister rust disease characteristics. OTUs associated with *Lophodermium* and *Paraphoma* spp. were negatively correlated with the overall disease severity of inoculated seedlings (df = 121; $R^2 = -0.216$, $P = 0.017$; $R^2 = -0.207$, $P = 0.021$; respectively). The second most abundant OTU belonging to *Ascomycota* was recovered from 102 seedlings and was positively correlated with both 3-carene and the number of needle spots (df = 121; $R^2 = 0.222$, $P = 0.013$; $R^2 = 0.182$, $P = 0.044$,

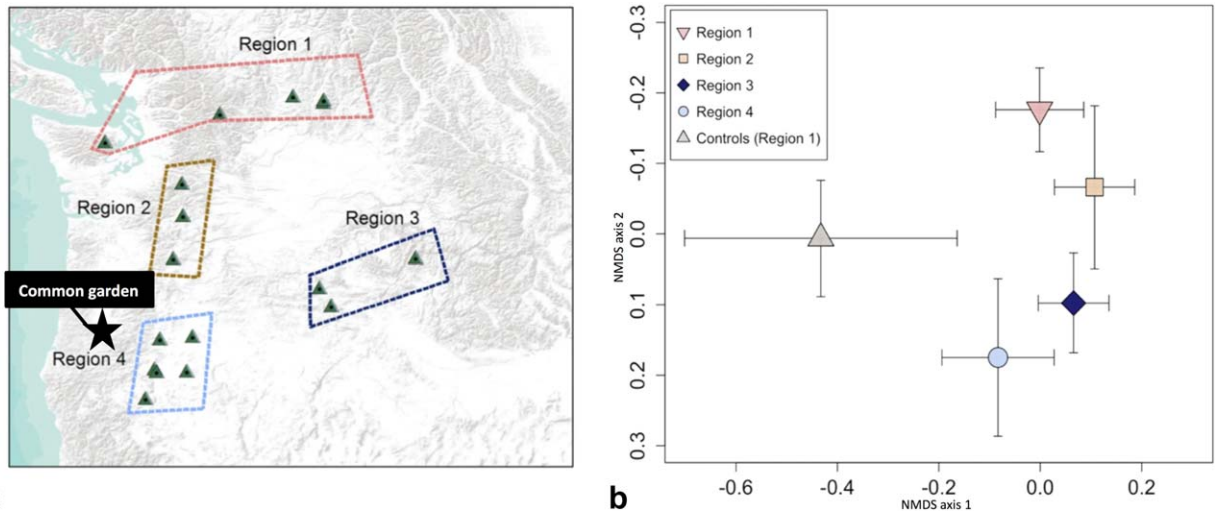

Fig. 2 (a) Map of seed family source locations in the Pacific Northwest grouped by geographical region from north to south. (b) Non-metric multidimensional scaling (NMDS) of fungal endophyte communities in *Pinus albicaulis* seedlings after inoculation with *Cronartium ribicola*. Shapes represent centroids for seed source regions and error bars represent standard errors (stress = 17.95; $n = 123$). Individual seed source populations are grouped by region, from north to south, where the most northern populations are represented here as Region 1 and the most southern populations as Region 4.

respectively). Most interestingly, the OTU matching 100% to the ITS2 region of *Metarhizium anisopliae* was recovered from 88 seedlings and its presence was negatively correlated with both the number of needle spots and the number of cankers on whitebark pine seedlings (df = 121; $R^2 = -0.233$, $P = 0.009$; $R^2 = -0.191$, $P = 0.034$; respectively). Another OTU belonging to *Helotiales* was recovered from 81 seedlings and was negatively correlated with 3-carene (df = 121, $R^2 = -0.187$, $P = 0.038$).

Measures of evenness (Pielou's J' evenness; $J' = H'/H'_{max}$) and rarefied richness of fungal communities also differed between seedling groups. We saw differences in evenness and richness of seedlings before blister rust inoculation compared with susceptible seedlings after inoculation (Fig. 4, $P = 0.003$ and $P = 0.006$, respectively). Richness decreased more in susceptible seedlings than in resistant seedlings or seedlings never inoculated, but endophyte composition was more even in susceptible seedlings than in any other group.

DISCUSSION

In this study, blister rust infection influenced the endophytic profiles in whitebark pine seedlings. Subsequent relationships between disease severity, fungal endophytes and terpene profiles of whitebark pine suggest that the interactions which take place inside needle tissue, at the point of infection, relate to the resulting pathology. Terpene profiles and fungal endophytes were also both influenced by host seed family, or the maternal parent identity, indicating that the genetic resistance observed in natural populations may actually be a combination of both endophytes and terpenes in needle tissue, in which the initial interactions between the host, endophytes and pathogen take place.

Terpenes correspond with disease outcome

The phenotypic characteristics of partial resistance to blister rust disease include fewer stem infections, fewer bark reactions and a higher survival rate (Sniezko *et al.*, 2014). The underlying

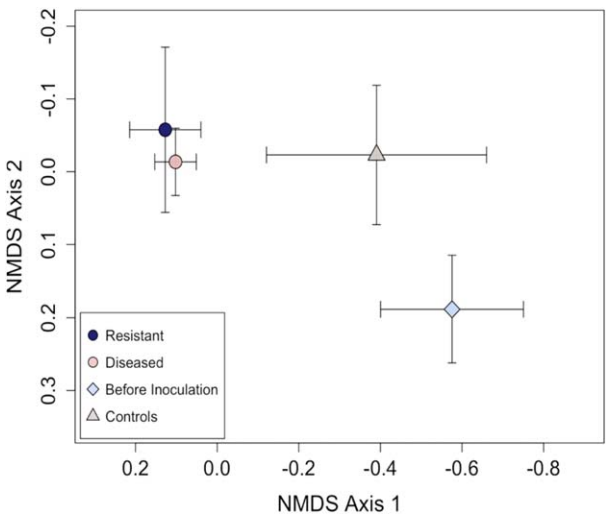

Fig. 3 Results of non-metric multidimensional scaling (NMDS) analysis of foliar fungal endophyte communities in seedlings inoculated with blister rust that were susceptible (red circle) or resistant (blue circle) to blister rust disease, control seedlings that were never inoculated (triangle) and all seedlings before inoculation (diamond). Shapes indicate centroids and error bars represent standard error (stress = 16.93; $n = 132$).

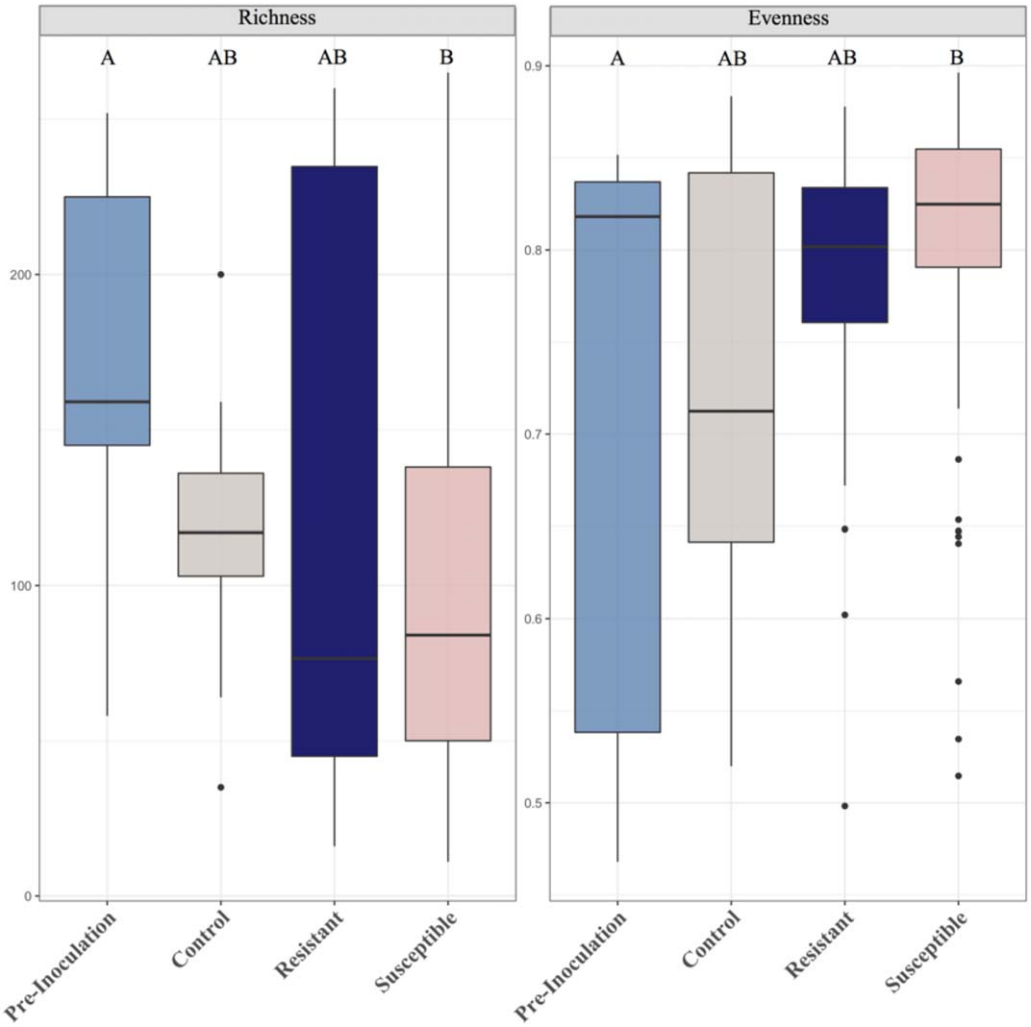

Fig. 4 Rarefied richness and evenness measurements of *Pinus albicaulis* seedlings before inoculation with *Cronartium ribicola* spores compared with susceptible and resistant seedlings after infection and control seedlings that were never inoculated.

mechanisms of these resistance traits are unknown in whitebark pine, but our study shows that resistance may be related to the terpene composition and endophyte communities within foliar tissue. The terpene (+)-limonene was more concentrated in resistant seedlings than in susceptible seedlings. (+)-Limonene was also positively correlated with the number of needle spots, but negatively correlated with overall disease severity later on, suggesting that initial infection severity may induce the production of (+)-limonene, inhibiting disease progression. (+)-Limonene is a common terpene in nature and is known to inhibit fungal growth (Duetz *et al.*, 2003).

Concentrations of (–)-α-pinene were higher in trees exposed to *C. ribicola* and highest in resistant seedlings (Fig. 1). A recent study by Burke and Carroll (2016) suggested that elevated levels of α-pinene might increase attack success and aggregation by mountain pine beetle. These results, combined with the current study, support the observations by Six and Adams (2007) and Jules *et al.* (2016) that mountain pine beetles appear to be more attracted to trees infected by blister rust disease, but it should be noted that, in the former study, the healthiest trees were avoided. Further investigations should be conducted to determine whether mountain pine beetles are indeed more attracted to rust-resistant phenotypes as a result of specific resistance characteristics, especially if levels of α-pinene remain elevated in resistant trees as a form of acquired resistance to blister rust disease.

We did not detect fungal DNA matching the ITS2 region of *C. ribicola* in the needle tissue of any seedlings sampled in this study. We do not believe that this was a result of the methods used, as we have detected *C. ribicola* in the needle tissue of five other needle pines 1 year after inoculation using the same methods. It is more likely that blister rust was undetectable because it had grown into branch and stem tissues or succumbed to tree

defences in needles, or both. As such, the differences in terpene concentrations in resistant vs. susceptible seedlings are perhaps even more striking, because this indicates that terpene concentrations persist even after the pathogen has senesced or been eliminated from needle tissues. This again may suggest that whitebark pine forms an acquired resistance to *C. ribicola*, or an increase in resistance to blister rust after exposure to the pathogen (Kloepper *et al.*, 1992; Sticher *et al.*, 1997). Alternatively, resistant trees may already maintain elevated levels of these terpenes before exposure to the pathogen as a constitutive defence.

As in Sampedro *et al.* (2010), in which terpenes were analysed in *Pinus pinaster* families, we also saw a significant effect of seed family on the terpene profile of individual seedlings (*Adonis*, $R^2 = 0.332$, $P < 0.001$). To our knowledge, this is the first evidence of genetic variation in terpenes in whitebark pine. As a result of the heritability of terpene composition, and because we did not analyse terpenes in seedlings both before and after inoculation, we can only speculate whether the terpene concentrations reported here represent constitutive or induced levels. We also hesitate to use control seedlings as a baseline, as they only represent two seed families, one of which has exhibited high resistance to blister rust when inoculated in other trials. However, when using mixed models in which we accounted for seed family identity, we still saw significant differences in terpene composition between resistant and susceptible seedlings.

Endophyte communities correlate with disease characteristics and host defensive chemistry

In accordance with our hypothesis that endophyte community composition would shift in response to blister rust infection, *C. ribicola* inoculations altered the foliar endophyte community composition of inoculated whitebark pine seedlings relative to controls and pre-inoculation seedlings (Figs 3, S2) by decreasing the overall richness and abundance of multiple taxonomic groups. After infection, we observed fewer fungal species in susceptible seedlings compared with all seedlings before inoculation (Fig. 4). A possible explanation for this observation is that altered host chemistry as a result of disease may filter the endophytic community, reducing low-abundant species that are already less tolerant of the host's chemical environment. This may explain the higher species evenness we see within susceptible seedlings, as well as the significant reduction in the abundance of many individual fungal species after *C. ribicola* inoculation (Table S2).

Host genotype is known to structure endophyte communities (Bálint *et al.*, 2013), and we found additional support for this. The strongest signal found for a change in endophyte communities was the half-sibling family of the host seedling. Using many of the same whitebark pine populations as used here, Liu *et al.* (2016) found similar geographical trends in population genetic structure to those found in endophyte community differences. Liu *et al.* (2016) also found that half-sibling family explained 27% of the genetic variation in whitebark pine populations, suggesting that family variation is a strong driver of genetic variation in whitebark pine. We would expect half-sibling families to exert a structural force on endophyte communities, but, in this case, we also found that this signal persisted through a common garden study in which all the seedlings were exposed to the same airborne fungal propagules. This indicates that host genotype filters the locally available fungal propagules and structures fungal endophyte communities.

In this study, we found that the structure and composition of endophytes may largely be a product of host filtering, with conspecific hosts supporting significantly different fungal communities based on their maternal genetics. For any endophyte to potentially alter the growth of *C. ribicola* in plant tissue or change the progress of blister rust disease in some other way, the endophyte must first show compatibility with the host's defensive chemistry at an individual tree level. This fact may contribute to the variability in endophyte–host assemblages amongst individuals in a species and, by extension, the same can be said of symbiont community variability amongst half-sibling families. Those endophytes able to colonize multiple whitebark pine seed families, and also having negative correlations with disease characteristics, may offer protection against blister rust and be good candidates for inoculations in future restoration efforts. Of the OTUs tested here, multiple fungal endophytes were correlated with disease characteristics and terpene concentrations in whitebark pine. Specifically, the presence of *Metarhizium anisopliae* was negatively correlated with both the number of needle spots and cankers on individual seedlings. *Metarhizium* is in the family Clavicipitaceae, which contains many well-known plant mutualists (Rodriguez *et al.*, 2009) and, to our knowledge, this is the first report of *Metarhizium* in the foliar tissue of conifers. Some *Metarhizium* spp. can produce secondary compounds toxic to both insects and other microbes, and have been widely demonstrated as effective biocontrols for pathogens of various hosts (Barelli *et al.*, 2016; Keyser *et al.*, 2016). In this study, seedlings with few or no cankers were associated with higher abundances of this fungus. This suggests that endophytes may act as complementary defences in conifers; higher abundances of this beneficial fungus may inhibit blister rust, but many other factors, including terpenes, can contribute to resistance or susceptibility. *Lophodermium* also negatively correlated with overall disease severity. *Lophodermium* belongs to the family Rhytismataceae, whose members are common in other white pine species (Ganley *et al.*, 2004), and have shown potential in reducing symptoms of white pine blister rust in previous studies (Ganley *et al.*, 2008). These and our results suggest that these endophytes have potential as biocontrol agents to protect whitebark pine from *C. ribicola* infection.

We detected little variation in the endophyte community composition between resistant and susceptible seedlings after

inoculation, which may be partly a result of the pool of available colonizers at DGRC. Naturally occurring whitebark pines are not found near the common garden, and the surrounding habitat at DGRC is much different from the whitebark pine habitat, which is restricted to high-elevation ecosystems. Many of the fungal species that most influence blister rust severity in whitebark pine may be absent from the surrounding environment at DGRC. However, whitebark pine seed families supported specific endophytic communities within needle tissue, and previous studies have shown evidence of genetically correlated resistance in natural whitebark pine populations (Retzlaff *et al.*, 2016; Sniezko *et al.*, 2011). Future studies of endophyte communities in naturally occurring, resistant whitebark pine exposed to blister rust may provide more information as to which endophytic species or clades are more likely to increase resistance to the pathogen.

Future applications

The results of this study highlight the ecological relationships between tree genetics, terpenes and foliar endophytes with implications for white pine blister rust resistance. Blister rust infection influenced both terpene and endophytic profiles of whitebark pine seedlings, and subsequent correlations of endophytes, terpenes and disease severity indicated that interactions that take place inside needle tissue can influence disease outcome. The response of both terpene and endophytic profiles to infection was additionally related to seed family. This suggests that the genetic resistance seen in natural populations may be a result of the additive effects of both endophytes and the composition of terpenes in needle tissue, in which initial interactions between endophytes, the host and *C. ribicola* take place. The quantification of terpene concentrations in whitebark pine exposed to blister rust demonstrates the potential physiological mechanisms of disease resistance, where higher or lower concentrations of certain terpenes indicate a resistant phenotype. Terpene profiles that can predict disease severity can then act as biomarkers for disease resistance in the future, and may indicate preferred parent trees as seed sources for replanting forests in areas threatened by white pine blister rust.

Recent next-generation sequencing (NGS) technology has highlighted the complex nature of plant microbiomes and has provided us with a much clearer understanding of endophytic community composition in plants than ever before. This is essential because, in order to understand the function of the fungal microbiome and to optimize its potential in disease resistance, we must first understand how different microbial species coexist together in natural settings, as well as the abiotic and biotic factors, such as host genetics, that shape these communities. This study, although limited in providing a direct mechanistic solution for disease resistance, provides the first evidence that both terpenes and endophyte communities in individual host trees may simultaneously contribute to disease resistance. In the future, direct manipulations of fungal endophyte communities (Bullington and Larkin, 2015), as well as investigations into the production of secondary compounds (e.g. terpenes) by fungal species, will help us to better understand the functional roles of specific fungi and the mechanisms underlying their effects on host plants. In addition, this study provides valuable information for experimental inoculations of nursery seedlings with beneficial fungal endophytes for out-planting into high-rust areas. Five-needle pines remain an ecologically and culturally important species across the western USA and Canada, and a comprehensive understanding of what constitutes a resistant or susceptible tree in natural settings will increase the success of future restoration outcomes.

EXPERIMENTAL PROCEDURES

Data collection

DGRC in Cottage Grove, OR, USA breeds five-needle pines to look for genetic resistance to rust infection (Sniezko and Koch, 2017; Sniezko *et al.*, 2014). Seeds collected from healthy, surviving trees are germinated and grown in an open-air glasshouse. After 1–2 years, seedlings are experimentally inoculated with *C. ribicola* spores present on infected leaves of the alternative host (*Ribes* plants) collected from natural *Ribes* populations. For this experiment, 141 seeds belonging to 20 whitebark pine seed families (half-siblings or seeds produced by the same parent tree) were sourced throughout the Pacific Northwest, from British Columbia to southern Oregon (Fig. 2a). All seedling families are currently undergoing rust resistance screening at DGRC. Seeds were stratified and sown directly into Cone-tainers in accordance with standard DGRC protocols (Riley *et al.*, 2007). After two growing seasons (~18 months), seedlings (four to seven seedlings per family) were placed in an inoculation chamber and inoculated with *C. ribicola* rust spores. Inoculation with *C. ribicola* spores also followed the standard DGRC protocol, as described in Sniezko *et al.* (2011). Briefly, infected *Ribes* leaves are suspended over whitebark pine seedlings in a fog chamber with high humidity. *Cronartium ribicola* spores then fall onto seedlings and hyphae enter the seedling through needle stomata. No mechanical wounding is necessary for infection. Ten seedlings, belonging to two of the same families as those that were inoculated, served as controls and were never inoculated (Table S1). After inoculation, all seedlings were out-planted into a common garden at DGRC. As only two seedling families were available for control treatments, we use caution when making direct comparisons with treated seedlings, and instead focus our results on seedling characteristics before and after inoculation with *C. ribicola*, and between resistant and susceptible seedlings.

Needles were sampled from all seedlings at two time periods. The first collection occurred immediately prior to blister rust inoculation on 11 September 2014. Both primary and secondary needles were collected from each seedling. The needle tissue of four to seven seedlings was pooled within each family to ensure sufficient sample volume. This resulted in a total of 22 pooled samples (20 treated families, two control families) before blister rust inoculation. Phenotypic characteristics associated with resistance to blister rust were assessed at 8 months (inspection 1) and 14

months (inspection 2) after inoculation. Inspection 1 included a count of needle spots per seedling caused by the pathogen *C. ribicola*. Data recorded at inspection 2 included the number of bark and stem cankers, height and severity of infection. Severity ranged from 0 to 9, with '0' representing seedlings with no symptoms and '9' representing seedlings dead from blister rust (Sniezko *et al.*, 2014). Approximately 1 month before inspection 2 we collected needle tissue from the same seedlings as sampled before (collection 2). In this sampling, each seedling represented one replicate and needles were pooled within seedlings for a total of 141 individual seedlings (131 inoculated and 10 control seedlings), sampled after the presentation of symptoms on inoculated seedlings. On each seedling we again collected both primary and secondary needles that appeared green and healthy with the exception of some needle spotting caused by blister rust disease on inoculated seedlings. Samples were kept on ice or at −20°C until processing.

Terpene analysis

At collection 2, subsamples of needle tissue from all seedlings were used to analyse tree defensive chemistry in the form of terpene composition. The needle tissue was ground in liquid nitrogen, weighed and sent on dry ice to the University of Alberta for terpene analysis. Extraction and analysis of terpenes were performed as described by Erbilgin *et al.* (2014) and Karst *et al.* (2015). The quantity of chemicals was calculated using response curves generated from analyses of a dilution sequence of known quantities of standards. Calibration with these standards allowed for the analysis of quantitative differences in terpenes amongst seedling groups. For some compounds (terpineol acetate, elemene, cadinene and germacrene), no standards were available, and so the peak area was compared for qualitative and quantitative differences amongst samples; however, concentrations between these compounds and all other compounds could not be compared.

Fungal endophyte community characterization

The remaining needle tissue from individual seedlings and the 22 pooled samples collected before blister rust inoculation were washed and surface sterilized according to Larkin *et al.* (2012). To verify sterilization efficacy, sterilized needles were imprinted on malt extract agar and monitored for fungal growth. No evidence of contamination was observed. Tissue was freeze–dried using a Labconco Freezone benchtop freeze dry system (Labconco, Kansas City, MO, USA). Dried needle tissue was then macerated to a fine powder using a 1600 MiniG® tissue homogenizer and cell lyser (Spex SamplePrep, Metuchen, NJ, USA)

Genomic DNA was extracted from ground needle tissue using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Bullington and Larkin, 2015). Fungal DNA was amplified directly from pine needle tissue and prepared for Illumina sequencing using a two-step polymerase chain reaction (PCR) protocol to first amplify our target region and then to attach unique identifiers. The ITS2 region was initially amplified using a mix of forward fungal primers fITS7 (Ihrmark *et al.*, 2012) and fl ITS7o (Kohout *et al.*, 2014) and the reverse primer ITS4 (White *et al.*, 1990). To reduce problems related to low sequence diversity at the conservative regions (Fadrosh *et al.*, 2014), we increased the sequence variability of our amplicon libraries using a heterogeneity spacer region (0–6 nucleotides) between target primers and 22-bp Fluidigm universal tags, CS1 and CS2, which act as sticky ends for the PCR2 reaction (Fluidigm Inc., San Francisco, CA, USA). Reactions were carried out in 12.5-μL reaction volumes containing 1 μL of template and 20 pmol of each primer in 1 × GoTaq® Green Master Mix (Green GoTaq® Reaction Buffer, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP and 1.5 mM $MgCl_2$; Promega, Madison, WI, USA). Each reaction was performed in a Techne TC-4000 thermocycler (Bibby Scientific, Burlington, NJ, USA) under the following conditions: 3 min at 94 °C, followed by 35 cycles of 60 s at 95 °C, 40 s at 57 °C and 40 s at 70 °C, and a final extension step of 7 min at 68 °C, before storage at 4 °C.

To confirm the presence of our target amplicon, all reactions were analysed by 1.5% agarose gel electrophoresis using a 100-bp ladder (O'GeneRuler DNA Ladder, Thermo Scientific, Waltham, MA, USA). PCR2 primer complexes consisted of Fluidigm tags (CS1 or CS2), 8-bp Illumina Nextera barcodes (Illumina Inc., San Diego, CA, USA) and Illumina adapters P5 and P7. PCR2 was carried out in 25-μL reaction volumes containing 1 μL of template and 20 pmol of each primer in 1 × GoTaq® Green Master Mix (Promega). Each reaction was performed under the following conditions: 95°C for 1 min; 10 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 1 min; and 68°C for 5 min. Samples were pooled on the basis of band intensities in 1.5% agarose gel electrophoresis of the PCR2 product. Sequencing was performed at the Institute for Bioinformatics and Evolutionary Studies (iBEST) genomics resources core at the University of Idaho (http://www.ibest.uidaho.edu/; Moscow, ID, USA). Amplicon libraries were sequenced using 2 × 300 paired-end reads on an Illumina MiSeq sequencing platform (Illumina Inc.).

Initial bioinformatics analyses were conducted using 'quantitative insights into microbial ecology' (QIIME version 1.9.1; Caporaso *et al.*, 2010). Paired reads were assembled using *fastq-join* (Aronesty, 2013) with a minimum overlap of 20 bp and allowing a maximum mismatch of 10% within the region of overlap. We followed primary quality filtering parameters as recommended from Bokulich *et al.* (2013) with the exception of the minimum acceptable Phred quality score, which we adjusted to 27 to ensure that only high-quality reads were included in our analyses. All trimmed and quality-filtered sequences were clustered via subsampled open-reference OTU picking using the QIIME implementation of SortMeRNA and the UNITE fungal ITS sequence database (Kõljalg *et al.*, 2013) as a reference database, followed by Sumaclust for *de novo* clustering (Kopylova *et al.*, 2012). OTUs were delineated at 97% pairwise similarity (Nilsson *et al.*, 2008). Chimera checking was performed on all sequences using USEARCH 6.1 chimera checking software in QIIME (Edgar, 2010; Edgar *et al.*, 2011). Taxonomic identification was determined using the QIIME-based SortMeRNA taxonomy assigner (Mercier *et al.*, 2013) and the UNITE fungal ITS sequence database (Kõljalg *et al.*, 2013). All taxonomic designations refer to assignments based on a minimum pairwise similarity of 97% to sequences within the UNITE fungal database with 90% sequence coverage. OTUs that did not meet these guidelines were subsequently searched against the National Center for Biotechnology Information (NCBI) database, which revealed sufficient (>80%) similarity and coverage to other known fungi as to be retained in our dataset, as they probably belong to fungal species that do not yet have a reference barcode. All OTUs that were represented by less than 0.001% of total sequences (16 sequences), or present in fewer than two seedlings, were removed to avoid potential PCR and sequencing artefacts. All sequence data have since been deposited into GenBank under the accession numbers MG206237–MG207680.

Data analysis

The relationships between terpenes, endophytes and disease phenotype of the same individual seedlings were analysed using R (version 3.3.1, R Core Team, 2017) with the *MASS*, *PMCMR*, *lme4*, *vegan, mvabund* and *afex* packages (Bates *et al.*, 2015; Oksanen *et al.*, 2017; Pohlert, 2014; Singmann *et al.*, 2017; Venables and Ripley, 2002; Wang *et al.*, 2016), unless otherwise noted. Significance was inferred at $P < 0.05$ for all tests.

We first performed a χ^2 test of independence to determine whether or not disease resistance was related to seed family identity or genetic variation using the chist.test function in the *MASS* package (Venables and Ripley, 2002). We also tested for genetic variation in overall terpene composition, as well as differences amongst seedling groups (control, resistant and susceptible seedlings), by performing permutational multivariate analyses of variance using the *adonis* function with 999 permutations on Bray–Curtis distances of terpene concentrations (Oksanen *et al.*, 2017). To then assess the relationship between tree defensive chemistry and *C. ribicola* infection in inoculated whitebark pine seedlings, we used a generalized linear mixed model (*glmer*, Bates *et al.*, 2015) with Poisson error distribution and a log-link function. We used this analysis as data were obtained from seedlings belonging to the same seed family, which was significantly related to terpene concentration and violates the assumption of sampling independence. We considered disease severity as the response, concentrations of each terpene as a fixed factor in individual models and the interaction between terpene concentration and seed family as a random effect. All models were individually assessed for overdispersion. Statistical significance was assessed using parametric bootstrapping and 999 permutations (*mixed* function, Singmann *et al.*, 2017). Only terpenes with available standards were considered in total terpene and total monoterpene analyses. Pearson correlations were conducted between individual terpene concentrations and phenotypic characteristics of disease, as well as relative abundances of endophytic fungal OTUs, to assess the relationships between endophytes, terpenes and disease. Trace compounds and rare OTUs were not included in these analyses.

For all endophyte community analyses, we rarefied sequencing depth to 900 sequences per seedling. Seedlings with fewer than 900 sequences were excluded from further analyses, including five pooled samples before inoculation, eight inoculated seedlings and one control seedling.

To identify the main factors influencing fungal communities, we examined seedling inoculation status (before or after inoculation), seedling group (resistant vs. susceptible seedlings), disease characteristics, seed family identity and seed source characteristics, including latitude, longitude and region, for significant relationships with endophyte composition using the *envfit* and *adonis* (permutational multivariate analysis of variance) functions with 999 permutations on Bray–Curtis distances (Oksanen *et al.*, 2017). To elucidate patterns in fungal community composition in all seedlings before and after inoculation, as well as amongst seedling groups (resistant, susceptible and control seedlings), we used non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distances of rarefied relative abundances of OTUs (*metaMDS* function, Oksanen *et al.*, 2017).

Kruskal–Wallis tests were performed on rarefied data for abundant individual OTUs and higher order taxonomic groups to look for differences in mean abundances between resistant and susceptible seedlings (those that developed cankers and those that did not), as well as before and after inoculation. *Post hoc* analyses were performed using Dunn's test for multiple comparisons with a Bonferroni correction using the *PMCMR* package (Pohlert, 2014).

OTU data were used to estimate foliar fungal species richness within seedlings and diversity indices (evenness, Fisher's alpha) in all samples using the *vegan* community ecology package (Oksanen *et al.*, 2017). Diversity was visualized using box and whisker plots, and analyses were performed using a one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) to assess *post hoc* contrasts amongst seedling groups.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

L.S.B. designed the research, performed the research, wrote the manuscript and analysed the data. Y.L. designed the research and extensively edited the manuscript. R.S. designed the research, contributed supplies, collected data and edited the manuscript. B.L. edited the manuscript and contributed statistics knowledge.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Hypothesized theoretical framework of potential interactions between host chemistry, blister rust and fungal endophytes. Host-imposed biotic and abiotic interactions shape microbial communities in host plants. Host plants are first exposed to a large diverse pool of microbes in the environment, including both pathogens and endophytes. Host chemistry downsizes the pool of microbes by inhibiting initial colonization (C, F). Subsequent fungal–fungal interactions that take place inside the plant after initial colonization further reduce the number of successful colonizers (A, B). In addition, host chemistry can mediate these interactions (G), and both endophytes and pathogens can, in turn, induce a chemical response in the host (D, E). Together, these interactions determine the realized microbial species assemblage of the host.

Fig. S2 Non-metric multidimensional scaling (NMDS) showing direct comparisons of the endophyte communities in seedling families 102 and 62 that were included in both inoculated and control treatments.

Table S1 Information on seed families used in this study, including coordinates of parent trees and the number of seedlings from each family that developed cankers (susceptible seedlings). Control seed families are shown in bold.

Table S2 Differences in mean abundance of individual fungal operational taxonomic units (OTUs) and taxonomic groups amongst *Pinus albicaulis* seedlings before inoculation, after inoculation and in controls.