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Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*

Rebecca J. Ganley [a,*], Richard A. Sniezko [b], George Newcombe [c]

[a] *Scion, New Zealand Forest Research Institute Limited, 49 Sala Street, Private Bag 3020, Rotorua, New Zealand*
[b] *USDA Forest Service, Dorena Genetic Resource Center, Cottage Grove, OR 97424, USA*
[c] *University of Idaho, Department of Forest Resources, Moscow, ID 83844-1133, USA*

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Abstract

Induced resistance responses, including fungal endophyte-mediated resistance, have been well studied in both agricultural crops and grass systems. Yet, the effect of these processes and symbionts in forest trees is poorly known. Fungal endophytes have been found in all conifer forest systems examined to date and have been hypothesised to be involved in resistance-mediated responses. However, in the absence of functional studies the influence of these endophytes on the extended phenotype of the host plant is unclear. In this study we demonstrate that fungal endophytes from *Pinus monticola* were effective at increasing survival in host plants against the exotic pathogen *Cronartium ribicola*, which is responsible for the devastating disease called white pine blister rust. Seedlings previously inoculated with fungal endophytes lived longer than endophyte-free seedlings and also showed some reduction in white pine blister rust disease severity. This endophyte-mediated resistance was found to be effective over time, indicating persistence, and is hypothesised to be a form of induced resistance. Overall, this suggests fungal endophytes may play a determinative role in the structure of biological communities and could provide a useful alternative or ancillary management tool for combating pests and diseases.

Keywords: *Pinus monticola*; *Cronartium ribicola*; White pine blister rust; Fungal endophytes; Endophyte-mediated resistance; Induced resistance

1. Introduction

Woody plants employ a variety of defence mechanisms to prevent invasion by fungal pathogens. Ordinarily, most plants function as non-hosts to a variety of fungi, and vulnerability is the exception. Two of the major types of resistance plants utilise to protect themselves against pathogens are host-specific and non-specific resistance (Ferreira et al., 2006). With host-specific resistance, plants typically possess major genes for resistance (*R*) to specialized fungal pathogens; an *R* gene conditions resistance when a corresponding avirulence (*Avr*) gene is present in the pathogen (Flor, 1971). *R*/*Avr* gene combinations are subject to frequency-dependent selection that maintains resistance at a fairly high level. However, when *R* genes are absent (e.g. chestnut blight (Kubisiak et al., 1997)) or at low frequency (white pine blister rust (Kinloch et al., 2003), catastrophic expansion of the pathogen host range can occur resulting in the decimation of host plant populations.

In addition to major-gene resistance, plants also contain an assortment of minor genes that function collectively to provide resistance, a mechanism commonly referred to as polygenic or quantitative resistance. This form of non-specific resistance is generally invoked when the genetic inheritance of resistance does not fit the major-gene pattern. Although demonstrated in many interactions, understanding of this process is limited due to the varied and complex interaction of genes involved (Parlevliet, 1978; Browning, 1974; Sztejnberg and Wahl, 1976). In comparison to major-gene resistance, polygenic resistance is thought to be effective regardless of the identity of the pathogen isolate and is considered a more durable form of resistance because of its multigenic nature.

In addition to these forms of resistance, induced resistance responses are also known. Induced resistance is an enhanced defensive capacity developed when a host plant is appropriately stimulated (Dealney, 1997). Stimulation factors can include, but are not limited to, pathogens, non-pathogenic microbes, host-incompatible races of pathogens and chemicals. It is

* Corresponding author. Tel.: +64 7 343 5767; fax: +64 7 343 5333.
E-mail address: Rebecca.Ganley@scionresearch.com (R.J. Ganley).

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possible that the stimulation factors trigger some of the pathways involved in polygenic resistance. Similar to polygenic resistance, induced resistance can provide resistance for a prolonged period once activated and against multiple pathogens (van Loon et al., 1998; van Wees et al., 1997). Thus, it is conceivable that in some cases the apparent inheritance of polygenic resistance may be a consequence of stimulation through the extended phenotype (Whitham et al., 2003).

Induced resistance mechanisms have been shown to be effective and are well utilised in the agricultural sector (Terry and Joyce, 2004). Despite this, very little is known about the systems present in forest trees, specifically in widely planted and utilised conifer species (Bonello et al., 2006). Systemic induced resistance responses stimulated by repeated pathogen attack have been documented in two pine species (Blodgett et al., 2007; Bonello et al., 2001; Swedjemark et al., 2007), as have similar responses induced by rhizobacteria (Enebak and Carey, 2000) and avirulent nematotodes (Kosaka et al., 2001). An apparent gap in this list is the influence of fungal endophytes on induced resistance mechanisms in conifer species. Fungal endophytes are fungi that live within their host without causing disease symptoms. Endophytic fungi are probably present to some extent in all plants, and a high diversity of fungal endophytes has been documented in temperate conifer species (Ganley and Newcombe, 2006; Kauhanen et al., 2006; Stefani and Bérubé, 2006). Although fungal endophytes in forest trees have been postulated to be involved in a plethora of host functions including mediated resistance, so far there is little evidence to support these claims.

Assemblages of fungal endophytes from the white pine species *Pinus monticola* (western white pine) have been identified and characterised within its range in the Rocky Mountain region, USA (Ganley and Newcombe, 2006; Ganley et al., 2004). Over 21 different genera of fungi were isolated from needles using culturing methods only. Of concern was the low diversity of fungal endophytes in nursery seedlings and managed forest plantations in comparison to endophytes obtained from seedlings and trees in old-growth forest settings (Ganley and Newcombe, 2006). *P. monticola* is largely a seral, temperate forest tree species of Northwest America. From the early 1900s the introduction of the exotic white pine pathogen *Cronartium ribicola* (white pine blister rust) has caused widespread mortality of this species through its native range. Resistance of white pines against *C. ribicola* is rare, although, a variety of responses have been observed (Fins et al., 2002). The existence of major-gene resistance has been identified in *P. monticola* (Kinloch et al., 1999), although this gene is undetectable within the Rocky Mountain region (Kinloch et al., 2003). In addition to major-gene resistance, resistance responses believed to be controlled by polygenic inheritance have also been observed. However, *P. monticola* F_2 plantations in Idaho have shown wide variation in mortality due to white pine blister rust, ranging from 2 to 95% infection, despite an initially reported 66% level of resistance (Muller, 2002).

To determine whether fungal endophytes play a role in host defense and could potentially account for the variability seen in the *P. monticola* Idaho F_2 stock, we investigated the potential for fungal endophytes from *P. monticola* to mediate resistance against *C. ribicola*. Endophyte-treated seedlings were examined for a reduction in blister rust symptoms in comparison to control seedlings and, in addition, the long-term effects of these endophytes on survival was also investigated to determine their overall influence on host fitness.

2. Methods

2.1. Seedling stock and culturing

Five experiments were performed to test the ability of fungal endophytes to mediate resistance in *P. monticola* seedlings against *C. ribicola*. *P. monticola* seedlings for experiments 1–3 and 5 (Table 1) were obtained from the Dorena Genetic Resource Center, OR. Seedlings for experiment 4 (Table 1) were obtained from the Dorena Genetic Resource Center, OR, from the Coeur d'Alene Nursery, ID and from the Moscow Seed Orchard, ID. The family identities were known for most of the 1-year seedlings used but for only some of the 2-year-old material. A composite of seedlings with low-known polygenic

Table 1
The number of seedlings used per treatment for the five experiments performed on *Pinus monticola* seedlings

Experiment (location, year inoculated, seedling year of growth)	Treatment [endophyte (E) or water (W) inoculated] [*Cronartium ribicola* (Cr) or water (W) inoculated]							
	1 [E] [Cr]	2 [E] [Cr]	3 [E] [Cr]	4 [E] [Cr]	5 [E] [Cr]	6 [E] [Cr]	Control1 [W] [Cr]	Control2 [W] [W]
1 (Dorena, 2001, first)	30	30	30	30	30	30	30	20
2 (Dorena, 2001, second)	25	25	25	25	25	25	25	15
Experiment (location, year inoculated, seedling year of growth)	Treatment [endophyte (E) or water (W) inoculated] [*Cronartium ribicola* (Cr) or water (W) inoculated]							
	A [E] [Cr]	B [E] [Cr]	A/B [E] [Cr]	–	–	–	Control1 [W] [Cr]	Control2 [W] [W]
3 (ID, 2002, first)	160	160	–	–	–	–	160	40
4 (ID, 2002, second)	180	180	–	–	–	–	180	40
5 (Dorena, 2002, first)	–	–	106	–	–	–	106	18

inheritance were preferentially selected and no seedlings containing known major genes for resistance were used. Seedlings from Moscow and Coeur d'Alene were from bulked seedlots but did not contain Idaho F_2 material. All seedlings were germinated and maintained in greenhouses, as stated, and subjected to regular watering and fertilization, both pre- and post-inoculation.

2.2. Experimental design

A randomised complete block design was employed for experiments 3 and 4. For experiment 3, 480 seedlings from 13 different families were equally divided among four replicates. Each replicate consisted of three treatments (treatment A, treatment B and control1), which each contained 40 seedlings from the 13 families (Table 1). Experiment 4 contained four lots of seedlings from three different locations: Dorena Genetic Resource Center, OR (1 seed lot sown 2000); Coeur d'Alene nursery, ID (1 seed lot sown 2000); and Moscow Seed Orchard, ID (2 seed lots; Moscow1, sown late 2001 and Moscow2, sown early 2000). Each of the four lots contained 135 seedlings, which were divided among nine replicates. The three treatments (treatment A, treatment B and control1) within the nine replicates each contained five seedlings from each of the four seedling lots (Table 1). In addition, a second control, control2, was included in both experiments to assess natural seedling mortality but was not used in the final analysis. Both experiment 3 and 4 were conducted in the greenhouses at the University of Idaho.

For experiments 1, 2 and 5 the seedlings were randomly divided between the treatments. Experiments 1 and 2 contained seven treatments (treatments 1–6 and control1) (Table 1) with each treatment containing 30 seedlings for experiment 1, and 25 seedlings for experiment 2. Experiment 5 contained two treatments (treatment A/B and control1), each containing 106 seedlings (Table 1). The number of different families used for experiments 1, 2 and 5 varied: 23 different families were used for experiments 1, 9 for experiment 2 and 19 for experiment 5. The number of seedlings used per family were equally distributed among the treatments and control. In addition, a second control, control2, was included in all three experiments to assess natural seedling mortality but was not used in the final analysis. Experiments 1, 2 and 5 were all conducted in the Dorena Genetic Resource Center greenhouses, OR.

2.3. Endophyte inoculation

The *P. monticola* seedlings were then inoculated with endophyte suspensions (treatments) or water (controls). Fungal endophytes used were isolated from surface-sterilized *P. monticola* needles and seeds (Ganley and Newcombe, 2006). The same group of endophytes were used for all experiments; however, the composition of endophytes within the treatments varied. Experiments 1 and 2 both contained six endophyte treatments (Tables 1 and 2). Experiments 3 and 4 contained two endophyte treatments, treatment A (non-*Rhytismataceae* isolates) and treatment B (*Rhytismataceae* isolates) (Tables 1 and 2). Experiment 5 contained one treatment, which consisted of all the 38 fungal endophytes used for both experiments 3 and 4 (Tables 1 and 2). The number of different species for each fungal taxon is unclear; the *Rhytismataceae* isolates comprise at least six different species (Ganley et al., 2004). Each experiment also contained two controls (control1 and 2), which were both sprayed with water only. As many of the endophytes used in this study did not sporulate in culture and sporulation in the field has also not been observed, a macerated suspension of endophyte mycelium was used for the treatments. Endophyte suspensions contained a final concentration of 0.5 mg wet weight mycelium per ml for each endophyte isolate. The same weight of mycelium for each endophyte was used irrespective of the total number of endophytes present in each treatment. The fungal tissue was blended in Milli Q H_2O using a Tissue Tearor™. The final volume of endophyte solution or water applied to each seedling was based on a volume of 0.25 ml of solution per cm of seedling height.

After inoculation, the seedlings were placed in a rooting chamber (temperature approximately 21 °C, relative humidity 70–100%, day:night = 15:9 h) for experiments 1, 2 and 5, or a growth chamber (temperature approximately 24 °C, relative humidity 80–100%, day:night = 14:10 h) for experiments 3 and 4. To maximize endophyte colonisation, the seedlings were re-inoculated with the original endophyte suspensions 48 h after the first inoculation. Endophyte suspensions were stored at 4 °C until approximately 1 h before the second inoculation; at that time, the suspensions were removed from the refrigerator and the endophyte suspensions were allowed to reach room temperature. The seedlings were then retained in the rooting or growth chamber for another 48 h before being returned to the greenhouses. Endophyte and control water suspensions were plated onto potato dextrose agar (PDA) prior to each inoculation to check endophyte viability and water sterility, respectively. Plates were sealed with parafilm, incubated at 18 °C for 7 days and then inspected for colony growth. All endophyte treatments were viable and conversely, the controls were sterile. Seedlings were inoculated with fungal endophytes 2–3 weeks prior to inoculation with *C. ribicola* to allow sufficient time for colonisation of the needles.

2.4. C. ribicola inoculation

All *C. ribicola* inoculations were performed at the Dorena Genetic Resource Center, OR. Seedlings were moved into the white pine blister rust inoculation chamber 2 days prior to inoculation to acclimatize. The seedlings were placed on boxes inside the room and wetted down. Temperature within the inoculation chamber was maintained at around 16.7 °C and relative humidity at 100% throughout the inoculation. *C. ribicola* inoculum, at the telial stage, was collected from *Ribes* species within Washington and Oregon. Infected leaves were placed telial side down on wire racks above the seedlings and were then covered with plastic tents to minimize air movement. *Ribes* leaves were removed after sufficient basidiospore inoculum density was reached (experiments 1–3 and

Table 2
Fungal endophyte taxa used for treatments 1–6 in Experiments 1 and 2, and treatments A and B in Experiments 3–5

Treatment	Taxa[a]	GenBank accession number[b] (number of isolates)[c]
1	*Cladosporium* sp., *Geopyxis* sp., *Hormonema* sp., *Zalerion* sp., *Rhizosphaera* sp.	AY465432 (4x), AY465433 (1x), AY465441 (1x), AY465453 (4x), AY465470 (1x), AY465472 (1x)
2	*Rhytismataceae* sp., *Xenochalara* sp.	AY465451 (1x), AY465471 (1x), AY465482 (1x), AY465483 (2x), AY465488 (2x), AY465489 (1x), AY465490 (1x), AY465491 (1x), AY465492 (1x), AY465497 (1x)
3	*Helotiales* sp., *Hormonema* sp., *Mycosphaerella* sp., *Rhytismataceae* sp., *Sarcosomataceae* sp., *Pezizales* sp.	AY465452 (1x), AY465453 (2x), AY465456 (2x), AY465473 (1x), AY465480 (1x), AY465485 (3x), AY465491 (1x), AY465503 (1x), AY465508 (1x), AY465511 (1x), AY465512 (1x), AY465513 (1x)
4	*Cladosporium* sp., *Helotiaceae* sp., *Rhytismataceae* sp., *Pezizales* sp.	AY465434 (1x), AY465448 (1x), AY465483 (2x), AY465491 (2x), AY465495 (1x), AY465499 (6x), AY465504 (1x), AY465506 (1x), AY465508 (1x), AY465509 (1x)
5	*Sarcinomyces* sp., *Rhytismataceae* sp., *Pezizales* sp.	AY465431 (1x), AY465474 (1x), AY465475 (1x), AY465483 (4x), AY465491 (1x), AY465496 (1x), AY465499 (8x), AY465505 (1x), AY465507 (1x)
6	*Nemania* sp., *Rhytismataceae* sp., *Pezizales* sp.	AY465458 (3x), AY465478 (1x), AY465481 (1x), AY465483 (5x), AY465488 (1x), AY465491 (1x), AY465492 (1x), AY465499 (2x), AY465507 (1x)
A[d] (non-*Rhytismataceae* isolates)	*Sarcinomyces* sp., *Cladosporium* sp., *Geopyxis* sp., *Helotiaceae* sp., *Helotiales* sp., *Hormonema* sp., *Mycosphaerella* sp., *Nemania* sp., *Zalerion* sp., *Rhizosphaera* sp., *Sarcosomataceae* sp.	AY465431 (1x), AY465433 (1x), AY465434 (1x), AY465441 (1x), AY465448 (1x), AY465452 (1x), AY465453 (1x), AY465456 (1x), AY465457 (1x), AY465458 (1x), AY465470 (1x), AY465472 (1x), AY465503 (1x), AY465504 (1x), AY465505 (1x), AY465506 (1x), AY465508 (1x), AY465509 (1x), AY465513 (1x)
B[d] (*Rhytismataceae* isolates)	*Rhytismataceae* sp.	AY465473–AY465476 (1x), AY465479–AY465481 (1x), AY465483 (1x), AY465485 (1x), AY465489–AY465492 (1x), AY465495 (1x), AY465496 (1x), AY465498 (1x), AY465499 (1x)

[a] Ganley and Newcombe (2006) and Ganley et al. (2004).
[b] GenBank accession numbers for endophyte taxa with unique ITS sequences.
[c] Number of different endophyte isolates used with the same ITS sequence.
[d] Endophyte taxa for treatment A/B in experiment 5 consisted of taxa listed in both treatments A and B.

5 = approximately 8000 spores/cm^2; experiment 4 = approximately 13,000/cm^2) and the seedlings were left in the inoculation chamber for approximately 48 h at 20 °C to ensure spore germination. The inoculation spore loads were very high to minimize escapes. Microscope slides, coated with rubber cement, were placed amongst the seedlings and were periodically examined for spore density and later, germination.

2.5. Confirmation of inoculations

To determine successful inoculation of the seedlings with fungal endophytes a selection of needles were analysed for the presence of the endophytes. Needles could not be taken from any of the seedlings used in the analyses as removal of needles containing fungal endophytes could potentially jeopardize a systemic response within the host. Instead, a selection of seedlings were inoculated with either the endophyte suspensions for each treatment or water, for each experiment. These seedlings were also inoculated with *C. ribicola*. As these seedlings were used to assay the success of the endophyte inoculation they were not included in the final analyses. A selection of the needles were removed from each of these seedlings, surface-sterilized, plated on PDA and monitored for the outgrowth of endophytic fungal colonies, according to the procedures described in Ganley and Newcombe (2006). The PDA medium used was not conducive for growth of *C. ribicola*. Confirmation of inoculation by *C. ribicola* was determined by the presence of white pine blister rust symptoms, either needle spots and/or stem swelling. The percentage of infected seedlings was recorded, except control2 seedlings which were inoculated with water only.

2.6. Data collection and statistical analyses

Data were collected regularly from each of the experiments after inoculation with *C. ribicola*. Appearance of the first blister rust spots on the needles was recorded and subsequently the density of blister rust spots for each seedling was monitored

using a foliage rating index: no spots = 0, 1–9 spots = 1, 10–19 spots = 2, 20–29 spots = 3, >30 spots = 4. Seedlings were also monitored for development of stem cankers/swelling and finally, mortality. Assessment times (days post-inoculation) varied for each experiment depending on the length of time taken for first development of needle spots and stem swelling in the seedlings. The general linear model (GLM) procedure was used to analyse spots, canker/swelling and mortality for each experiment using SAS 8.4 (SAS Institute Inc., Cary, NC) and significant differences between treatments were determined using least squares means (LSMs). The interaction between family or location where the seedlings were grown and treatments was also investigated for experiments 3 and 4. In addition, survival analyses were performed on the mortality data using Systat 9.0 (Systat Software Inc., San Jose, CA).

3. Results

3.1. Confirmation of inoculation

The efficiency of the endophyte inoculation procedure was confirmed by outgrowth, from surface-sterilized needles, of a selection of the endophytes that had been inoculated into the seedlings. Both the water- and endophyte-treated seedlings contained a few greenhouse-propagated endophytes, as have been previously observed in greenhouse-raised seedlings (Ganley and Newcombe, 2006), and that belong to genera or species different from what was applied in the endophyte treatments. These greenhouse endophytes were morphologically easily distinguished from the inoculated endophytes used in the treatments (data not shown). *C. ribicola* inoculations of the *P. monticola* seedlings were also successful, with total infection levels, measured as the presence of needles spots and/or stem lesions, in seedlings in their first year of growth (>99% infection) exceeding those in their second year (93–96% infection). Control2 seedlings, which were neither pre-treated nor inoculated with *C. ribicola* (Table 1), remained healthy for the duration of the experiments and did not display symptoms of white pine blister rust.

3.2. Incidence of needle spots and stem swelling

The incidence of disease symptoms in endophyte-treated seedlings versus control1 varied among experiments for both needle spot density and stem swelling. In experiments 3 and 5 needle spot density in the endophyte treatments did not differ significantly from that of the controls, whereas the endophyte treatments resulted in significantly fewer needle spots in experiments 1, 2 and 4 (Table 3). For stem swelling, the only experiment in which endophyte treatments provided a significant reduction in the number of stem swellings versus the control was experiment 3 ($P = 0.061$) (Table 3). However, as disease severity was not always found to be directly linked with mortality (e.g., one spot could result in seedling mortality whereas a seedling with multiple spots might not die), the incidence of needle spots or stem swellings was not used as a measure of host fitness. Instead, survival time was used as the preferred measure of host fitness.

3.3. Survival analysis

Analysis of mortality for *P. monticola* seedlings infected with *C. ribicola* showed that, more often than not, survival time was increased in seedlings pre-treated with endophytes in comparison to those pre-treated with water (Table 4 and Fig. 1). This difference was significant for the experiments with 1-year-old seedlings (experiments 1, 3, and 5) (Table 4). For experiment 1, treatments 1, 2, and 4–6 survived significantly longer than the control, as did treatment A for experiment 3 and treatment A/B for experiment 5 (Table 4 and Fig. 1). For all of the experiments, increased seedling survival percentage among endophyte treatments ranged between 3 and 16% over the percentage of seedlings that survived in the controls (Table 4). For some of the treatments, endophyte-inoculated seedlings were predicted to have increased survival up to 147 days longer than the controls (Table 4). For experiment 2 with 2-year-old seedlings, mean survival time of the controls was shorter at 668 days than all but one of the six endophyte treatments, although these differences were not significant. Experiment 4, also with 2-year-old seedlings was similar (Table 4).

The interaction between family, when known, and treatments was also investigated. In experiment 3 there was a significant interaction between these two factors ($P < 0.0001$, $F = 2.02$, d.f. = 24). Conversely, the interaction between the location where the seedlings were grown (Moscow, ID; Coeur d'Alene, ID; Dorena, OR) and treatments for experiment 4 was not significant ($P = 0.69$, $F = 0.65$, d.f. = 6).

4. Discussion

The results from this study indicate that fungal endophytes can mediate resistance against *C. ribicola* and thereby increase host fitness in *P. monticola*. Five independent experiments conducted in two separate locations showed that pre-inoculation of *P. monticola* seedlings with fungal endophytes could increase survival against the exotic pathogen *C. ribicola*. The increase in survival percentage in the endophyte treatments versus the control was found to be significant in the 1-year-old seedlings (experiments 1, 3, and 5) and the same pattern was also observed in the 2-year-olds, although the difference was not statistically significant (experiments 2 and 4) (Table 4). The majority of endophyte treatments had reduced mortality levels in comparison to the control and survival analysis also showed an increase in the mean survival time (Table 4). It is unlikely that all of the endophytes used in these experiments were responsible for inducing resistance against white pine blister rust. Many of them would be involved in a suite of different functions in the host but determining these many and varied roles for individual endophytes would be a considerable undertaking. Gene expression studies in *Theobroma cacao* between host seedlings and *Trichoderma* endophytes have shown that profiles produced during colonisation by the endophytes are dependent on the isolate colonising the seedling

Table 3
Incidence of needle spot density and stem swelling in *Pinus monticola* seedlings pre-treated with fungal endophytes and inoculated with *Cronartium ribicola*

Experiment	Mean number of spots (±S.E.)[a]							Days post-inoculation[b]	Significance of factors (ANOVA)		
	Treatment								*P*-Values	*F* values	Degrees of freedom
	1	2	3	4	5	6	Control1				
1	2.36 (±0.62)	3.11 (±0.85)	3.11 (±0.95)	3.21 (±0.68)	2.86 (±0.58)	2.43 (±0.57)	3.11 (±0.58)	91	<0.0001	16.02	6
2	2.91 (±1.25)	2.54 (±1.28)	2.65 (±1.53)	3.25 (±0.91)	2.25 (±1.36)	2.15 (±1.39)	2.58 (±1.07)	91	0.097	1.83	6

Experiment	Mean number of spots (±S.E.)[a]							Days post-inoculation[b]	Significance of factors (ANOVA)		
	Treatment								*P*-Values	*F* values	Degrees of freedom
	A	B	A/B	–	–	–	Control1				
3	2.10 (±0.56)	2.07 (±0.53)	–	–	–	–	2.14 (±0.61)	14	0.46	0.78	2
3	3.08 (±0.51)	3.09 (±0.39)	–	–	–	–	3.14 (±0.34)	81	0.40	0.92	2
4	0.48 (±0.80)	0.51 (±0.82)	–	–	–	–	0.76 (±0.79)	22	0.0017	6.45	2
4	2.14 (±0.98)	2.09 (±1.04)	–	–	–	–	2.30 (±1.00)	267	0.14	1.95	2
5	–	–	3.89 (±0.32)	–	–	–	3.88 (±0.36)	145	0.84	0.04	1

Experiment	Percentage of seedlings with stem swellings							Days post-inoculation[b]	Significance of factors (ANOVA)		
	Treatment								*P*-Values	*F* values	Degrees of freedom
	1	2	3	4	5	6	Control1				
1	100	100	97	100	97	97	100	166	0.55	0.83	6
2	88	72	76	88	84	64	84	249	0.53	0.85	6

Experiment	Percentage of seedlings with stem swellings							Days post-inoculation[b]	Significance of factors (ANOVA)		
	Treatment								*P*-Values	*F* values	Degrees of freedom
	A	B	A/B	–	–	–	Control1				
3	91	86	–	–	–	–	94	122	0.061	2.83	2
4	56	52	–	–	–	–	57	267	0.46	0.77	2
5	–	–	87	–	–	–	90	145	0.52	0.41	1

[a] Foliage rating index used for spot counts: 0 = no spots, 1 = 1–9 spots, 2 = 10–19 spots, 3 = 20–29 spots and 4 = >30 spots.
[b] Post-inoculation with *Cronartium ribicola*; experiments 3 and 4 were measured at two different post-inoculation time points.

Table 4
Survival time of pre-treated *Pinus monticola* seedlings inoculated with *Cronartium ribicola*

Experiment	Mean survival time (days)[a] [percentage of live seedlings][b]							Significance of factors (ANOVA)		
	Treatment							*P*-Values	*F* values	Degrees of freedom
	1	2	3	4	5	6	Control1			
1	611 [0][c]	594 [3][c]	550 [3]	585 [0][c]	578 [7][c]	580 [0][c]	479 [0]	0.0007	4.43	6
2	681 [16]	709 [8]	815 [21]	665 [4]	751 [12]	781 [20]	668 [9]	0.45	0.97	6
Experiment	Mean survival time (days)[a] [percentage of live seedlings][b]							Significance of factors (ANOVA)		
	Treatment							*P*-Values	*F* values	Degrees of freedom
	A	B	A/B	–	–	–	Control1			
3	313 [22][c]	267 [10]	–	–	–	–	264 [6]	0.0001	9.14	2
4	431 [37]	456 [28]	–	–	–	–	432 [21]	0.15	2.63	2
5	–	–	412 [58][c]	–	–	–	412 [48]	0.047	4.02	1

[a] Estimated mean survival time determined by Systat survival analysis.
[b] As assessed at the last data collection time point.
[c] Significantly longer survival time than control1, in terms of LSM.

(Bailey et al., 2006). This suggests complex and specific interaction between individual hosts and fungal endophytes. As the interaction of fungal endophytes in their host is unclear, a multiplex approach was undertaken in this study to include realistic combinations of different fungi that might be required to elicit a resistance response. The endophyte treatments applied contained combinations of endophyte assemblages similar to what was observed in the field. Treatments that did not show increased survival times against the control are not likely to have contained fungal endophytes effective against white pine blister rust. However, these fungi may be effective in mediating resistance against other fungal pathogens of *P. monticola*.

For the majority of treatments, endophytes were shown to reduce levels of pathogen damage. However, survival was not always associated with reduced pathogen symptoms. For instance, experiment 3 showed no variation in needle spot density among the treatments or control, yet the survival time for treatment A (non-rhytismataceous endophytes) seedlings were significantly greater than both treatment B (rhytismataceous endophytes) and control1 seedlings (Tables 3 and 4). This

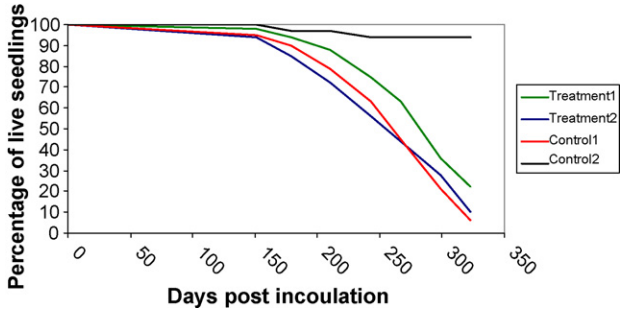

Fig. 1. Mortality in pre-treated *Pinus monticola* seedlings inoculated with *Cronartium ribicola*. Results from experiment 3 for treatments A (non-rhytismataceous) and B (rhytismataceous), pre-treated with fungal endophytes and then inoculated with *C. ribicola* and two controls (control1 and 2). Control1 was pre-treated with water only and inoculated with *C. ribicola*. Control2 was pre-treated with water but not inoculated with *C. ribicola*.

same trend was also apparent at the individual seedling level in many of the experiments. Seedlings with only one needle spot could end up dying, whereas a seedling with multiple needle spots could survive. The use of stem swelling as an indication of disease presence was also problematic. A variety of different types of stem lesions were observed including: slight stem swelling, blackened spots associated with stem swellings, and apparent lesions on stem swellings. Determining whether the stem was swollen, as well as distinguishing between the different types of stem swellings, was subjective. As a result, severity of disease symptoms was not used as a factor for evaluating the effects of endophytes on host fitness. Survival was easily quantified, and it is ultimately the determining factor for this disease in the field.

There was no evidence from this study that any of the endophyte treatments were overtly pathogenic or detrimental to the *P. monticola* seedlings, which would have resulted in a significantly decreased mean survival time in comparison to the control. For some experiments seedling mortality was higher than observed in the control during the course of the study (Fig. 1, treatment B vs. control1), although this difference was not statistically significant. For other experiments, treatments had final live seedling percentages or mean survival times equivalent to the control. For experiment 5, the mean survival time for treatment A/B was the same as control1 despite this treatment having a significantly greater number of seedlings survive than the control. The low number of treatments for this experiment (treatment A/B and control1 only) could be why the same survival times were estimated by the survival analysis software. In experiment 2, treatments 2 and 4 had lower seedling survival rates than control1. However, this difference was not significant and the estimated mean survival time was greater than control1 for both of these treatments. Similarly, for experiment 1, the majority of seedlings were dead after 2 years, yet the estimated mean survival time of these seedlings was considerably more that of the control (>550 days for the treatments vs. 479 days for control1) (Table 4), and after 1 year the seedling survival percentages had been much higher than

control1, from 18 to 39% higher (data not shown). So, although many of the seedlings may have eventually died, it took much longer for them to do so. The ability of these endophyte treatments to significantly extend survival times in the seedlings over time indicates persistence in this form of endophyte-mediated resistance. The final percentages of live seedlings were assessed at the last data collection, which varied between experiments depending on the year the experiment was set up (2001 were analysed for over 2 years and the 2002 experiments up to 1.5 years) and the onset of mortality (delayed in 2-year-old seedlings). It is possible that the survival rate of the treated seedlings could have been increased with a re-application of the endophyte treatments. In the absence of further treatment, the mortality rates in the second year of experiments (experiments 3–5) would be expected to match those obtained for experiments 1 and 2.

Endophyte treatments that prolonged seedling survival in the 1-year-old seedling experiments (experiments 1, 3, and 5) were not always as effective in the 2-year-old seedling experiments (experiments 2 and 4) and vice versa (Table 4). In view of this difference in the response of endophytes with age, it is possible that as the tree matures variations in leaf chemical or physiological factors may stimulate or arrest certain suites of fungi causing activation of specific, but potentially different, resistance pathways or responses. Alternatively, older material may also be more difficult for some types of fungal endophytes to colonise, providing a different form of age-specific endophyte responses. The endophytes used in this study came from both mature trees (of different ages) and seedlings of *P. monticola*. Thus, the persistence of resistance in the endophyte-treated seedlings may have been increased not only with a re-application of endophyte treatments but also with application of specific fungal endophyte assemblages more suited to older plant material. Ontogenetic (mature) resistance, a form of resistance expressed by the host during the mature stages of its life, has been observed in several *Pinus* species including *P. monticola* and is believed to be akin to polygenic resistance, although no genetic mechanism has been identified (Kinloch and Byler, 1981; Gibson, 1972). It is conceivable that ontogenetic resistance may actually be a form of endophyte-mediated resistance.

How these fungal endophytes function to reduce disease symptoms and delay mortality in their hosts is unknown. In general, there are three common hypotheses on how endophytes may function in providing resistance: induced resistance, direct antagonism and competitive habitat exclusion. In view of the results obtained from this study, we think that endophytes in woody plants mediate resistance in their hosts via an induced response. It is unlikely that the results observed were from the endophytes acting antagonistically against the pathogen, such as occurs with some endophytes in *Festuca* grasses (Schardl et al., 2004), as reduced needle spot density or stem swelling was not evident in all experiments despite increased survival rates. Secondly, endophytes are abundant within their host and *P. monticola* needles have been shown to contain up to at least 14 culturable fungal isolates (Ganley and Newcombe, 2006). If these endophytes predominantly functioned by direct antagonism then the abundance and diversity of endophytes per needle within *P. monticola* would be expected to be lower than what has been observed (Ganley and Newcombe, 2006) and/or an individual tree would be dominated by one isolate growing systemically through the plant, such as commonly occurs in *Festuca* grasses (Schardl et al., 2004). For the same reasons, competitive habitat exclusion is also an unlikely factor as fungal endophytes are restricted to discrete portions of the needle tissue where they remain in a slow growing state (Suske and Acker, 1986). Thus, exclusion over an entire leaf would be difficult. Instead, it is hypothesised that the fungal endophyte-mediated resistance observed in this study is a form of induced resistance.

Induced resistance has been identified in many plant species and can provide a persistent form of resistance against a wide variety of pathogens. Induced resistance mechanisms have been well studied and utilised in agricultural crop systems (Terry and Joyce, 2004). In contrast, only a handful of studies have shown the effectiveness of these resistance responses in woody plant species. Inoculation of conifers species with fungal pathogens has been demonstrated to increase host resistance (Blodgett et al., 2007; Bonello et al., 2001; Swedjemark et al., 2007). Of these systems, the best studied have been the interaction of the fungal pathogens *Sphaeropsis sapinea* and *Diplodia scrobiculata* in *P. nigra* and the fungal pathogen *Fusarium circinatum* in *P. radiata*. Overall the mechanisms of resistance have been shown to be durable, organ-dependent and reciprocal (Bonello et al., 2001; Blodgett et al., 2007). Similarly, in the tropical plant species *T. cacao* evidence of reduced pathogen damage due to endophyte-mediated resistance against *Phytophthora* spp. is also indicative of an induced resistance response, as *in vitro* studies showed that the majority of endophytes in this system were not antagonistic against three different fungal pathogens (Arnold et al., 2003). It is possible that the endophyte-mediated resistance observed in this study is similar to the induced resistance observed in *T. cacao*, and thus, could be functional against other pathogens of *P. monticola*. However, further testing would be required to determine if the endophyte-mediated resistance observed is a form of induced resistance.

If endophyte-mediated resistance functions by stimulating defense gene expression then it is possible that in some cases inherited forms of polygenic resistance could be confounded with endophyte-mediated resistance. Interestingly, the level of increased resistance for some of the endophyte treatments after 1 year are equivalent to survival percentages of resistant pine material in artificial screening tests against white pine blister rust and corresponds to what would be selected for in polygenic screening trials (Kegley and Sniezko, 2004; Zsuffa, 1981; Sniezko, 2006). In view of this, it is possible that endophyte-mediated resistance could explain the variation in resistance observed in the Idaho F_2 *P. monticola* progeny, at least in part. Although the Idaho F_2 progeny were predicted to have 66% increased resistance, field trials showed considerable variation ranging between 2 and 95% infection (McDonald and Dekker-Robertson, 1998; Fins et al., 2002; Muller, 2002). In Canada, Idaho F_2 material has also shown different levels of resistance in interior plantations versus coastal regions, which has been

attributed to environmental effects (Hunt, 2004). It is possible that variations in the endophyte assemblages that F_2 material was exposed to in Idaho and Canada could have impacted resistance thus contributing to the ''environmental'' influence, i.e. the extended phenotype (Whitham et al., 2003). The variation in endophyte response with age would also fit in with an induced resistance response, with different endophytes triggering resistance at different life stages and potentially, against different pathogens. Likewise, the increase in survival of some seedlings for an extended period of time indicates a persistent form of resistance for the host plant rather than a localised, transitory response.

The spore load and level of white pine blister rust infection in this study would most likely be equivalent to a high hazard blister rust stand. While the high level of mortality observed in this study is common among artificial white pine blister rust trials (Kegley and Sniezko, 2004; Zsuffa, 1981), the spore inoculation load in these experiments was very high. Inoculum levels of 8000 or 13,000 spores/cm^2, versus traditional levels of 3000 spores/cm^2, were used to ensure minimal escape (Kegley and Sniezko, 2004). The higher than usual level of spore inoculum may have exerted a stronger selective pressure than what would usually be observed in the field and it is possible that this increase in selective pressure may have overwhelmed some of the resistance responses in the seedlings. Field testing of the endophyte treatments is required to confirm the resistance responses observed in this study under natural conditions. The effectiveness and potential long-term contribution of these symbionts to their hosts will also only be determined through field testing. It is expected that endophyte treatments that confer increased resistance or slowed the mortality on very small seedlings in greenhouse trials could provide potentially a much larger benefit to trees in the field. For instance, endophytes might slow canker development just enough to allow the tree to outgrow it. It is likely that many of the small seedlings in this study died before some of the potential resistance responses had a chance to be expressed. Significant improvements to the application and timing of the endophyte inoculations could also increase the effectiveness of this method of resistance, as would further understanding of individual endophyte functions.

Although diverse arrays of fungal endophytes are known to be present in native stands of *P. monticola* (Ganley and Newcombe, 2006), baseline information is lacking. It is possible that the majority of the endophytes present when *C. ribicola* was introduced to North America were not effective in inducing resistance against this disease. After many decades of selective pressure, there may now be more endophytes present in *P. monticola*, and other white pine populations, that are effective at mediating resistance against this disease. Conversely, many beneficial endophytes involved in resistance or other ecological functions may well have been lost from these populations. The impact of this on *P. monticola* and other white pines is unknown. It is also conceivable that endophyte-mediated resistance responses were unheralded factors in the survival of host individuals that would otherwise have died.

The form of endophyte-mediated resistance observed in this study, along with other methods of induced resistance, provides an alternative means to enhance resistance and stabilize traditional breeding practices by applying multiple defenses for primary or other secondary pathogens to overcome. This study highlights the potential contribution that fungal endophytes could make to disease management in forest ecosystems. However, determining the interaction of the endophytes and mechanisms involved in these resistance-mediated responses, as well as in other ecosystems processes, is paramount. Further investigation is also required to determine whether the resistance response observed is indeed an induced systemic reaction and if so, the connection of endophyte-mediated resistance to traditional forms of genetic host resistance or other methods of induced resistance.

5. Conclusion

Fungal endophytes from *P. monticola* were effective at reducing pathogen damage but, more importantly, at increasing survival (or slowing the rate of mortality) in seedlings challenged with the fungal pathogen, *C. ribicola*. This indicates that these endophytes are involved in host defense in *P. monticola*, and potentially could be central to its restoration. These results provide further evidence that fungal endophytes play an important role in disease resistance and they highlight the need for further investigation of endophytes as tools in forest disease management.

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